Innovative platform technologies for stabilization and controlled release of proteins from polymer depots

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The work described in this thesis is the result of co-operations of the Department of Pharmaceutical Technology and Biopharmacy of the University of Groningen and InnoCore Pharmaceuticals, Groningen, within the framework of the Northern Drug Targeting and Delivery Cluster (EFRO Grant).

Printing of this thesis was supported by the Department of Pharmaceutical Technology and Biopharmacy, University of Gronignen, Groningen Graduate Shool of Science and InnoCore Pharmaceuticals, Groningen.

ISBN: 978-90-367-7328-7 (printed version)

978-90-367-7329-4 (electronic version)

Cover design: Milica Stanković and Stefan Buchberger

Layout and print: Medivest KT, Niš

university of groningen

Innovative platform technologies for stabilization and controlled release of proteins from polymer depots

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Monday 8 December 2014 at 11.00 hours

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Contents

“The important thing is not to stop questioning.
Curiosity has its own reason for existing”

Dedicated to my family, for their endless support, love and encouragement

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General introduction

Milica Stanković

General introduction

During the past few decades there has been an expanding interest in the application of proteins for the treatment of various diseases and disorders. To this date, there are more than 150 proteins approved by the US Food and Drug Organization (FDA), and many more are currently under development [1].

The advantages of therapeutic proteins over the small drug molecules are numerous. They have a highly specific set of functions that cannot be easily achieved with smaller molecules. Furthermore, many proteins used as therapeutics are naturally produced by the body, which increases their tolerance and prevents side effects such as the occurrence of undesired immune responses [2]. Additionally, the development of therapeutic proteins is faster than small drug molecules [3].

However, the delivery of proteins has introduced certain challenges that limit their wide application. Because proteins easily degrade in the gastrointestinal tract and do not easily pass the intestinal wall, the preferred route of the administration is parenteral. However, also in the circulation proteins exhibit a short serum half-life due to fast biodegradation, metabolism and excretion. Therefore, in order to maintain therapeutically effective levels in plasma frequent injections are often required, which increases patient discomfort and introduces high costs of the treatment. For this reason, the development of injectable sustained release depot preparations in which proteins are encapsulated in polymer matrices is currently an important field of interest of many researchers. Sustained release of proteins reduces injection frequency, toxicity, enables local delivery, and therefore enables the use of drug in lower doses. These depot systems are designed to achieve predictable, reliable or desired drug release profiles.

The parenteral sustained release formulations offer a number of advantages over conventional injections [4]:

- Improved drug pharmacokinetics
- Prolonged duration of action or maintaining a high drug concentration in blood circulation
- Reduction of side effects by maintaining a constant drug level
- Decreased systemic side effects due to targeted drug delivery

Protein instability in controlled release systems

A number of polymer based formulations for the sustained release of peptides have already reached the market. E.g. with the currently marketed formulations for goserelin and leuprorelin acetate the frequency of injections is reduced to once every one to three months [5,6]. However, despite extensive research on protein delivery depots, to this date there are no marketed products, implying a lot of challenges involved in the development of controlled or sustained release system for proteins. Proteins are highly organized and complex entities and in order to function properly they need to maintain their chemical and physical integrity and structure. However, encapsulation of proteins into depot formulations often expose them to harsh conditions such as aqueous / organic interfaces, hydrophobic surfaces, high temperatures, shear stresses. Another great obstacle in the development of depot systems is the need to retain the structure and biological activity of proteins during the time anticipated for complete release in the body. Additionally, the polymeric environment can have a significant effect on the stability of the proteins. In particular, polymer degradation products can cause covalent binding of the protein to the polymer surface [7] and they can cause a substantial pH drop in the medium surrounding the protein resulting in deterioration of the protein [8,9].

Different approaches have been proposed to stabilize proteins. Proteins can be chemically modified in order to minimize their susceptibility to chemical degradation, e.g. by PEGylation [10]. Further, proteins can be stabilized by spray or freeze drying them in the presence of sugars like inulin and trehalose, prior to encapsulation into polymer matrix [11,12]. Proteins can be further stabilized and encapsulated into the polymeric depot with a help of basic additives, with the aim to decrease the pH drop caused by polymer degradation [13,14]. In addition, using encapsulation methods that are not exposing proteins to water/organic solvent interphases are favorable. Some of those methods are injection molding, solvent extrusion and hot melt extrusion [15–17].

The aim of this thesis was to use the spray drying technology to stabilize proteins in the sugar glasses and then to encapsulate the sugar-stabilized proteins into the polymer matrices by Hot Melt Extrusion (HME). Further, novel biodegradable hydrophilic multiblock copolymers were evaluated as a matrix to prepare sustained-release depot formulations. Various factors crucial for the development of the platform technology for the successful encapsulation and release of the active proteins were explored.

Outline of the thesis

Chapter 2 is the review on the HME technique with focus on the different biodegradable and non-biodegradable polymers used for the encapsulation of active pharmaceutical ingredients by HME. The application of HME and characterization of drug delivery systems prepared using this technique has been described.

In **chapter 3**, the role of the two mechanisms of protein stabilization by sugar glasses has been investigated: the vitrification theory and the water replacement theory. Even though stabilization of proteins has often been ascribed to either one of these two mechanisms, in this study the role of both mechanisms has been addressed. The model protein alkaline phosphatase was incorporated into a glassy matrix of inulin and trehalose using spray drying. Ammediol buffer, a glass former, was used as a plasticizer; thus, by varying the sugar/buffer mass ratio, spray dried products with different glass transition temperatures could be prepared. The protein storage stability at different glass transition temperatures was studied.

The extensively studied poly(lactide-co-glycolide) (PLGA) represents the gold standard of biodegradable polymers. However, for the preparation of the polymer depot formulation by HME, this polymer requires rather high temperatures (>85 °C), which can be detrimental for the encapsulated proteins. To study whether the high temperatures used for the extrusion of PLGA can be overcome in **chapter 4** a novel hydrophilic multiblock copolymer with a relatively low melting temperature, was synthetized and characterized. This copolymer was composed of two phase-separated blocks, namely semi-crystalline poly(ε-caprolactone) (PCL) and amorphous poly(ethylene glycol)–poly(ε-caprolactone) block (PCL-PEG). This polymer composition was selected because, based on the relatively low melting temperature of PCL, it was anticipated that it could be extruded at much lower temperatures than PLGA. The model protein lysozyme was spray dried with inulin and then encapsulated into the polymer matrix by HME which indeed could be accomplished at a relatively low temperature of 55 °C. Furthermore, it was investigated whether inulin can be used as a pore forming excipient, thus tailoring the release kinetics by changing the lysozyme/inulin ratio. Furthermore, the protein activity after extrusion and during 260 days of the release period was assessed. Additionally, the influence of loading and the size of encapsulated lysozyme(/inulin) particles on the release kinetics was evaluated.

In **chapter 5**, we investigated the degradation behavior of the multiblock copolymer introduced in the previous chapter and whether the degradation rate of the polymer and thus the release kinetics of encapsulated proteins can be tailored by changing the [PCL-PEG]/[PCL] block ratio. Moreover, by using five different

proteins, the effect of the molecular weight of the encapsulated proteins on the release kinetics was investigated.

In **chapter 6**, multiblock copolymers with a modified molecular architecture were synthetized and evaluated. These copolymers were composed of poly-D,L-lactide-PEG (PDLA-PEG) instead of (PCL-PEG) in the amorphous block and (PCL) in the semi-crystalline block. The [PDLA-PEG]/PCL] block ratio was varied. The influence of the block ratio on both the degradation rate of these copolymers and the release of model proteins lysozyme and bovine serum albumin was evaluated. Additionally, possible interactions between the proteins and the polymer degradation products formed during release were investigated.

In **chapter 7** the results of the studies described in this thesis are summarized and the perspectives are discussed.

References:

[1] http://www.drugbank.ca/, (n.d.).

[2] B. Leader, Q.J. Baca, D.E. Golan, Protein therapeutics: a summary and pharmacological classification, Nat. Rev. Drug Discov. 7 (2008) 21–39.

[3] J.M. Reichert, Trends in development and approval times for new therapeutics in the United States, Nat. Rev. Drug Discov. 2 (2003) 695–702.

[4] D.J. Burgess, D.J. A. Crommelin, A.S. Hussain, M.-L. Chen, Assuring quality and performance of sustained and controlled release parenterals: EUFEPS workshop report., AAPS J. 6 (2004) 100–111.

[5] A.S. Dutta, B.J.A. Furr, F.G. Hutchinson, The discovery and development of Goserelin (Zoladex®), Pharmaceut. Med. 7 (1993) 9–28.

[6] H. Okada, Y. Doken, Y. Ogawa, H. Toguchi, Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers, Pharm Res. 11 (1994) 1143–1147.

[7] R.E. Johnson, L.A. Lanaski, M.J. Griffin, T.E. Needham, H. Zia, Stability of atriopeptin III in poly- (D,L-lactide-co-glycolide) microspheres, J. Control Release. 17 (1991) 61–68.

[8] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-co-glycolide) Microspheres Stabilizes Camptothecins, Pharm. Res. 16 (1999) 241–248.

[9] K. Fu, D.W. Pack, A.M. Klibanov, R. Langer, Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres, Pharm. Res. 17 (2000) 100–106.

[10] M. Diwan, T.G. Park, Pegylation enhances protein stability during encapsulation in PLGA microspheres, J Control Release. 73 (2001) 233–244.

[11] N. Grasmeijer, M. Stanković, H. de Waard, H.W. Frijlink, W.L.J. Hinrichs, Unraveling protein stabilization mechanisms: vitrification and water replacement in a glass transition temperature controlled system, Biochim. Biophys. Acta. 1834 (2013) 763–769.

[12] W.L.J. Hinrichs, M.G. Prinsen, H.W. Frijlink, Inulin glasses for the stabilization of therapeutic proteins, Int. J. Pharm. 215 (2001) 163–174.

[13] J. Kang, O. Lambert, M. Ausborn, S.P. Schwendeman, Stability of proteins encapsulated in injectable and biodegradable poly(lactide-co-glycolide)-glucose millicylinders, Int. J. Pharm. 357 (2008) 235–243.

[14] G. Zhu, S.R. Mallery, S.P. Schwendeman, Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide), Nat. Biotechnol. 18 (2000) 52–57.

[15] L. Zema, G. Loreti, A. Melocchi, A. Maroni, A. Gazzaniga, Injection Molding and its application to drug delivery, J. Control. Release. 159 (2012) 324–331.

[16] X. Zhang, U.P. Wyss, D. Pichora, B.G. Amsden, M.F.A. Goosen, Controlled release of albumin from biodegradable poly (DL-lactide) cylinders, J. Control Release. 25 (1993) 61–69.

[17] M.A. Repka, S. Shah, J. Lu, S. Maddineni, J. Morott, K. Patwardhan, et al., Melt extrusion: process to product, Expert Opin. Drug Deliv. 9 (2012) 105–125.

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Polymeric formulations for drug release prepared by Hot Melt Extrusion

Application and characterization

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Manuscript submitted

Abstract

Over the last few decades hot melt extrusion (HME) has emerged as a powerful processing technology for the production of pharmaceutical solid dosage forms in which an active pharmaceutical ingredient (API) is dispersed into polymer matrices. With the HME process a large variety of dosage forms and formulations can be prepared such as granules, pellets, tablets, ophthalmic inserts, implants, stents and transdermal systems. It has been shown that formulations using HME can provide time controlled, sustained and targeted drug delivery, and improved bioavailability of poorly soluble drugs. Because of the solvent free nature of the process, an improved content uniformity of the product due to enhanced mixing, a reduced number of unit operations, the adaptability to continuous manufacturing setups and the suitability of the process to apply quality by design and process analytical technology tools, HME is gaining an increasingly prominent role in pharmaceutical manufacturing of advanced dosage forms.

In this review the basic principles of HME process are described together with an overview of some of the mostly used biodegradable and non-biodegradable polymers for the preparation of different formulations using this method. Further, the application of HME in drug delivery and analytical techniques employed to characterize HME products are addressed.

1. Introduction

Since the 1930s, hot melt extrusion (HME) has found its place as an established process in the plastic and food industry. In the 1980s, HME has been used for the first time in the formulation of pharmaceuticals [1]. From that time on, this technique has emerged as a potent processing technology for the development of solid dosage forms, in which the active pharmaceutical ingredient (API) is dispersed into polymer matrices. It has been demonstrated that formulations using HME are able to provide time controlled, extended and targeted drug delivery, and improved bioavailability of poorly soluble drugs. Several aspects of HME techniques have been extensively reviewed [2–6]. Further, the number of HME-based patents has been growing in the last decades.

HME technology offers numerous advantages over the traditional emulsification based microencapsulation methods: fewer processing steps, reduced process time, continuous operation, absence of solvents or water during processing and superior mixing [3]. Additionally, although HME is often used as a batch process, it can be readily adapted for continuous manufacturing [7,8]. The combination of HME with other techniques, such as powder coating, complexation with cyclodextrines, supercritical carbon dioxide technology has demonstrated the adaptability of this technology [9–11]. On the other hand, HME is a thermal process, which might compromise the integrity of the API, polymer stability; especially when these are heat and/or shear sensitive molecules. The process requires high energy input coming from the used shear forces combined with high temperatures and additionally, it requires sufficient material flow properties for processing. These requirements limit the process to a restricted number of available polymers [12]. Taken together, HME is achieving an important and challenging role in the formulation science.

This review appraises the role of HME in the formulation development and provides an overview of some of the mostly used biodegradable and non-biodegradable polymers in preparation of various formulations by HME, the application of HME in drug delivery and characterization techniques employed in evaluation of the HME products.

2. Hot melt extrusion process

HME is a process in which material melts or softens under elevated temperatures and is further forced through a die, usually with the help of one or two conveyer screws in a barrel. The process itself can be divided into several steps, involving heating of the material, feeding, mixing and conveying, flow through the die and downstream

processing of material. Each of these steps can be controlled and affect the final properties of the product.

Commercially available extruders normally consist of a feeder (hopper) and optionally a side feeder, a conveyer barrel with one or two screws and a die that shapes the melt pumped through the extruder to provide the desired dimensions at a specific throughput rate [13] (**Figure 1**). The barrel of large commercially available extruders is usually divided in sections, which are clamped together. However, mini lab twin-screw extruders often used in preparation of smaller batch sizes consist of only one barrel and two screws (**Figure 2**). Accessories may comprise of a heating or cooling device for barrels, a caterpillar with conveyer belt for stretching and cooling the product, an *in-line* laser, and a solvent delivery pump [13]. The downstream processing equipment may be coupled to the extrusion die and can form either the final dosage form (injection molding, shaping callender) or intermediate product for further processing (strand cutting, film extrusion, cooling callender, die-face pelletizing) [8]. In some extrusion processes, co-extrusion is used to process two or more materials which flow through different channels but are fed through the same die in order to produce a multilayered product, where each layer has specific properties. Co-extrusion can optimize product performance by combining multiple carriers with different properties [13–15].

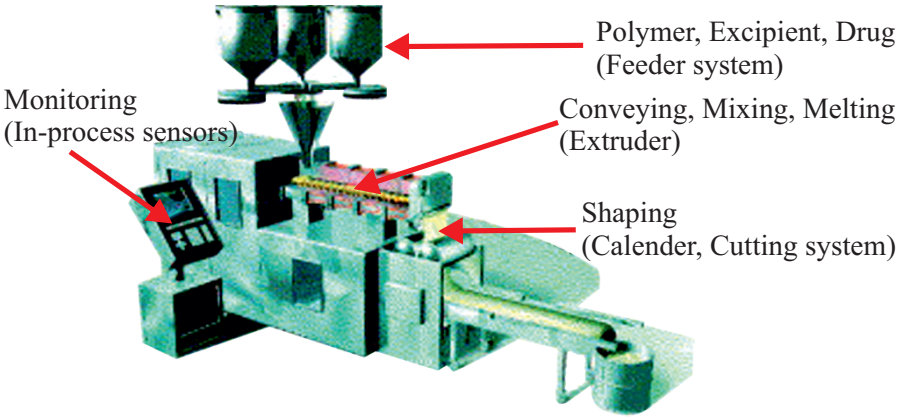


Figure 1. Schematic representation of a pharmaceutical hot melt extruder. Published from [2] with permission from Elsevier.

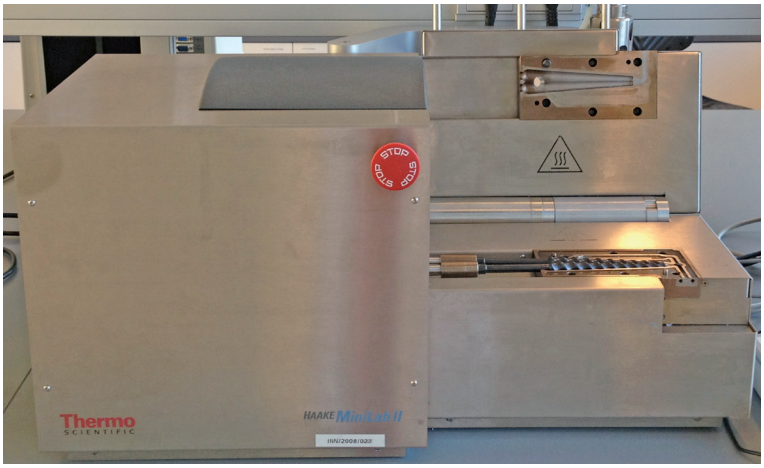


Figure 2. HAAKE MiniLab Rheomex CTW5 (Thermo-Electron) co-rotating mini-lab twin-screw extruder.

The extruder can have one or two screws. The twin-screw extruder has become the preferred device due to its better mixing capability. The twin-screw extruder utilizes two screws, arranged side by side and allowing a number of different configurations to be obtained in all zones, from the feeder until the die. Screws can rotate in the same (co-rotating) or in the opposite direction (counter rotating, used when the high shear forces are required). The friction between the barrel, mixture, and rotating screws provides the driving force for the material to reach the die.

Depending on the extruder design, the material can be fed at different locations. E.g. additives can be added to the melt at several downstream locations, using a side feeder. Liquids can also be introduced using a liquid pump and liquid injection system [13]. After the material enters the transition zone it melts or fuses due to increased temperature in the barrel. The material further blends with the API with a help of screws and it moves along the barrel towards the die. When the material reaches the metering zone in the form of a homogenous melt or dispersion, it is delivered through the die cavity and sized to obtain its final shape. The material extruded from the die in the form of for example a strand will be further referred to as an "extrudate" in this review.

The cooling of an extrudate can be done by air, water or by a contact with a cold surface. Semi-crystalline polymers have a very sharp melting point and consequently a very sharp solidification temperature. Choosing the cooling rates is important when extruding semi-crystalline polymers in order to obtain a product with the required crystallinity; rapid cooling would lead to the formation of small crystals and a relatively low overall crystallinity, while annealing would result in additional crystal growth and higher overall crystallinity. Thus, when a high crystallinity is preferred,

the extrudate should be cooled slowly, with a rate determined by throughput rate and the temperature of the cooling medium (air, roll temperature of caterpillar) (**Figure 3**) [13]. Amorphous polymers do not have a distinct temperature below which they solidify, it is rather a transition that extends over a certain temperature range. The midpoint of the transition is usually referred to as the glass transition temperature (*Tg*). For more information about the crystalline versus amorphous polymers, the readers are referred to a book of Giles et al. [13].

Figure 3. Extrudate pulled by a caterpillar equipped with an in-line laser.

The quality of the product from the extrusion process is affected by different parameters, such as the viscosity of the material, the interrelation of the viscosity with shear rate and temperature and the elasticity. Therefore, requirements on reproducibility demand close monitoring of various process conditions. Nowadays, extruders allow in-line monitoring and control of the process parameters such as temperature, melt pressure, screw speed, torque, feed rate, etc. The melt pressure largely depends on the temperature, feed rate, screw speed, polymer viscosity, and miscibility of polymer, drug and other excipients [2]. Additionally, factors such as ambient temperature, relative humidity, temperature and moisture level of the feed entering the extruder are of importance for the HME process [16,17].

The concept of process analytical technology (PAT) was initiated in 2004 by the American Food and Drug Administration (FDA) [18], with the intention to improve the understanding of the process by designing, analyzing, and controlling different unit operations through timely measurements with the aim to better ensure

the quality of the final products. Rheological behavior of the polymer depends largely upon temperature, process history and pressure. Rheological data obtained during an extrusion process can provide information on the macromolecular structure of the melt and its morphology. Covas et al. introduced a concept of *on-line* monitoring of the polymer rheology during the extrusion process along the extruder [19,20]. Raman spectroscopy has been used in HME to monitor ethylene-co-vinyl acetate melt composition [21,22] or to analyze the clotrimazole and ketoprofen content in hot melt extruded poly(ethylene oxide) (PEO) films [23]. Furthermore, *Near-Infrared Spectroscopy* (NIR) was successfully implemented into various HME processes, offering real-time information on the physical and chemical characteristics of the materials during the process. Tumuluri et al. used FT-NIR for quantitative analysis of clotrimazole in PEO during HME [24]. Application of various PAT tools for HME has recently been thoroughly discussed and reviewed [25,26].

3. Polymers used in HME

An important prerequisite of materials used in HME is their thermal stability even though due to the relatively short residence time (0.5 - 5 minutes) in the extruder not all thermolabile compounds are excluded for use in HME. Further, depending on the requirements of the final product, materials should be able to mix on a molecular level or should remain phase separated [27].

To prepare a drug delivery system (DDS) by HME, the API is embedded in a carrier containing one or more compounds or other functional excipients able to melt or fuse at a certain temperature (and pressure). Carriers used in HME can be either polymers or lipids. Polymeric materials can be either biodegradable or non-biodegradable [28] and will be further addressed in this review. To be extrudable, the polymer must exhibit thermoplastic characteristics next to its thermal stability in the required extrusion temperature range. In order to improve the processing conditions during the manufacturing (e.g. lowering the extrusion temperature) of the DDS, often plasticizers need to be incorporated. These are typically low molecular weight compounds able to improve processing conditions by increasing the free volume between polymer chains and thus lowering the melt viscosity or the *Tg* of the polymer. Consequently, they can soften the polymer and improve the flexibility and properties of the final product [29]. Commonly used plasticizers include triethyl citrate, tributyl citrate, triacetin, poly(ethylene glycol) and propylene glycol [30]. Other functional excipients such as diluents, release and pH modifiers, antioxidants, processing aids, surfactants and stabilizers can also be incorporated into the DDS during the HME process to improve its efficiency [31].

3. 1. Synthetic biodegradable polymers used in HME

In the last few decades, the delivery of APIs from polymers has attracted considerable attention. Amongst others, developments in tissue engineering, gene therapy, regenerative medicine and controlled drug delivery raised the need of using biodegradable polymer excipients requiring no surgical removal. The general principle in the field of biomaterials is to use a material that is able to perform a specific therapeutic task (e.g. the controlled release of an API) and which degrades over time into non-toxic metabolites that are eliminated via regular physiological elimination pathways. Next to their ability to perform the required therapeutic task, biodegradable polymers should be biocompatible, which means that they should be non-toxic, endotoxin free, non-carcinogenic and non-immunogenic. In addition, they should have suitable mechanical, physical, chemical and thermal properties. Besides, they should have appropriate degradation kinetics and resistance to sterilization methods [32,33]. Synthetic polymers allow tailoring of many of the physicochemical properties that determine the performance of the products made of these polymers [34]. Therefore, the development and application of novel synthetic polymers for therapeutic applications is rapidly expanding.

3. 1. 1. Aliphatic polyesters

The most extensively investigated synthetic polymers with available clinical and toxicological data are aliphatic polyesters. Starting from 1960's, they have been used for the broad range of applications such as sutures, scaffolds for tissue engineering, bone screws and drug delivery devices. Aliphatic polyesters have been comprehensively reviewed [32,35–40]. They can be developed from a variety of monomers using various synthetic routes, resulting in polymers of variable molecular weight and degradation kinetics such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε–caprolactone) (PCL) or copolymers such as poly(lactide-co-glycolide) with different lactide/glycolide ratios [41–48] and copolymers of ε-caprolactone and L,D-lactide or glycolide [49–51].

Aliphatic polyesters degrade mainly by bulk erosion, usually having non-linear and discontinuous erosion kinetics [52], which makes it difficult to predict the drug release kinetics. Polymer chains are cleaved by hydrolysis of the ester bonds ultimately resulting in the formation of monomeric acids, which can further catalyze the degradation. Moreover, during the degradation of aliphatic polyesters in a DDS, an acidic microenvironment can be formed inside the DDS, which can influence the stability of encapsulated molecules and may induce inflammation at the site of implantation

3. 1. 2. Poly(ortho esters)

Poly(ortho esters) (POE) have been developed to overcome several of the disadvantages of bulk eroding polymers. Since 1970's, four different families of these polymers have been synthesized, these include POE I – POE IV [53]. The application of poly(ortho esters) has been systematically reviewed by Heller et al. [54].

Degradation of poly(ortho esters) starts by hydrolysis of the polymer backbone resulting in the formation of fragments containing carboxylic acid end groups, which further catalyze the reaction similar to the degradation of aliphatic polyesters. In contrast to aliphatic polyesters, the weight loss and polymer erosion occurs predominantly at the surface even though also bulk erosion occurs to a certain extent [53]. However, because the polymer is highly hydrophobic, the water concentration in the bulk is lower and therefore the rate of hydrolysis is limited by the amount of available water, which is higher on the polymer surface. The degradation rate, pH sensitivity and *Tg* of these polymers can be controlled by using diols with variable chain flexibility [55]. The rate of drug release from poly(ortho esters) is predominately controlled by the rate of polymer hydrolysis, which can be catalyzed by the incorporation of acidic or alkaline excipients.

3. 1. 3. Polyurethanes

Next to polyesters, polyurethanes have been broadly investigated as tissue engineering scaffolds and long term medical implants, such as cardiac pacemakers, vascular grafts and vaginal rings [56,57]. Polyurethanes are multiblock copolymers containing soft and hard segments through which they have thermoplastic and elastic properties. Polyurethanes are produced by a reaction between polyols (polyethers or polyesters) and diisocyanates. Most types of polyurethanes are considered non-biodegradable due to the long duration necessary for complete degradation, the release time scale is often negligible compared to the degradation time scale [58]. Therefore, although there are some examples of drug releasing polyurethanes based coatings [59] and osmotic implants with semi-permeable polyurethane membranes [60] non-degradable polyurethanes are less suitable for drug release purposes and will not be further considered in this review. However, due to their good biological performances (in other applications), mechanical properties and processability, efforts have been made to prepare polyurethanes that degrade faster. Biodegradable poly(ester urethanes) have been prepared from lysine diisocyanate (LDI) with D,L-lactide, ε-caprolactone and other monomers [56,61,62], where aliphatic polyesters form the soft segments and polypeptides the hard segments of the polymer.

The aliphatic ester linkages in poly(ester urethanes) are known to be susceptible to hydrolysis. Depending on their hydrophilicity, these polyurethanes exhibit either bulk degradation or surface erosion [63]. Poly(ether urethanes) are susceptible to mechanical degradation involving crack formation and propagation occurring in areas of the device where the mechanical stress level on polymer is high [64]. Comprehensive review on degradation of polyurethanes has been published by Santerre et al. [64]. Owing to their favorable properties, degradable polyurethanes are of a great interest for the preparation of drug releasing devices by HME.

3. 1. 4. Polyanhydrides

Polyanhydrides are copolymers of sebacic acid and erucic acid dimer specially designed and developed for drug delivery applications with the intention that the material should degrade within the time frame of their application.

Polyanhydrides are hydrophobic in nature, which prevents water penetration into the bulk and therefore mainly allows degradation of the hydrolytically labile backbone at the surface [65]. The degradation starts by water uptake, followed by hydrolysis and finally erosion of the polymer matrix. HME was used to prepare PolyAspirin™, which is a fiber composed of a poly(anhydride-ester) synthesized by copolymerization of salicylate monomer, carboxypenoxydecanoate (CPD) and para-carboxyphenoxyhexane (pCPH). In an aqueous environment, PolyAspirin™ undergoes hydrolysis which results in the formation and release of salicylic acid [66].

3. 2. Synthetic non-biodegradable polymers used in HME

Non-degradable polymers have been applied for the fabrication of oral dosage forms, transdermal films, implants, coatings, medical devices, materials for tissue engineering applications and regenerative medicine. Choices for specific polymers in various applications are often based on their physicochemical properties, such as aqueous solubility, viscosity or melting temperature. Polymers used in drug delivery should have adequate durability and mechanical strength during the intended *in-vivo* application.

3. 2. 1. Poly(vinyllactam) polymers

The most often used type of polyvinyl lactams are poly(vinylpyrrolidones), (PVPs; marketed products are Povidones®, Kollidon®, and Polyplasdone®), which can be purchased in large range of molecular weights (2.500-1.250.000 Da). PVPs are made by polymerization of N-vinylpyrrolidone; they are highly soluble in water and moderately soluble in organic solvents. The *Tg* of PVP is in the range of 90-156°C,

depending on its molecular weight [67]. These polymers are mainly used as binders in tablet formulations or as matrices to increase the bioavailability of poorly water-soluble drugs by improving their dissolution rate. Copovidone® is a copolymer consisting of the monomers vinylpyrrolidone and vinylacetate (VA), which has a molecular weight of around 55 000 Da and a *Tg* of 101°C [67].

Poly(vinylcaprolactam) - poly(vinyl acetate) - poly(ethylene glycol) graft copolymer (Soluplus®) was developed by BASF in 2009 with the intention of using it in particular for HME. With a molecular weight of 118.000 Da and relatively low *Tg* (70°C), this polymer is an excellent candidate for HME [68]. Properties and applications of various types of poly(vinyllactam) polymers have been described by Kolter [67].

3. 2. 2. Ethylene-co-vinyl acetate

Ethylene-co-vinyl acetate (EVA) is a water-insoluble copolymer of ethylene and VA. This copolymer can be well processed via HME and adjustment of the hydrophobicity of this polymer, by changing the ethylene/VA ratio, allows tailored release. EVA polymer has been successfully used in wide spectrum of applications, including preparation of sustained release tablets, intravaginal rings, scleral implants [69].

3. 2. 3. Acrylic polymers – poly(acrylic acid)

Poly(acrylic acid) (PAA) is a water-soluble polymer with various industrial applications. Block copolymers of PAA and PEG or poly(propylene oxide) offer a wide range of medical applications. Random copolymers derived from esters of acrylic and methacrylic acid (Eudragit®) have physicochemical properties and physical forms determined by their functional groups. These polymers are extensively used as coating material for oral products, but they have also found their use in a variety of formulations prepared by HME [70–73].

3. 2. 4. Poly(ethylene glycol) / Poly(ethylene oxide)

Poly(ethylene glycol) (PEG) is a synthetic polymer suitable for biological applications, relevant properties of the polymer are its high solubility in water and low intrinsic viscosity. This polymer is often used in combination with aliphatic polyesters as a part of block copolymers. Due to its high hydrophilicity, it has been widely used to enhance the hydrophilicity of block copolymers, to increase water uptake, increase porosity and thereby influence the release/degradation properties of the prepared formulations [74,75]. In HME, PEG was successfully used to enhance solubility of oral dosage forms, films, or as a plasticizer [76–78].

Poly(ethylene oxide) (PEO) has the same repeating units as PEG, i.e. -[CH_2CH_2O]-. The difference is that the PEG has hydroxyl groups at both ends of the polymer chain while PEO only at one end. In addition, PEO can be obtained at much higher molecular weights (up to 5.000.000 Da) than PEG (up to 40.000 Da).

Copolymerization of monomers into random or block copolymers offers the incorporation of combined properties of at least two different monomers into the material, leading to improved copolymer stability, tailored degradation and released properties.

3. 3. Natural polymers

Even though biologically derived polymers, i.e. natural polymers and derivatives thereof, are valuable sources that have been used in a variety of biomedical applications, their degradation is usually enzymatic, occurring at a rate that is hard to predict, which implies difficulties in establishing *in-vitro – in-vivo* correlations. Further, they can have an inherent biological activity, which may cause side effects such as immune responses [51].

3. 3. 1. Cellulose derivatives - hydroxypropyl cellulose and hydroxypropylmethyl cellulose

Cellulose derivatives have been developed to alter the properties of cellulose, in particular its insolubility in water and its poor thermoplasticity. Thanks to their excellent biocompatibility cellulose derivatives have been used in wide range of applications. Hydroxypropyl cellulose (HPC) is non-ionic water-soluble and pH insensitive cellulose ether, which can be used as tablet binder, as a modified release carrier, film-coating polymer or carrier for film preparation. The processing temperatures of this polymer depend on its molecular weight and are in the range of 120 °C - 200 °C [79]. Commercially available HPC, Klucel™ HPC polymers have been successfully used as matrix formers and solubility-enhancing agents [80].

Hydroxypropylmethyl cellulose (HPMC) is also a non-ionic water-soluble polymer, which is widely used for the preparation of immediate or controlled release tablets, microparticles and films by HME [81–83]. The release of drugs from this polymer can be tailored by altering the polymer molecular weight and the degree of substitution. It has been shown that higher molecular weights HPMC gave faster release profile due to higher swelling capacity [84].

3. 3. 2. Starch, chitosan and xanthan gum

The starch molecule consists of both amylose and amylopectin, which has been extensively evaluated and processed by HME for the encapsulation of bioactive

agents [85]. Depending on the amylose content, physical properties of starch differ in melt viscosity or die pressure. Chitosan is a linear hydrophilic polysaccharide composed of poly(D-glucosamine), which is derived from chitine, the main component of the exoskeletons of species like shrimps and crabs. The driving force in the application of chitosan originates from its biocompatibility, biodegradability and non-toxicity. This polymer has been studied in a variety of controlled release dosage forms. High molecular weight chitosans have been used as matrix tablet retardants, while low molecular weight chitosans have been used as drug release enhancers for poorly water-soluble drugs. Xanthan gum is hetero-polysaccharide composed of β-D-glucose, mannose and glucuronic acid and it has been used as an excipients in controlled release applications. The use of chitosan and xanthan gum as a matrix in HME oral formulation for sustained release has been previously described [86,87]. The use of these and other polysaccharides in HME has been recently reviewed by Wolf [88].

4. Application of HME in drug delivery

Over the past decades the interest in HME as a tool to produce drug delivery devices has increased. HME using commercially available dies results in the formation of strands or films, which can be cut to the required size [89]. However, the extrusion process may be executed using specific downstream equipment setups, which produce a wide array of specific dosage forms including pellets, granules, spheres, immediate and controlled release tablets [84,90,91] oral fast dissolving films, transdermal and transmucosal DDS's [92] implants [47,74,75,93,94] stents and ophthalmic inserts [95]. The end products made by HME can be adminstered orally or parenterally via subcutaneous or intra-muscular injection, with the use of customized applicators [96] (**Figure 4**).

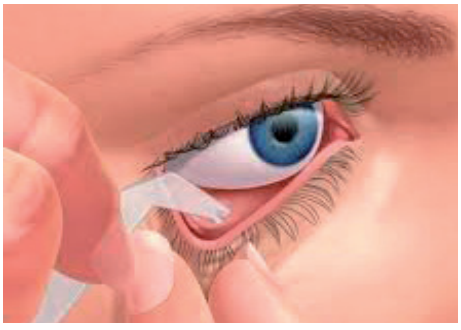
a)  b) 

Figure 4 a) An example of an ocular insert (3.5 mm) and its application b) a contraceptive implant (4 cm) and its applicator for subcutaneous administration (downloaded from internet site: a) http://www.lacrisert.com/using.php and b) http://drpinna.com/contraceptive-alert-unwanted-pregnancies-14455 on 22.05.2014).

4. 1. Oral drug delivery

HME has proved its potential in producing various solid oral dosage forms for immediate or sustained release using water-soluble polymers. Furthermore, HME has been used to mask the bitter taste of API [97–99].

4. 1. 1. Immediate / Enhanced oral drug delivery

Immediate release formulations can be in form of effervescent granules, rapid release granules, orally disintegrating tablets and have been thoroughly reviewed by Repka et al. [100]. Further, HME technology has been widely applied to increase dissolution rate and thereby the bioavailability of poorly water soluble drugs after oral administration by dispersing the drug in a matrix of a highly water soluble polymer [101–105]. These so-called solid dispersions are two or more component systems in which the drug is molecularly dispersed or dispersed as nanoparticles in the crystalline or amorphous state in a hydrophilic carrier [99,106]. Suitable polymeric carries are PVP, PVP-VA, PEG, HPMC, polymethacrylate derivatives and a polyvinyl caprolactam – polyvinyl acetate – polyethylene glycol graft copolymer (Soluplus®). PVP has been formulated by HME into DDS's for numerous poorly water-soluble drug substances among which indomethacin, nifedipin, lacidipine, tolbutamide [107,108]. Also Copovidone® has been used for the production of solid dispersions by HME [109] to improve the bioavailability of many poorly water-soluble drugs [110,111]. Verreck et al. prepared amorphous solid dispersion by HME to increase the dissolution rate and thereby the oral bioavailability of the itraconazole dispersed in HPMC [112,113]. An extensive review addressing melt extrusion for the preparation of solid dispersions has been recently published [6].

4. 1. 2. Sustained and targeted oral drug delivery

Besides for the production of DDS with enhanced drug release, HME can also be used to prepare products based on hydrophilic polymers for slow or targeted release in the gastro-intestinal tract. EVA copolymers or Eudragit® polymers are widely used in applications for intestinal targeting, due to their good gastric-resistance [71]. The drug release from the water-soluble tablet matrices depends largely on factors such as the erosion rate of the matrix system (for drugs with poor aqueous solubility), the infiltration rate of medium into the matrix (for drugs with reasonable aqueous solubility), drug loading, drug molecular size and solubility and the presence of the disintegration agents [114]. Fukuda et al. investigated the influence of sodium bicarbonate on the physicochemical and the floating properties of controlled release hot melt extruded tablets containing different Eudragit® polymers as release

retardants. The authors showed that the drug release rate from floating tablets was controlled by the incorporation of Eudragit® E PO into the matrix tablet and the diameter of the die used for the extrusion process [71]. Verhoeven et al. prepared sustained release mini-matrices using ethyl-cellulose as a sustained-release agent and different concentrations and molecular weights of the hydrophilic polymers PEG or PEO as drug-release enhancers. It has been shown that the release rate of a model drug was faster with higher concentrations of hydrophilic polymers, irrespective of the polymer molecular weight, while the influence of the molecular weight was dependent on the polymer concentration [115]. Next to hydrophilic polymers as release enhancers, citric acid has been used as an acidifying agent in solid oral dosage forms and it has been shown to increase dissolution rate of the poorly soluble drug diltiazem hydrochloride [116].

4. 2. Parenteral drug delivery

Next to the oral delivery, HME has been successfully investigated for parenteral delivery via subcutaneous, intra-muscular, intra-ocular or intraosseous administration. A large variety of different drug release profiles from depot DDS have been reported. Drug release kinetics largely depends on many aspects, e.g. polymer characteristics like composition, degradation behavior, molecular weight, crystallinity, swellability in water, nature of the encapsulated substance (hydrophilic/hydrophobic), drug load and presence of additives. Additionally, size, porosity, shape and density of the device along with the composition affect drug release from the DDS. The effect of some of these factors has been reviewed by Fredenberg et al. [117].

Biodegradable water-insoluble polymers as PLA, PGA, PLGA or copolymers of ε-caprolactone and L,D lactide or glycolide [41,42,44–49,118] and further poly(ortho esters), polyanhydrides, polyurethanes have been extensively investigated for the preparation of depot formulations by HME.

Encapsulation of proteins into PLGA polymers was extensively investigated, showing that lysozyme could be completely recovered from implants in an active form and that complete release could be achieved [45,119]. In contrast, in another study of the same group, bovine serum albumin (BSA) encapsulated into PLGA implants by HME resulted in an incomplete release. Ghalanbor et al. revealed that the reason for an incomplete release of BSA from implants was acylation of BSA by thioester formation [120]. A complete and faster release was achieved by increasing the porosity and loading of implants [120] or by the incorporation of plasticizers, which increased the free-volume of the polymer [47]. An extensive review about the mechanisms of drug release from PLGA based drug-delivery systems has been

published by Fredenberg et al. [117]. Stanković et al. prepared implants by low temperature extrusion (55 °C), using a novel multiblock copolymer composed of PCL in the semi-crystalline block and PCL-PEG in the amorphous block (PCL-PEG/PCL). The authors have shown that lysozyme can be encapsulated into these polymer implants with preserved integrity during extrusion and during 180 days of release [75]. Furthermore, it was shown that factors such as protein particle size and drug loading are highly important in controlling the release rate of the protein from the polymer matrix. In another study, the authors showed that the peptide/protein release rate from the same multiblock copolymer implants appeared to be highly dependent on both the protein molecular weight and PEG content of the polymer [74]. In the study of Rosenberg et al., the release of hydrophilic low molecular weight compounds (i.e. nicotine and caffeine) from PCL occurred by slow diffusion through the polymer matrix and water filled pores and was largely influenced by the drug load [121]. The mechanism of drug release from poly(D,L-lactide) polymer was found to be temperature dependent. At temperatures below the *Tg* of the DDS, the degradation of the polymer was restricted to the surface and the drug release was governed by surface erosion, while at temperatures above the *Tg,* the polymer degradation was a bulk process and the release was diffusion controlled [122].

As previously mentioned, copolymerization with PEG offers the possibility to modify the polymer properties and influence the release of encapsulated API. Li et al. used HME to prepare implants based on PLA / PEG-poly(propylene glycol) -PEG copolymers with controlled release of dexamethasone [123]. In another study, it has been shown that changing the composition of the PEG/PCL blends could effectively alter the release of praziquantel from polymer implants prepared by HME [44]. Septacin™ is the polyanhydride based implant prepared by HME for the local delivery of gentamicin to the infected bone. Septacin™ has been shown effective in delivering high doses of gentamicin to the infected sites while keeping the systemic exposure to the minimum [124]. It has also been shown that the release of gentamicin from this implant was largely affected by the storage temperature and the storage time. It was found that storage temperatures below 0°C were required for long-term stability of the implant. For implants stored for longer periods at higher temperatures the drug released substantially slower than from non-aged samples. Poly(ortho esters) have been successfully processed by HME and allowed sustained release of the model protein BSA during 60 days [125]. It has been found that the release from poly(ortho) esters is also governed by polymer degradation and erosion kinetics.

Non-biodegradable water-insoluble polymers can be processed into reservoir type devices (usually by co-extrusion) and matrix type devices (conventional HME), which can be used for local administration in form of transdermal patches, vaginal

rings or implants. In reservoir-type devices, the drug is incorporated into the bulk of a polymer that is covered by a thin layer of another polymer that acts as a permeable membrane. The release rate from these devices can be relatively constant, driven by dissolution of drug into the bulk polymer followed by the diffusion through the membrane polymer and depends mainly on the thickness and permeability of the polymeric membrane [126]. To achieve the constant release, the diffusion rate of the drug in the bulk polymer should be higher than that in the membrane polymer. Matrix-type devices can be used for long-term drug release, where drug release is driven by diffusion which depends on the concentration gradient, diffusion pathway and degree of swelling [58,127]. Since it is often challenging to achieve controlled-release from matrix type of devices, the rate-limiting membrane can be added, yielding reservoir–type devices. The release mechanisms from non-degradable polymers has been reviewed by Yao and Kao [58]. A contraceptive non-degradable water-insoluble implant: Implanon® is an example of the reservoir type of device, containing etonogestrel embedded in EVA bulk and covered by a thin-layer EVA membrane [128].

Sustained drug release in the eye has been achieved using both biodegradable (PLA, PGA, PLGA) [95,129] and non-biodegradable polymers (PVA) [130]. However, the major drawback for the use non-degradable type of intraocular devices is the need to remove the polymer after the complete release has been achieved. Furthermore, serious complications as cataract, vitreous hemorrhage, retinal detachment or hypotony were observed with implantation of this kind of devices, which requires eye-surgery [131,132].

4. 3. Transmucosal, transdermal, transungual drug delivery

The application of HME for preparation of transdermal, transmucosal and transungual DDS is gaining considerable attention in the last years. Melt extrusion process can overcome the disadvantages of the solvent casting method as presence of the residual organic solvents, long processing times, batch waste etc. Polymers such as HPC, PEO and their blends have extensively been investigated for these formulations. The application of HME in the DDS for these applications has been reviewed by Repka et al. [4].

Further, HME has been used for preparation of both reservoir and matrix type of devices for **intravaginal drug delivery** involving EVA copolymers [94,133] and variety of polyurethanes [56,134,135].

4. 4. HME products on the market

A number of HME products have entered the market (**Table 1**). A few of them will be described here. **Ozurdex®** is an implant for intravitreal application, which has been approved by the FDA in 2009 for the treatment of macular edema and non-infectious uveitis. It consists of dexametasone embedded in a PLGA matrix and shows an offset of 2 months after application and effects that last longer than 3 months. The implant is inserted into the intravitreal region using a special applicator. **Lacrisert®** is also an ocular implant for daily application consisting of hydroxypropyl cellulose and acts as a moisturizer that is used to treat dry eye syndrome [136] **(Figure 4a)**. **Zoladex®**, subcutaneous implant containing goserelin acetate embedded in a PLGA matrix, has been developed to treat prostate cancer. Two strengths of this implant have been manufactured, with sustained release of goserelin in duration of 28 days and 12 weeks. Even though application of biodegradable polymers for implants is preferable, because they require no surgical removal, also non-biodegradable polymers have often been used for the sustained release purposes. E.g. in 2006, the FDA approved **Implanon®,** an implant applied subcutaneously consisting of the contraceptive etonogestrel incorporated in EVA. The sustained release from this implant could be controlled during 3 years, after which the implant requires surgical removal [137] (**Figure 4b**). As an alternative, the EVA based intravaginal ring, **NuvaRing®**, for the controlled release of the contraceptives etonogestrel and ethylvinyl estradiol over 21 days, has been developed, which obviously does not require surgical intervention to remove it.

Meltrex® technology has been developed with the aim to prepare solid dispersions with well defined controlled release characteristics in a single manufacturing process [138]. This technology has been used to embed drugs in the amorphous state into polymer matrices with adjustable release profiles. The single basic prerequisite of the polymer to be used in Meltrex® technology is its thermoplasticity, allowing many polymers to be processable by this technique, resulting in the formation of granules, tablets or thin films. A number of solid dispersions with a drug load ranging from 30% to 60% have been developed [138].

An overview of currently marketed drug products developed using HME is shown in the **Table 1**.

*Table 1. Currently marketed HME products. * Implant acts as a wetting agent.*

Name / Company/Drug	Indication, route of administration	Polymer	Duration of the release	Product size and shape	Reference
Lacrisert®, Valeant (no drug)*	- Dry eye syndrome - Ocular insertion	HPC	1 day	Rod-shaped implant 1.27 mm x 3.5 mm	[136]
Ozurdex®, Allergan (Dexametazone)	- Macular edema; uveitis - Ocular insertion	PLGA	3 months	Rod-shaped implant 0.46 mm x 6 mm	[139]
Zoladex®, AstraZeneca (Goserelin acetate)	- Prostate cancer - Subcutaneous insertion in the anterior abdominal wall below the navel line	PLGA	28 days or 12 weeks	Rod-shaped implant 1.2 mm x 10-12 mm or 1.5 mm x 16-18 mm	[140]
Implanon®, Merck (Etonogestrel)	- Contraceptive -Subcutaneous implantation in the inner side of the upper arm	EVA	3 years	Rod-shaped implant 2 mm x 40 mm	[137]
NuvaRing®, Merck (Etonogestrel/ Ethylvinyl estradiol)	- Contraceptive - Intravaginal ring	EVA	21 days	A ring with an outer diameter of 54 mm and a cross-sectional diameter of 4 mm.	[141]
Norvir®, Abbott (Ritonavir)	- Viral infection (HIV) - Oral tablet	PEG-glyceride	12 hours	Oval-shaped tablet	[142]
Kaletra®, Abbott (Lopinavir/ritonavir)	- Viral infection (HIV) - Oral tablet	PVP/PVA	6 hours	Oval-shaped tablet	[143]
Onmel®, Merz (Itraconazole)	- Onychomycosis - Oral tablet	HPMC	1 day	Oval-shaped tablet	[144]
Gris-PEG®, Pedinol (Griseofulvin)	- Onychomycosis - Oral tablet	PEG	1 day	Oval-shaped tablet	[145]
Covera- HS®, Pfizer (Verapamil HCL)	- Hypertension and angina pectoris - Oral tablet	HPC	24 hours	Round-shaped tablet	[146]
Nurofen (Meltlets lemon®), Reckitt Benckiser Healthcare (Ibuprofen)	- Analgetic - Oral tablet	HPMC	4 hours	Round-shaped tablet	[147]
Eucreas®, Novartis (Vildagliptin / Metformin HCL)	- Diabetes type 2 - Oral tablet	HPC	12 hours	Oval-shaped coated tablet	[148]
Zithromax®, Pfizer (Azithromycin enteric-coated multiparticulate prepared by HME and melt congealing)	- Bacterial infection - Oral tablet	Pregela-tinized starch	24 hours	Oval-shaped tablets	[149]

4. 5. Sterilization of parenteral HME products

Sterilization of DDS prepared from polymers intended for parenteral administration is not a straightforward process. The two major routes to obtain sterile products are sterilization after production and aseptic production. Sterilization of polymeric formulations is easier to control than production of the formulation under aseptic conditions, but still it is often very challenging. Polymers are usually susceptible to heat and moisture, making steam sterilization and dry- heat sterilization usually impossible. Polymeric formulations are often sterilized by gamma radiation, electron beams or X-rays. Nonetheless, radiation can degrade, cross-link or recombine polymer chains and therefore involves a compromise between inactivation of the potential contaminating microorganism and damage to the product being sterilized [150]. However, gamma irradiation has been proved useful for many pharmaceutical products [151]. Gasses such as ethylene oxide and propylene oxide can be also used to sterilize polymeric devices; however, the major problems associated with these agents relate to toxic residues within the polymer, which were found to be carcinogenic, mutagenic or cause allergic reactions. Additionally, these agents were found to decrease the polymer molecular weight and initial strength, thereby affecting the release characteristics of the formulation and handling properties and to chemically modify the encapsulated drugs in particular when these drugs are proteins or peptides [152,153].

Due to all these reasons, aseptic processing provides an interesting alternative to obtain sterile polymeric formulations. Moreover, since the process can be carried out under water-free conditions and the product itself contains no water. Polymers are usually highly soluble in a number of organic solvents and they can be sterilized by filtration. Since there is no flawless sterilization method for polymers, the selection of the technique should be based on the particular application, material, and encapsulated drug.

5. Characterization of HME products

After the production of the final dosage form by HME (and optionally further processing steps), it is of utmost importance to perform physicochemical characterization of the products. Many physicochemical techniques have been used to characterize polymers in the solid state. Additionally, an adequate combination of techniques should be used to determine the physicochemical state and structural integrity of the API and to provide relevant information for the development of the DDS.

5. 1. Determination of the API content uniformity and integrity

After preparation of drug-loaded implants, one of the established methods to confirm the drug content uniformity and structural integrity is to bring the drug in the dissolved state after which the obtained solution is analyzed. Normally, the drug-loaded implant is dissolved in a solvent or solvent mixture in which both the polymer and drug dissolve. As an alternative, the polymer can be extracted with a solvent in which the drug does not dissolve. The suspended undissolved drug is subsequently recovered by centrifugation or filtration and then dissolved in a suitable solvent. This method is in particular suitable when the API is a protein or a peptide incorporated in a water-insoluble polymer [45,75]. However, it has to be realized that exposure to an organic solvent could be detrimental to protein or peptide and proper control experiments should be performed to validate the method.

Many techniques can be used to monitor protein structural integrity after extraction from polymeric matrices. To name a few**, circular dichroism**, **fluorescence**, **optical rotation**, **UV**, **nuclear magnetic resonance spectroscopy (NMR)** can be used. These techniques are able to detect the signal differences between unfolded and folded state of proteins [154]. **High Performance Liquid Chromatography (HPLC)** is often used for quantitative determination of the drug concentration and identification of possible impurities, or degradation products; in case of macromolecules it can give a good indication of degradation or aggregation events, which can be further analyzed using size-exclusion chromatography. **Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)** can identify protein/peptide aggregation. **Infrared and Raman spectroscopy** are useful techniques to analyze secondary structures, while refractometry can detect protein-substrate interactions [154]. To monitor the protein activity, biological assays based on protein function are required. A good activity assay should be reproducible and sensitive enough to detect small changes in protein activity.

To quantify the API in the HME products while avoiding process steps that may be detrimental to vulnerable APIs like proteins, more and more research is devoted to the applicability of on-line and off-line analytical tools to measure the API content uniformity during or after the HME process. Recent studies showed the potential of Raman spectroscopy to quantitatively study on-line and off-line the protein content of HME products [155–157]. Further, **Raman spectroscopy** complemented by **Fourier Transformed Infrared (FTIR)** has been successfully used to study interactions between API's and polymers prepared by HME [158–160]. To investigate distribution of API's within the polymer matrix in the final dosage form **confocal Raman spectroscopy** and **confocal laser scanning microscopy** has been used [161,162]. Moreover, ATR-FTIR has been used to determine the distribution

of an API in a polymer matrix [163], to investigate solid-solid interactions between API and polymer [164] and to identify the presence of phase separation in the HME samples [163].

To quantitatively investigate the chemical composition of the polymer surface on an atomic level regarding chemical contamination or to identify the adsorption or binding of the API to the polymer surface, **X-ray photoelectron spectroscopy (XPS)** can be used [165,166]. Similarly, to analyze the chemical composition of the polymer surface and to obtain information about the presence of API on the polymer surface or to observe the distribution of the API in the polymer (by using cross-sections) **energy-dispersive X-ray analysis (EDX)** can be utilized [43,120,167].

5. 2. Determination of polymer / drug crystallinity and thermal properties of the hot melt extruded drug delivery systems

To characterize crystalline properties of HME dosage forms, **X ray powder diffraction (XRPD)** can be used. Provided that the fingerprints of API and carrier are not superimposed, both the degree of crystallinity of the drug, the polymer and, if applicable, other excipients following HME can be determined semi-quantitatively. Drawbacks of the XRPD technique are limited sensitivity and difficulties in identifying crystallinity changes of less than 10%. Further, **differential scanning calorimetry (DSC)** has been widely used to study the thermal properties of materials processed by HME. DSC can be used for both qualitative and quantitative detection of thermal events of the drug and polymer (e.g. melting point, *Tg*) to identify the amorphous / crystalline nature of the materials and to study drug/polymer interactions [168,169]. The absence of melting transitions in a DSC scan may indicate that the drug is present in the amorphous or molecularly dispersed state. The disadvantage of the DSC method is that it is a destructive technique. Furthermore, when different thermal events occur at a similar temperature, thermograms may be difficult to interpret as different signals overlap. In addition, when a crystalline drug is dispersed in a polymer with a *Tm* or *Tg* lower than the *Tm* of the drug the drug may dissolve in the polymer during the DSC run. As a consequence, the absence of a melting endotherm of the drug in the thermogram may lead to the erroneous conclusion that the drug was incorporated in the amorphous state [170]. Therefore, XRPD is a highly valuable complementary technique to DSC, because heating of the sample is avoided and can thus reveal significant information on the crystalline or amorphous nature of the drug.

Moreover, **hot stage microscopy (HSM)** can be used to visually follow thermal events as a function of temperature and time. HSM has been used to study the

dissolution process of drug particles in a molten polymer [169] and the dispersibility of an API in a polymer matrix [159,164]. Thus, HSM can be used to aid the design of an extrusion process. In addition, it can be used as a complementary technique to DSC. Further, to detect crystallinity in polymeric formulations, observe morphology, color, crystals and crystal habit, **polarized light microscopy (PLM)** is widely used in combination with several other analytical techniques. However, PLM provides qualitative rather than quantitative information. **^{13}C solid-state nuclear magnetic resonance (^{13}C-NMR)** has been used to investigate the crystallinity of materials and to distinguish between a molecularly dispersed drug and amorphous drug material, which cannot always be easily done with other above mentioned techniques [171].

5. 3. Determination of the extrudate morphology, surface properties and porosity

Microscopy is one of the most used methods to study to morphology of extrudates. **Light microscopy** gives information about particle size, particle morphology and crystallinity of the sample. ***Scanning electron microscopy*** (SEM) can be used to access the extent of aggregation of the particles before and after extrusion [82], the porosity of the extrudates immediately after preparation, and during in-vitro release and dissolution [69,75]. **Atomic force microscopy** (AFM) can be used to study the fine morphology and physicochemical properties of the material on a nanometer scale, and to elucidate the presence of amorphous or crystalline domains [173]. This technique delivers three-dimensional surface pictures and is able to image and manipulate atoms on a variety of surfaces.

The porosity of the HME products can be determined on the basis of the apparent and the true density of the material. Data such as total pore volume, total pore surface area, pore diameters, volume pore size distribution can be obtained using the methods such as **mercury porosimetry** [43], **gas picnometry** [174], **X ray tomography** [69], or physical adsorption of gas molecules on the solid surface and further calculating the amount of the gas adsorbed using the **Brunauer-Emmet-Teller (BET) theory** [174].

6. Conclusion and perspectives

During the last three decades, HME has offered a “platform” for the development of a variety of solid dosage forms covering oral, parenteral and topical applications and has thus found its place in research and manufacturing within the pharmaceutical industry. Successful products based on this technology have already been marketed

(table 1), while a number of other pharmaceutical formulations and medical devices are in the development pipeline. Next to the solvent-free nature of the technique, the relatively short residence time in the extruder, the simple scale-up and the option to operate it as a continuous process make the technology attractive. HME find its application in the production of dosage forms with improved bioavailability of poorly soluble drugs, taste masking, and products with controlled release. HME can also be combined with other production techniques such as injection molding, co-extrusion, melt granulation, formation or development of co-crystals or extrusion with supercritical fluids (foaming polymers). However, the development of production processes for pharmaceutical products using this technique requires comprehensive knowledge in the material science field as well as in the process-engineering field.

The major drawbacks of the HME are the high shear forces and temperatures that occur during the process. Therefore, even though the research in the last decades is showing some promising results in the encapsulation of peptides and proteins using HME, it still remains challenging to readily use this process to encapsulate thermo-labile compounds as well as complex macromolecular compounds. Hence, the future development should focus in the direction of overcoming the drawbacks of the high-energy input required for the extrusion by involving the process engineering in the equipment design and manufacturing. Alongside, innovations in the formulation science and polymer chemistry, further developments of PAT tools and equipment will ensure HME an important front place in pharmaceutical manufacturing.

References

[1] H.H. Görtz, R. Klimesch, K. Lämmerhirt, S. Lang, A. Sanner, R. Spengler, Process for the preparation of solid pharmaceutical forms, EP 0240904 B1, 1992.

[2] J. Breitenbach, Melt extrusion: from process to drug delivery technology., Eur. J. Pharm. Biopharm. 54 (2002) 107–117.

[3] M.A. Repka, S.K. Battu, S.B. Upadhye, S. Thumma, M.M. Crowley, F. Zhang, et al., Pharmaceutical applications of hot-melt extrusion: Part II, Drug Dev. Ind. Pharm. 33 (2007) 1043–1057.

[4] M.A. Repka, S. Majumdar, S.K. Battu, R. Srirangam, S.B. Upadhye, Applications of hot-melt extrusion in drug delivery, Expert Opin. Drug Deliv. 5 (2008) 1357–1376.

[5] M.A. Repka, S. Shah, J. Lu, S. Maddineni, J. Morott, K. Patwardhan, et al., Melt extrusion: process to product, Expert Opin. Drug Deliv. 9 (2012) 105–125.

[6] S. Shah, S. Maddineni, J. Lu, M. a Repka, Melt extrusion with poorly soluble drugs., Int. J. Pharm. 453 (2013) 233–252.

[7] D. Djuric, P. Kleinebudde, Impact of Screw Elements on Continuous Granulation With a Twin-Screw Extruder, J. Pharm. Sci. 97 (2008) 4934–4942.

[8] D. Treffer, P. Wahl, D. Markl, G. Koscher, E. Roblegg, J.G. Khinast, Hot Melt Extrusion as a Continuous Pharmaceutical Manufacturing Process, in: M.A. Repka, N. Langley, J. DiNunzio (Eds.), Melt Extrus. Mater. Technol. Drug Prod. Des., Springer New York, 2013: pp. 363–396.

[9] D. Sauer, W. Zheng, L.B. Coots, J.W. McGinity, Influence of processing parameters and formulation factors on the drug release from tablets powder-coated with Eudragit L 100-55, Eur. J. Pharm. Biopharm. 67 (2007) 464–475.

[10] M. Fukuda, D.A. Miller, N.A. Peppas, J.W. McGinity, Influence of sulfobutyl ether beta-cyclodextrin (Captisol) on the dissolution properties of a poorly soluble drug from extrudates prepared by hot-melt extrusion., Int. J. Pharm. 350 (2008) 188–196.

[11] M. Sauceau, J. Fages, A. Common, C. Nikitine, E. Rodier, New challenges in polymer foaming: A review of extrusion processes assisted by supercritical carbon dioxide, Prog. Polym. Sci. 36 (2011) 749–766.

[12] S. Singhal, V.K. Lohar, V. Arora, Hot Melt Extrusion Technique, WebmedCentral Pharm. Sci. WMC001459. 2 (2011) 1–20.

[13] H.F.J. Giles, J.R.J. Wagner, M.M.I. Eldrige, Extrusion : The Definitive Processing Guide, William Andrew, New York, 2005.

[14] L. Liu, T.Z. Jin, D.R. Coffin, K.B. Hicks, Preparation of antimicrobial membranes: coextrusion of poly(lactic acid) and Nisaplin in the presence of Plasticizers., J. Agric. Food Chem. 57 (2009) 8392–8398.

[15] L. Dierickx, J.P. Remon, C. Vervaet, Co-extrusion as manufacturing technique for multilayer mini-matrices with dual drug release., Eur. J. Pharm. Biopharm. 85 (2013) 1157–1163.

[16] V. Taubner, R. Shishoo, Influence of Processing Parameters on the Degradation of Poly(L-lactide) During Extrusion, J. Appl. Polym. Sci. 79 (2001) 2128–2135.

[17] C. Rauwendaal, Understanding Extrusion, 2nd ed., Hanser publications, Cincinnati, Cincinnati, 2010.

[18] D.C. Hinz, Process analytical technologies in the pharmaceutical industry: the FDA's PAT initiative., Anal. Bioanal. Chem. 384 (2006) 1036–1042.

[19] J. a. Covas, O.S. Carneiro, P. Costa, A.V. Machado, J.M. Maia, Online monitoring techniques for studying evolution of physical, rheological and chemical effects along the extruder, Plast. Rubber Compos. 33 (2004) 55–61.

[20] J.A. Covas, J.M. Maia, A.V. Machado, P. Costa, On-line rotational rheometry for extrusion and compounding operations, J. Nonnewton. Fluid Mech. 148 (2008) 88–96.

[21] P.D. Coates, S.E. Barnes, M.G. Sibley, E.C. Brown, H.G.M. Edwards, I.J. Scowen, In-process vibrational spectroscopy and ultrasound measurements in polymer melt extrusion, Polymer (Guildf). 44 (2003) 5937–5949.

[22] S.E. Barnes, M.G. Sibley, H.G.M. Edwards, P.D. Coates, Process monitoring of polymer melts using in-line spectroscopy, Trans. Inst. Meas. Control. 29 (2007) 453–465.

[23] V.S. Tumuluri, M.S. Kemper, I.R. Lewis, S. Prodduturi, S. Majumdar, B. a Avery, et al., Off-line and on-line measurements of drug-loaded hot-melt extruded films using Raman spectroscopy., Int. J. Pharm. 357 (2008) 77–84.

[24] S.V.S. Tumuluri, S. Prodduturi, M.M. Crowley, S.P. Stodghill, J.W. McGinity, M.A. Repka, et al., The use of Near-Infrared Spectroscopy for the Quantification of a Drug in Hot-Melt extruded films, Drug Dev. Ind. Pharm. 30 (2004) 505–511.

[25] A. Gryczke, Hot-Melt Extrusion Process Design Using Process Analytical Technology, in: M.A. Repka, N. Langley, J. Dinunzio (Eds.), Melt Extrus. Mater. Technol. Drug Prod. Des., Springer, 2013: pp. 397–431.

[26] M. Watari, A Review of Online Real-Time Process Analyses of Melt-State Polymer Using the Near-Infrared Spectroscopy and Chemometrics, Appl. Spectrosc. Rev. 49 (2014) 462–491.

[27] K. Kolter, M. Karl, A. Gryczke, Hot-Melt Extrusion with BASF Pharma polymers. Extrusion Compendium 2nd Revised and Enlarged Edition, 2nd ed., BASF SE Pharma Ingredients & Services 67056 Ludwigshafen, Germany October, 2012.

[28] G. Sax, G. Winter, Mechanistic studies on the release of lysozyme from twin-screw extruded lipid implants, J. Control. Release. 163 (2012) 187–194.

[29] S.M. Aharoni, Increased Glass Transition Temperature in Motionally Constrained Semicrystalline Polymers, Polym. Adv. Technol. 9 (1998) 169–201.

[30] S. Madan, S. Madan, Hot melt extrusion and its pharmaceutical applications, Asian J. Pharm. Sci. 7 (2012) 123–133.

[31] S. Sejal, M.A. Repka, Melt Extrusion in drug delivery: three decades of progress, in: M.A. Repka, N. Langley, J. DiNunzio (Eds.), Melt Extrus. Mater. Technol. Drug Prod. Des., Springer New York, New York, NY, 2013.

[32] M. Vert, Aliphatic Polyesters : Great Degradable Polymers That Cannot Do Everything, Biomacromolecules. 6 (2005) 538–546.

[33] J.H. Park, M. Ye, K. Park, Biodegradable polymers for microencapsulation of drugs., Molecules. 10 (2005) 146–161.

[34] N. Saito, N. Murakami, J. Takahashi, H. Horiuchi, H. Ota, H. Kato, et al., Synthetic biodegradable polymers as drug delivery systems for bone morphogenetic proteins., Adv. Drug Deliv. Rev. 57 (2005) 1037–1048.

[35] A.-C. Albertsson, I.K. Varma, Aliphatic Polyesters : Synthesis , Properties and Applications, Adv. Polym. Sci. 157 (2002) 1–40.

[36] T.A. Holland, A.G. Mikos, Review: Biodegradable Polymeric Scaffolds. Improvements in Bone Tissue Engineering through Controlled Drug Delivery, Adv. Biochem. Eng. Biotechnol. 102 (2006) 161–185.

[37] R.A. Jain, C.T. Rhodes, A.M. Railkar, A.W. Malick, N.H. Shah, Comparison of various injectable protein-loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices: in-situ-formed implant versus in-situ-formed microspheres versus isolated microspheres, Pharm Dev Technol. 5 (2000) 201–207.

[38] M. Labet, W. Thielemans, Synthesis of polycaprolactone: a review., Chem. Soc. Rev. 38 (2009) 3484–3504.

[39] H. Seyednejad, A.H. Ghassemi, C.F. Van Nostrum, T. Vermonden, W.E. Hennink, Functional aliphatic polyesters for biomedical and pharmaceutical applications, J. Control. Release. 152 (2011) 168–176.

[40] Z.Wei, Y. Fenqyun, G. Chen, C. Qu, P.Wang, W.Zhang et al., Nonisothermal crystallization and melting behavior of poly(ε-caprolactone)-b-poly(ethylene glycol)-b-poly(ε-caprolactone) by DSC analysis, J. Appl. Pol. Sci, 114 (2009) 1133-1140..

[41] R. Bhardwaj, J. Blanchard, In vitro characterization and in vivo release profile of a poly (D, L-lactide-co-glycolide) - based implant delivery system for the h -MSH analog , melanotan-I, 170 (1998) 109–117.

[42] P. Viitanen, E. Suokas, P. Tormala, N. Ashammakhi, Release of diclofenac sodium from polylactide-co-glycolide 80/20 rods, J Mater Sci Mater Med. 17 (2006) 1267–1274.

[43] S. Ozkan, D.M. Kalyon, X. Yu, C.A. McKelvey, M. Lowinger, Multifunctional protein-encapsulated polycaprolactone scaffolds: fabrication and in vitro assessment for tissue engineering, Biomaterials. 30 (2009) 4336–4347.

[44] L. Cheng, L. Lei, S. Guo, In vitro and in vivo evaluation of praziquantel loaded implants based on PEG/PCL blends, Int. J. Pharm. 387 (2010) 129–138.

[45] Z. Ghalanbor, M. Körber, R. Bodmeier, Improved Lysozyme Stability and Release Properties of Poly (lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion, Pharm. Res. 27 (2010) 371–379.

[46] M. Gosau, B.W. Muller, Release of gentamicin sulphate from biodegradable PLGA-implants produced by hot melt extrusion, Pharmazie. 65 (2010) 487–492.

[47] Z. Ghalanbor, M. Körber, R. Bodmeier, Interdependency of protein-release completeness and polymer degradation in PLGA-based implants., Eur. J. Pharm. Biopharm. 85 (2013), 624-630.

[48] K. Rajagopal, J. Wood, B. Tran, T.W. Patapoff, T. Nivaggioli, Trehalose Limits BSA Aggregation in Spray-Dried Formulations at High Temperatures: Implications in Preparing Polymer Implants for Long-Term Protein Delivery, J. Pharm. Sci. 102 (2013) 2655–2666.

[49] Y. Lemmouchi, E. Schacht, P. Kageruka, R. De Deken, B. Diarra, O. Diall, et al., Biodegradable polyesters for controlled release of trypanocidal drugs: in vitro and in vivo studies., Biomaterials. 19 (1998) 1827–1837.

[50] M. Okada, Chemical syntheses of biodegradable polymers, Prog. Polym. Sci. 27 (2002) 87–133.

[51] H. Tian, Z. Tang, X. Zhuang, X. Chen, X. Jing, Progress in Polymer Science Biodegradable synthetic polymers : Preparation , functionalization and biomedical application, Prog. Polym. Sci. 37 (2012) 237–280.

[52] A. Göpferich, Polymer Bulk Erosion, Macromolecules. 9297 (1997) 2598–2604.

[53] J. Heller, J. Barr, Poly(ortho esters)--from concept to reality., Biomacromolecules. 5 (2004) 1625–1632.

[54] J. Heller, J. Barr, S.Y. Ng, H.R. Shen, K. Schwach-Abdellaoui, S. Einmahl, et al., Poly(ortho esters) - their development and some recent applications., Eur. J. Pharm. Biopharm. 50 (2000) 121–128.

[55] L.S. Nair, C.T. Laurencin, Biodegradable polymers as biomaterials, Prog. Polym. Sci. 32 (2007) 762–798.

[56] T.J. Johnson, K.M. Gupta, J. Fabian, T.H. Albright, P.F. Kiser, Segmented polyurethane intravaginal rings for the sustained combined delivery of antiretroviral agents dapivirine and tenofovir., Eur. J. Pharm. Sci. 39 (2010) 203–212.

[57] M.R. Clark, T.J. Johnson, R.T. Mccabe, J.T. Clark, A. Tuitupou, H. Elgendy, et al., A Hot-Melt Extruded Intravaginal Ring for the Sustained Delivery of the Antiretroviral Microbicide UC781, J. Pharm. Sci. 101 (2012) 576–587.

[58] F. Yao, W.J. Kao, Drug Release Kinetics and Transport Mechanisms of Nondegradable and Degradable Polymeric Delivery Systems, Expert Opin Drug Deliv. 7 (2011) 429–444.

[59] Q. Guo, P.T. Knight, P.T. Mather, Tailored drug release from biodegradable stent coatings based on hybrid polyurethanes., J. Control. Release. 137 (2009) 224–233.

[60] C.L. Stevenson, F. Theeuwes, J.C. Wright, Osmotic implantable delivery systems, in: D. Wise (Ed.), Handb. Pharm. Control. Release Technol., Dekker, New York, New York, 2000: pp. 225–254.

[61] Q. Meng, J. Hu, Y. Zhu, Shape-Memory Polyurethane / Multiwalled Carbon Nanotube Fibers, J. Appl. Polym. Sci. 106 (2007) 837–848.

[62] B. Saad, T.D. Hirt, M. Welti, G.K. Uhlschmid, P. Neuenschwander, U.W. Suter, Development of degradable polyesterurethanes for medical applications: in vitro and in vivo evaluations., J. Biomed. Mater. Res. 36 (1997) 65–74.

[63] J. Pan, G. Li, Z. Chen, X. Chen, W. Zhu, K. Xu, Alternative block polyurethanes based on poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(ethylene glycol)., Biomaterials. 30 (2009) 2975–2984.

[64] J.P. Santerre, K. Woodhouse, G. Laroche, R.S. Labow, Understanding the biodegradation of polyurethanes: from classical implants to tissue engineering materials., Biomaterials. 26 (2005) 7457–7470.

[65] A. Göpferich, J. Tessmar, Polyanhydride degradation and erosion., Adv. Drug Deliv. Rev. 54 (2002) 911–931.

[66] K. Whitaker-Brothers, K. Uhrich, Poly(anhydride-ester) fibers: role of copolymer composition on hydrolytic degradation and mechanical properties., J. Biomed. Mater. Res. A. 70 (2004) 309–318.

[67] K. Kolter, Properties and Applications of Polyvinyllactam Polymers, in: M.A. Repka, N. Langley, J. DiNunzio (Eds.), Melt Extrus. Mater. Technol. Drug Prod. Des., Springer New York, 2013: pp. 83–105.

[68] B.H. Hardung, D. Djuric, S. Ali, Combining HME & Solubilization: Soluplus® - The Solid Solution, Drug Deliv. Technol. 10 (2010).

[69] A. Almeida, S. Possemiers, M.N. Boone, T. De Beer, T. Quinten, L. Van Hoorebeke, et al., Ethylene vinyl acetate as matrix for oral sustained release dosage forms produced via hot-melt extrusion., Eur. J. Pharm. Biopharm. 77 (2011) 297–305.

[70] C.R. Young, J.J. Koleng, J.W. McGinity, Production of spherical pellets by a hot-melt extrusion and spheronization process, Int. J. Pharm. 242 (2002) 87–92.

[71] M. Fukuda, N.A. Peppas, J.W. McGinity, Floating hot-melt extruded tablets for gastro-retentive controlled drug release system, J. Control. Release. 115 (2006) 121–129.

[72] Y. Zhu, N.H. Shah, A.W. Malick, M.H. Infeld, J.W. McGinity, Influence of thermal processing on the properties of chlorpheniramine maleate tablets containing an acrylic polymer, Pharm Dev Technol. 7 (2002) 481–489.

[73] C.R. Young, C. Dietzsch, M. Cerea, T. Farrell, K.A. Fegely, A. Rajabi-Siahboomi, et al., Physicochemical characterization and mechanisms of release of theophylline from melt-extruded dosage forms based on a methacrylic acid copolymer., Int. J. Pharm. 301 (2005) 112–120.

[74] M. Stanković, J. Tomar, C. Hiemstra, R. Steendam, H.W. Frijlink, W.L.J. Hinrichs, Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly (ε-caprolactone) multiblock-copolymer implants, Eur. J. Pharm. Biopharm. 87 (2014) 329–337.

[75] M. Stanković, H. De Waard, R. Steendam, C. Hiemstra, J. Zuidema, H.W. Frijlink, et al., Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery, Eur. J. Pharm. Sci. 49 (2013) 578–587.

[76] S.U. Schilling, J.W. McGinity, Novel application of hot-melt extrusion for the preparation of monolithic matrices containing enteric-coated particles, Int J Pharm. 400 (2010) 24–31.

[77] S.U. Schilling, N.H. Shah, A. Waseem Malick, J.W. McGinity, Properties of melt extruded enteric matrix pellets, Eur J Pharm Biopharm. 74 (2010) 352–361.

[78] M.A. Repka, T.G. Gerding, S.L. Repka, J.W. McGinity, Influence of plasticizers and drugs on the physical-mechanical properties of hydroxypropylcellulose films prepared by hot melt extrusion, Drug Dev. Ind. Pharm. 25 (1999) 625–633.

[79] E. Pinto, T. Düring, Cellulose Ethers for Extrusion Application, in: M.A. Repka, N. Langley, J. DiNunzio (Eds.), Melt Extrus. Mater. Technol. Drug Prod. Des., Springer New York, New York, NY, 2013: pp. 123–144.

[80] N.N. Mohammed, S. Majumdar, A. Singh, W. Deng, N.S. Murthy, E. Pinto, et al., Klucel™ EF and ELF polymers for immediate-release oral dosage forms prepared by melt extrusion technology., AAPS PharmSciTech. 13 (2012) 1158–1169.

[81] B. Rambali, G. Verreck, L. Baert, D.L. Massart, Itraconazole formulation studies of the melt-extrusion process with mixture design., Drug Dev. Ind. Pharm. 29 (2003) 641–652.

[82] D.A. Miller, J.T. McConville, W. Yang, R.O. Williams 3rd, J.W. McGinity, Hot-melt extrusion for enhanced delivery of drug particles, J. Pharm. Sci. 96 (2007) 361–376.

[83] M.A. Repka, K. Gutta, S. Prodduturi, M. Munjal, S.P. Stodghill, Characterization of cellulosic hot-melt extruded films containing lidocaine, Eur. J. Pharm. Biopharm. 59 (2005) 189–196.

[84] C. De Brabander, C. Vervaet, J.P. Remon, Development and evaluation of sustained release mini-matrices prepared via hot melt extrusion., J. Control. Release. 89 (2003) 235–247.

[85] D. Henrist, J.. P. Remon, Influence of the process parameters on the characteristics of starch based hot stage extrudates, Int. J. Pharm. 189 (1999) 7–17.

[86] M. Fukuda, N.A. Peppas, J.W. McGinity, Properties of sustained release hot-melt extruded tablets containing chitosan and xanthan gum, Int J Pharm. 310 (2006) 90–100.

[87] T. Phaechamud, G.C. Ritthidej, Sustained-release from Layered Matrix System Comprising Chitosan and Xanthan Gum, Drug Dev Ind Pharm. 33 (2008) 595–605.

[88] B. Wolf, Polysaccharide functionality through extrusion processing, Curr. Opin. Colloid Interface Sci. 15 (2010) 50–54.

[89] A.N. Ghebremeskel, L.T. Spada, Sustained release latanoprost implant, US 2012/0276186 A1, 2012.

[90] S.U. Schilling, H.L. Lirola, N.H. Shah, A. Waseem Malick, J.W. McGinity, Influence of plasticizer type and level on the properties of Eudragit S100 matrix pellets prepared by hot-melt extrusion, J Microencapsul. 27 (2010) 521–532.

[91] N.R. Trivedi, M.G. Rajan, J.R. Johnson, A.J. Shukla, Pharmaceutical Approaches to Preparing Pelletized Dosage Forms Using the Extrusion-Spheronization Process, Crit. Rev. Ther. Drug Carr. Syst. 24 (2007) 1–40.

[92] J.O. Morales, J.T. McConville, Manufacture and characterization of mucoadhesive buccal films., Eur. J. Pharm. Biopharm. 77 (2011) 187–199.

[93] A. Rothen-Weinhold, N. Oudry, K. Schwach-Abdellaoui, S. Frutiger-Hughes, G.J. Hughes, D. Jeannerat, et al., Formation of peptide impurities in polyester matrices during implant manufacturing, Eur. J. Pharm. Biopharm. 49 (2000) 253–257.

[94] J.A.H. van Laarhoven, M.A.B. Kruft, H. Vromans, In vitro release properties of etonogestrel and ethinyl estradiol from a contraceptive vaginal ring., Int. J. Pharm. 232 (2002) 163–173.

[95] S.S. Lee, P. Hughes, A.D. Ross, M.R. Robinson, Biodegradable implants for sustained drug release in the eye., Pharm. Res. 27 (2010) 2043–2053.

[96] W. C. Jeremy, B. Diane, Long acting injections and implants, Springer Netherlands, 2012.

[97] D. Douroumis, Practical approaches of taste masking technologies in oral solid forms., Expert Opin. Drug Deliv. 4 (2007) 417–426.

[98] M. Maniruzzaman, J.S. Boateng, M. Bonnefille, A. Aranyos, J.C. Mitchell, D. Douroumis, Taste masking of paracetamol by hot-melt extrusion: an in vitro and in vivo evaluation., Eur. J. Pharm. Biopharm. 80 (2012) 433–442.

[99] P. Srinarong, H. De Waard, H.W. Frijlink, Improved dissolution behavior of lipophilic drugs by solid dispersions : the production process as starting point for formulation considerations, Expert Opin Drug Deliv. 8 (2011) 1121–1140.

[100] M.A. Repka, S. Majumdar, S.K. Battu, R. Srirangam, S.B. Upadhye, S. Kumar Battu, Applications of hot-melt extrusion for drug delivery, Expert Opin Drug Deliv. 5 (2008) 1357–1376.

[101] C. Leuner, J. Dressman, Improving drug solubility for oral delivery using solid dispersions., Eur. J. Pharm. Biopharm. 50 (2000) 47–60.

[102] E. Reitz, C. Vervaet, R.H.H. Neubert, M. Thommes, Solid crystal suspensions containing griseofulvin - Preparation and bioavailability testing., Eur. J. Pharm. Biopharm. 83 (2012) 193–202.

[103] S. Weatherley, B. Mu, M.R. Thompson, P.J. Sheskey, K.P. O'Donnell, Hot-melt granulation in a twin screw extruder: effects of processing on formulations with caffeine and Ibuprofen., J. Pharm. Sci. 102 (2013) 4330–4336.

[104] J. Albers, R. Alles, K. Matthee, K. Knop, J.S. Nahrup, P. Kleinebudde, Mechanism of drug release from polymethacrylate-based extrudates and milled strands prepared by hot-melt extrusion, Eur. J. Pharm. Biopharm. 71 (2009) 387–394.

[105] A. Kalivoda, M. Fischbach, P. Kleinebudde, Application of mixtures of polymeric carriers for dissolution enhancement of fenofibrate using hot-melt extrusion., Int. J. Pharm. 429 (2012) 58–68.

[106] T. Vasconcelos, B. Sarmento, P. Costa, Solid dispersions as strategy to improve oral bioavailability of poor water soluble drugs., Drug Discov. Today. 12 (2007) 1068–1075.

[107] A. Forster, J. Hempenstall, I. Tucker, T. Rades, Selection of excipients for melt extrusion with two poorly water-soluble drugs by solubility parameter calculation and thermal analysis., Int. J. Pharm. 226 (2001) 147–161.

[108] A. Forster, J. Hempenstall, T. Rades, Characterization of glass solutions of poorly water-soluble drugs produced by melt extrusion with hydrophilic amorphous polymers, J. Pharm. Pharmacol. 53 (2001) 303–315.

[109] L.S. Ranzani, J. Font, F. Galimany, A. Santanach, A.M. Gomez-Gomar, G. Casadevall, et al., Enhanced in vivo absorption of CB-1 antagonist in rats via solid solutions prepared by hot-melt extrusion, Drug Dev Ind Pharm. 37 (2011) 694–701.

[110] F. Jijun, X. Lishuang, W. Xiaoli, Z. Shu, T. Xiaoguang, Z. Xingna, et al., Nimodipine (NM) tablets with high dissolution containing NM solid dispersions prepared by hot-melt extrusion, Drug Dev. Ind. Pharm. 37 (2011) 934–944.

[111] H. He, R. Yang, X. Tang, In vitro and in vivo evaluation of fenofibrate solid dispersion prepared by hot-melt extrusion, Drug Dev. Ind. Pharm. 36 (2010) 681–687.

[112] G. Verreck, K. Six, G. Van den Mooter, L. Baert, J. Peeters, M.E. Brewster, Characterization of solid dispersions of itraconazole and hydroxypropylmethylcellulose prepared by melt extrusion—part I, Int. J. Pharm. 251 (2003) 165–174.

[113] K. Six, H. Berghmans, C. Leuner, J. Dressman, K. Van Werde, J. Mullens, et al., Characterization of solid dispersions of itraconazole and hydroxypropylmethylcellulose prepared by melt extrusion, Part II., Pharm. Res. 20 (2003) 1047–1054.

[114] K. Tahara, K. Yamamoto, T. Nishihata, Overall mechanism behind matrix sustained release (SR) tablets prepared with hydroxypropyl methylcellulose 2910, J. Control. Release. 35 (1995) 59–66.

[115] E. Verhoeven, T.R. De Beer, E. Schacht, G. Van den Mooter, J.P. Remon, C. Vervaet, Influence of polyethylene glycol/polyethylene oxide on the release characteristics of sustained-release ethylcellulose mini-matrices produced by hot-melt extrusion: in vitro and in vivo evaluations, Eur J Pharm Biopharm. 72 (2009) 463–470.

[116] S.U. Schilling, C.D. Bruce, N.H. Shah, A.W. Malick, J.W. McGinity, Citric acid monohydrate as a release-modifying agent in melt extruded matrix tablets, Int. J. Pharm. 361 (2008) 158–168.

[117] S. Fredenberg, M. Wahlgren, M. Reslow, A. Axelsson, The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems--a review., Int. J. Pharm. 415 (2011) 34–52.

[118] S. Ozkan, D.M. Kalyon, X. Yu, C.A. McKelvey, M. Lowinger, Multifunctional protein-encapsulated polycaprolactone scaffolds: fabrication and in vitro assessment for tissue engineering, Biomaterials. 30 (2009) 4336–4347.

[119] Z. Ghalanbor, M. Körber, R. Bodmeier, Improved Lysozyme Stability and Release Properties of Poly (lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion, 27 (2010) 371-379.

[120] Z. Ghalanbor, M. Körber, R. Bodmeier, 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 (2012) 302–306.

[121] R. Rosenberg, W. Devenney, S. Siegel, N. Dan, Anomalous release of hydrophilic drugs from poly(e-caprolactone) matrices., Mol. Pharm. 4 (2007) 943–948.

[122] Y. Aso, S. Yoshioka, A. Li Wan Po, T. Terao, Effect of temperature on mechanisms of drug release and matrix degradation of poly(d,l-lactide) microspheres, J. Control. Release. 31 (1994) 33–39.

[123] D. Li, G. Guo, X. Deng, R. Fan, Q. Guo, M. Fan, et al., PLA/PEG-PPG-PEG/dexamethasone implant prepared by hot-melt extrusion for controlled release of immunosuppressive drug to implantable medical devices, Part 2: in vivo evaluation., Drug Deliv. 20 (2013) 134–142.

[124] L.C. Li, J. Deng, D. Stephens, Polyanhydride implant for antibiotic delivery - from the bench to the clinic., Adv. Drug Deliv. Rev. 54 (2002) 963–986.

[125] A. Rothen-Weinhold, K. Schwach-Abdellaoui, J. Barr, S.Y. Ng, H.R. Shen, R. Gurny, et al., Release of BSA from poly(ortho ester) extruded thin strands., J. Control. Release. 71 (2001) 31–37.

[126] L.K. Fung, W.M. Saltzman, Polymeric implants for cancer chemotherapy, Adv. Drug Deliv. Rev. 26 (1997) 209–230.

[127] J. Siepmann, F. Siepmann, Mathematical modeling of drug delivery., Int. J. Pharm. 364 (2008) 328–343.

[128] H. Croxatto, Progestin implants, Steroids. 65 (2000) 681–685.

[129] J.-G. Shiah, R. Bhagat, W.M. Blanda, T. Nivaggioli, L. Peng, D. Chou, et al., OCULAR IMPLANT MADE BY A DOUBLE EXTRUSION PROCESS, US 8,034,366 B2, 2011.

[130] J.L. Bourges, C. Bloquel, A. Thomas, F. Froussart, A. Bochot, F. Azan, et al., Intraocular implants for extended drug delivery: therapeutic applications., Adv. Drug Deliv. Rev. 58 (2006) 1182–1202.

[131] J.I. Lim, R.A. Wolitz, A.H. Dowling, H.R. Bloom, A.R. Irvine, D.M. Schwartz, Visual and anatomic outcomes associated with posterior segment complications after ganciclovir implant procedures in patients with AIDS and cytomegalovirus retinitis., Am. J. Ophthalmol. 127 (1999) 288–293.

[132] P.J. Kappel, A.C. Charonis, G.N. Holland, R. Narayanan, A.D. Kulkarni, F. Yu, et al., Outcomes associated with ganciclovir implants in patients with AIDS-related cytomegalovirus retinitis., Ophthalmology. 113 (2006) 683.e1–8.

[133] A. Loxley, M. Mitchnick, O. Okoh, J. McConnell, L. Goldman, C. Morgan, et al., Ethylene vinyl acetate intravaginal rings for the simultaneous delivery of the antiretroviral UC781 and contraceptive levonorgestrel, Drug Deliv. Transl. Res. 1 (2011) 247–255.

[134] K.M. Gupta, S.M. Pearce, A.E. Poursaid, H.A. Aliyar, P.A. Tresco, M.A. Mitchnik, et al., Polyurethane intravaginal ring for controlled delivery of dapivirine, a nonnucleoside reverse transcriptase inhibitor of HIV-1., J. Pharm. Sci. 97 (2008) 4228–4239.

[135] J.T. Clark, T.J. Johnson, M.R. Clark, J.S. Nebeker, J. Fabian, A.L. Tuitupou, et al., Quantitative evaluation of a hydrophilic matrix intravaginal ring for the sustained delivery of tenofovir., J. Control. Release. 163 (2012) 240–248.

[136] D. Darougar, S. Darougar, Lacrisert Merc & Co. US patent 4,343,787, 1982.

[137] A.P. Sam, Controlled release contraceptive devices: a status report, J. Control. Release. 22 (1992) 35–46.

[138] J. Breitenbach, J. Lewis, Two concepts, one Technology: controlled-release and solid dispersions with Meltrex, in: M.J. Rathbone, J. Hadgraft, M.S. Roberts, M.E. Lane (Eds.), Modif. Drug Release Technol., 2nd ed., Informa Healthcare, London, 2008: pp. 125–134.

[139] Allergan, Ozurdex implant- product characteristics, OZURDEX 700 Micrograms Intravitreal Implant Appl. (2010) 1–35.

[140] AstraZeneca, Zoladex (goserelin acetate implant 3.6mg) - prescribing information, (2010).

[141] J.A.H. van Laarhoven, M. a B. Kruft, H. Vromans, Effect of supersaturation and crystallization phenomena on the release properties of a controlled release device based on EVA copolymer, J Control Release. 82 (2002) 309–317.

[142] E.M. Sherman, J.G. Steinberg, Heat-stable ritonavir tablets: a new formulation of a pharmacokinetic enhancer for HIV., Expert Opin. Pharmacother. 12 (2011) 141–148.

[143] C.E. Klein, Y.L. Chiu, W. Awni, T. Zhu, R.S. Heuser, T. Doan, et al., The tablet formulation of lopinavir/ritonavir provides similar bioavailability to the soft-gelatin capsule formulation with less pharmacokinetic variability and diminished food effect, J. Acquir. Immune Defic. Syndr. 44 (2007) 401–410.

[144] L.E.C. Baert, G. Verreck, D. Thoné, Antifungal compositions with improved bioavailability. US Patent 6,509,038 B2, 2003.

[145] Gris-PEG®, (griseofulvin ultramicrosize) Tablets, USP 125 mg; 250 mg, Pedinol Pharmacal Inc. (n.d.) 3–7.

[146] Pfizer, COVERA-HS® Monograph (verapamil hydrochloride) Extended-Release Tablets, 2011.

[147] A. Gryczke, S. Schminke, M. Maniruzzaman, J. Beck, D. Douroumis, Development and evaluation of orally disintegrating tablets (ODTs) containing Ibuprofen granules prepared by hot melt extrusion, Colloids Surf. B. Biointerfaces. 86 (2011) 275–284.

[148] Y. Joshi, J. Kowalski, P. Lakshaman, Jay, E. Royce, Alan, W.-Q. Tong, M. Vasanthavada, Formulation comprising metformin and vildagliptin, WO/2007/041053, 2007.

[149] Pfizer, Zitromax® (azithromycin tablets)- labeling information, ZITHROMAX® (azithromycin Tablets). (2013) 1–35.

[150] E. Cottam, D.W.L. Hukins, K. Lee, C. Hewitt, M.J. Jenkins, Effect of sterilisation by gamma irradiation on the ability of polycaprolactone (PCL) to act as a scaffold material., Med. Eng. Phys. 31 (2009) 221–226.

[151] F. Hasanain, K. Guenther, W.M. Mullett, E. Craven, Gamma sterilization of pharmaceuticals - a review of the irradiation of excipients, active pharmaceutical ingredients, and final drug product formulations., PDA J. Pharm. Sci. Technol. 68 (2014) 113–137.

[152] P. Puolakkainen, J. Ranchalis, D. Strong, D. Twardzik, The effect of sterilization on transforming growth factor beta isolated from demineralized human bone, Transfusion. 33 (1993) 679–685.

[153] M. Jo Doherty, R.A.B. Mollan, D.J. Wilson, Effect of ethylene oxide sterilization on human demineralized bone, Biomaterials. 14 (1993) 994–998.

[154] W. Wang, Instability, stabilization, and formulation of liquid protein pharmaceuticals., 1999.

[155] L. Saerens, L. Dierickx, B. Lenain, C. Vervaet, J.P. Remon, T. De Beer, Raman spectroscopy for the in-line polymer-drug quantification and solid state characterization during a pharmaceutical hot-melt extrusion process., Eur. J. Pharm. Biopharm. 77 (2011) 158–163.

[156] L. Saerens, L. Dierickx, T. Quinten, P. Adriaensens, R. Carleer, C. Vervaet, et al., In-line NIR spectroscopy for the understanding of polymer-drug interaction during pharmaceutical hot-melt extrusion., Eur. J. Pharm. Biopharm. 81 (2012) 230–237.

[157] A. Almeida, L. Brabant, F. Siepmann, T. De Beer, W. Bouquet, L. Van Hoorebeke, et al., Sustained release from hot-melt extruded matrices based on ethylene vinyl acetate and polyethylene oxide., Eur. J. Pharm. Biopharm. 82 (2012) 526–533.

[158] A.M. Agrawal, M.S. Dudhedia, A.D. Patel, M.S. Raikes, Characterization and performance assessment of solid dispersions prepared by hot melt extrusion and spray drying process., Int. J. Pharm. 457 (2013) 71–81.

[159] L. Li, O. AbuBaker, Z.J. Shao, Characterization of poly(ethylene oxide) as a drug carrier in hot-melt extrusion, Drug Dev. Ind. Pharm. 32 (2006) 991–1002.

[160] G.P. Andrews, O. Abu-Diak, F. Kusmanto, P. Hornsby, Z. Hui, D.S. Jones, Physicochemical characterization and drug-release properties of celecoxib hot-melt extruded glass solutions, J. Pharm. Pharmacol. Pharmacol. 62 (2010) 1580–1590.

[161] J. Breitenbach, W. Schrof, J. Neumann, Confocal Raman-spectroscopy: analytical approach to solid dispersions and mapping of drugs, Pharm. Res. 16 (1999) 1109–1113.

[162] J.-B. Park, C.-Y. Kang, W.-S. Kang, H.-G. Choi, H.-K. Han, B.-J. Lee, New investigation of distribution imaging and content uniformity of very low dose drugs using hot-melt extrusion method., Int. J. Pharm. 458 (2013) 245–253.

[163] S. Qi, A. Gryczke, P. Belton, D.Q.M. Craig, Characterisation of solid dispersions of paracetamol and EUDRAGIT E prepared by hot-melt extrusion using thermal, microthermal and spectroscopic analysis., Int. J. Pharm. 354 (2008) 158–167.

[164] J. Djuris, I. Nikolakakis, S. Ibric, Z. Djuric, K. Kachrimanis, Preparation of carbamazepine-Soluplus solid dispersions by hot-melt extrusion, and prediction of drug-polymer miscibility by thermodynamic model fitting., Eur. J. Pharm. Biopharm. 84 (2013) 228–237.

[165] J. Chesko, J. Kazzaz, M. Ugozzoli, M. Singh, D.T. O'Hagan, C. Madden, et al., Characterization of antigens adsorbed to anionic PLG microparticles by XPS and TOF-SIMS, J. Pharm. Sci. 97 (2008) 1443–1453.

[166] J. Wan, Y. Yang, T. Chung, D. Tan, S. Ng, POE – PEG – POE triblock copolymeric microspheres containing protein II . Polymer erosion and protein release mechanism, J. Control. Release. 75 (2001) 129–141.

[167] K. Campbell, S. Qi, D.Q. Craig, T. McNally, Paracetamol-loaded poly(epsilon-caprolactone) layered silicate nanocomposites prepared using hot-melt extrusion, J. Pharm. Sci. 98 (2009) 4831–4843.

[168] M.A. Repka, S. Prodduturi, S.P. Stodghill, Production and characterization of hot-melt extruded films containing clotrimazole, Drug Dev. Ind. Pharm. 29 (2003) 757–765.

[169] Z. Guo, M. Lu, Y. Li, H. Pang, L. Lin, X. Liu, et al., The utilization of drug-polymer interactions for improving the chemical stability of hot-melt extruded solid dispersions., J. Pharm. Pharmacol. 66 (2014) 285–296.

[170] P. Srinarong, S. Kouwen, M.R. Visser, W.L.J. Hinrichs, H.W. Frijlink, Effect of drug-carrier interaction on the dissolution behavior of solid dispersion tablets, Pharm. Dev. Technol. 15 (2010) 460–468.

[171] S.J. Byard, S.L. Jackson, A. Smail, M. Bauer, D.C. Apperley, Studies on the crystallinity of a pharmaceutical development drug substance., J. Pharm. Sci. 94 (2005) 1321–1335.

[172] K.M. Shakesheff, M.C. Davies, C.J. Roberts, S.J.B. Tendler, A.G. Shard, A. Domb, In situ Atomic Force Microscopy Imaging of Polymer Degradation in an Aqueous Environment, Langmuir. 10 (1994) 4417–4419.

[173] S.N. Magonov, D.H. Reneker, Characterization of Polymer Surfaces With Atomic Force Microscopy, Annu. Rev. Mater. Sci. 27 (1997) 175–222.

[174] Z. Dong, A. Chatterji, H. Sandhu, D.S. Choi, H. Chokshi, N. Shah, Evaluation of solid state properties of solid dispersions prepared by hot-melt extrusion and solvent co-precipitation., Int. J. Pharm. 355 (2008) 141–149.

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Unraveling protein stabilization mechanisms: vitrification and water replacement in a glass transition temperature controlled system

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Biochimica et Biophysica Acta 2013, 1834(4): 763-769

Abstract

The aim of this study was to elucidate the role of the two main mechanisms used to explain the stabilization of proteins by sugar glasses during drying and subsequent storage: the vitrification and the water replacement theory. Although in literature protein stability is often attributed to either vitrification or water replacement, both mechanisms could play a role and they should be considered simultaneously. A model protein, alkaline phosphatase, was incorporated in either inulin or trehalose by spray drying. To study the storage stability at different glass transition temperatures, a buffer, which acts as a plasticizer, ammediol, was incorporated in the sugar glasses. At low glass transition temperatures (<50 ºC), the enzymatic activity of the protein strongly decreased during storage at 60 °C. Protein stability increased when the glass transition temperature was raised considerably above the storage temperature. This increased stability could be attributed to vitrification. A further increase of the glass transition temperature did not further improve stability. In conclusion, vitrification plays a dominant role in stabilization at glass transition temperatures up to 10 to 20 °C above storage temperature, depending on whether trehalose or inulin is used. On the other hand, the water replacement mechanism predominately determines stability at higher glass transition temperatures.

1. Introduction

Proteins are applied more and more as therapeutic agents. Unfortunately, many of these proteins are labile and need to be stabilized. Proteins can be stabilized by drying a sugar-containing solution of the protein, thereby incorporating the protein into a matrix of the sugar in the glassy state. Two mechanisms of stabilization have been described, namely the water replacement theory and the glass dynamics hypothesis or vitrification theory [1–3]. Although in literature protein stability is often attributed to either vitrification or water replacement, both could have an effect and should be considered simultaneously.

The water replacement theory states that in solution, the conformation of the protein is maintained by the interaction with water, mainly due to hydrogen bonding. Upon drying, this interaction is lost and replaced by hydrogen bonds between the protein and the sugar by which the protein structure is maintained upon drying [4]. To maximize the hydrogen bonding with the protein, the sugar molecules should closely fit the irregular surface of the protein and should thus be in the amorphous state (and not in the crystalline state). A closely related theory is the water entrapment theory, which states that rather than forming hydrogen bonds directly with the sugar, the protein is coupled to the amorphous sugar matrix through water molecules entrapped at the interface [5]. Although the water replacement theory and water entrapment theory describe fundamentally different mechanisms, the stabilization in both theories is mediated by hydrogen bonding. It is not within the aim of this study to discern between these two theories and therefore, further discussion regarding the water replacement theory could also hold true for the water entrapment theory, unless stated otherwise.

The second theory, the vitrification theory, dictates that the protein will be immobilized inside the sugar matrix, preventing translational molecular movements and thereby degradation. The translational molecular mobility within the sample is mainly determined by the thermodynamic state of the sugar. For an amorphous sugar, the translational molecular mobility is determined by the difference between the glass transition temperature and the storage temperature. Although the translational molecular mobility of an amorphous sugar in its glassy state is greatly reduced compared to a sugar in its rubbery state, it is not completely absent. As a rule of thumb, the glass transition temperature should be at least 50 °C above the storage temperature for the translational molecular mobility to become “insignificant over the lifetime of a typical pharmaceutical product” [6].

Although such a rule of thumb is useful for designing a stable protein formulation, further optimization requires more detailed knowledge of the importance of the glass

transition temperature for protein stabilization. Even though it is generally accepted that the glass transition temperature is important for the stability of the protein, there is little detailed overview of the relation between the stability and the glass transition temperature of a given system [7]. To investigate this, typical 3-component systems, consisting of a model protein (alkaline phosphatase), a buffer (ammediol), and either inulin or trehalose, were spray dried. These sugars were selected because trehalose is often considered as the gold standard in protein stabilization [8], while previous studies have shown that inulin also has good stabilizing properties [9–13]. Besides the fact that both sugars have a high glass transition temperature, both sugars are also hydrophilic, have good hydrogen bonding capacity, are non-toxic and have no reducing groups, which makes them excellent candidates for the stabilization of proteins [9]. Ammediol was chosen as a buffer because it is also a good glass former, just like the sugars. Due the low glass transition temperature of ammediol, it will act as a plasticizer [14]. This enables adjustment of the glass transition temperature, allowing the stability to be determined for a wide range of glass transition temperatures, which in turn enabled us to study different system mobilities, without changing the type of stabilizing sugar. In this study, we investigated whether this strategy can be used to study the different roles in protein stabilization of the water replacement and vitrification mechanism.

2. Materials and methods

2. 1. Materials

Alkaline phosphatase from bovine intestinal mucosa (10-30 Units/mg, ~160 kDa), bovine serum albumin, ammediol, and para-nitrophenylphosphate were obtained from Sigma-Aldrich Co. (St. Louis, Missouri). Magnesium chloride was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Trehalose was obtained from Cargill B.V. (Amsterdam, The Netherlands) and inulin with a degree of polymerization of 23 from Sensus (Roosendaal, The Netherlands). All experiments were performed with millipore water, type 1.

2. 2. Spray drying

Protein containing powders were produced by dissolving alkaline phosphatase at a concentration of 2.5 mg/mL in a 50 mM ammediol at pH 9.8. Either inulin or trehalose was added in varying concentrations to obtain a range of samples with different sugar/buffer mass ratios and thus with different glass transition temperatures. The solutions were spray dried with a B-290 spray dryer (Büchi Labortechnik AG, Flawil,

Switzerland). The inlet air temperature was set at 100 °C, the aspirator at 100 %, pump speed at 7 %, and atomizing airflow at 50 mm. These settings were chosen such, that the outlet temperature (around 58 - 64 °C) was similar to the storage temperature. Samples were stored in a desiccator over silica for one night at room temperature and then immediately analyzed or stored at 60 °C for various periods of time. To minimize the moisture content of the samples, open vials were used during storage, while the relative humidity varied between 4.5 % and 6 %. The composition of the different dried powders is shown in **Table 1**.

Table 1. Stabilized protein sample compositions with varying glass transition temperature.

	Component mass fractions							
Protein/ sugar mass ratio	**1/1**	**1/3**	**1/5**	**1/7**	**1/10**	**1/12**	**1/15**	**1/20**
Sugar	0.24	0.49	0.62	0.69	0.76	0.79	0.83	0.87
Protein	0.24	0.16	0.12	0.10	0.08	0.07	0.06	0.04
Buffer	0.51	0.34	0.26	0.21	0.16	0.14	0.12	0.09

2. 3. Differential Scanning Calorimetry (DSC)

Modulated DSC measurements were done with a Q2000 and DSC 2920 differential scanning calorimeter (TA Instruments, New Castle, United States). Dry samples were measured after spray drying, while humidified inulin and trehalose sugar glasses were prepared by storing the samples at a relative humidity of 22 %, 33 %, and 52 % in a desiccator over a saturated aqueous solution of CH_3COOK, $MgCl_2 \cdot 6H_2O$, and $Na_2Cr_2O_7 \cdot 2H_2O$, respectively, or at 45%, and 60 % in a climate chamber for 1 - 3 weeks. Dry samples were weighed in open aluminum pans at ambient conditions and then preheated at 70 °C for 5 min to remove any moisture, after which scanning was performed at 2.5 °C/min modulated with a period of 40 s and amplitude of 0.8 °C. Humidified samples were weighed in closed aluminum pans, then cooled to -20 °C, and finally heated at a rate of 2 °C/min with a modulation period of 60 s and amplitude of 0.316 °C. The glass transition temperature was taken as the inflection point of the transition

2. 4. Enzymatic Activity of Alkaline Phosphatase

The enzymatic activity of alkaline phosphatase was determined using a kinetic assay based on a method described by Eriksson et al. [14]. The sample was reconstituted in water to a protein concentration of about 0.03 mg/mL, of which 20 µL

was transferred in triplicate to a BSA coated 96 wells plate, together with 190 µL 50 mM ammediol with 0.1 mg/mL MgCl2. After warming the solution to 37 °C, 20 µL of a 5 mg/mL p-NPP solution was added to start the reaction. The absorption at a wavelength of 415 nm was measured on a Benchmark plate reader (BioRad, Hercules, California). Samples were mixed for 15 s and measured every 30 s for 5.5 min. To calculate the remaining activity, the rate of conversion was compared to a standard of unprocessed alkaline phosphatase.

2. 5. Dynamic Vapor Sorption (DVS) analysis

The water sorption isotherms of different powders were measured at ambient pressure and 25 °C using a DVS-1000 water sorption instrument (Surface Measurement Systems Limited, London, UK). To estimate the glass transition temperature of protein containing samples, the moisture content of protein/ammediol containing powders was determined at a relative humidity of 4.5 % or 6.0 %, dependent on the conditions during the stability study, for samples with an initial mass of approximately 35 mg. To determine the influence of water on the glass transition temperature of sugar glasses, the moisture content of humidified sugar glasses without protein/ammediol was measured at relative humidity's of 0 – 90 % with steps of 10 %, for samples with an initial mass of around 10 mg. After subjecting the samples to the specified humidity equilibrium was assumed when the change in mass was less than 0.9 µg during 10 min.

3. Results

3. 1. Glass transition temperature

In an attempt to determine the glass transition temperature by DSC, it was found that alkaline phosphatase interfered with the measurements. DSC measurements performed with different modulations or higher scan rates of up to 20 °C/min, did not improve the result. Therefore, the glass transition temperature of the sugar/buffer matrix, without the protein, was used as the glass transition temperature of the sample, thereby neglecting the influence of the protein on the glass transition temperature. However, it is well known that the glass transition temperature can be greatly influenced by residual moisture or moisture adsorbed from the environment, which should be taken into account by estimating the glass transition temperature of a sugar-buffer-water mixture [15]. This was achieved by using the Gordon-Taylor

equation, extended for a ternary mixture (Eq. (1)), which describes the relation between the composition of a three component mixture (with mass fractions w_s, w_b, and w_w) and its glass transition temperature (T_g) [16]. Besides the mass fraction of the components, the glass transition temperature of the mixture is also dependent on the glass transition temperature of the individual components ($T_{g.s}$, $T_{g.b}$, and $T_{g.w}$) and two component-dependent Gordon-Taylor constants (k_{sb} and k_{sw}). The subscripts *s*, *b*, and *w* are used for sugar, buffer, and water, respectively. To calculate the glass transition temperature of both the inulin-ammediol-water and trehalose-ammediol-water mixtures, the glass transition temperatures of the components, the Gordon-Taylor constants and the mass fraction of water were determined.

$$T_g = \frac{w_s \cdot T_{g.s} + k_{sb} \cdot w_b \cdot T_{g.b} + k_{sw} \cdot w_w \cdot T_{g.w}}{w_s + k_{sb} \cdot w_b + k_{sw} \cdot w_w} \quad (1)$$

The glass transition temperature of pure inulin, trehalose, and ammediol was determined with DSC and found to be 155 °C, 121 °C, and -53 °C, respectively. For water, a glass transition temperature of -109 °C was used, which is the average of recently published values [17–19]. Although this value is substantially higher than the conventionally accepted value of -137 °C [17–19], our calculations indicate that the choice of either of these glass transition temperatures of water did not have large influence on the calculated glass transition temperature of the final samples (data not shown).

The Gordon-Taylor constant, k_{sb}, was determined for both the inulin-ammediol and trehalose-ammediol mixtures by fitting the Gordon-Taylor equation, for binary mixtures with $w_w = 0$, with glass transition temperatures of different compositions measured with DSC. The best fit was determined by using the least squares method. It was noted that glass transition temperatures below 90 °C were difficult to determine, due to large shifts in the baseline. These shifts can most likely be attributed to a highly energetic solid-solid phase transition of the ammediol buffer at 80 °C, as described by M. Barrio et al. [20]. If the glass transition temperature of the system is close to the temperature at which this solid-solid phase transition occurs, the translational molecular mobility might be high enough to allow the phase transition to start before the glass transition temperature is reached, in turn distorting the measurement and obscuring the glass transition temperature. Therefore, to determine the Gordon-Taylor constants, only samples with a glass transition temperature higher than 90 °C were used.

The Gordon-Taylor constants for the inulin-ammediol and trehalose-ammediol samples were found to be 1.87 and 1.64, respectively. The correlation coefficient of 0.999 and 1.000 for both inulin-ammediol (n = 8 points) and trehalose-ammediol

(n = 8 points) samples, respectively, indicate a perfect fit of the Gordon-Taylor equation with the experimental data, showing that the buffer was monomolecularly dispersed within the sugar.

The Gordon-Taylor constant, k_{sw}, for trehalose-water and inulin-water mixtures was also determined by fitting the Gordon-Taylor equation for binary mixtures, with $w_b = 0$, with glass transition temperatures of humidified sugar glasses measured with DSC. The moisture content of sugar glasses that were humidified at specific relative humidities was determined with DVS analysis. The Gordon-Taylor constants for the trehalose-water and inulin-water mixtures were found to be 7.90 and 6.57, respectively. The Gordon-Taylor constant for a trehalose-water mixture was found to be higher than values found in literature (i.e. 5.2, 6.5, and 7.3), due to the higher glass transition temperature of water we used [21–23].

The mass fraction of water, w_w, in the samples was estimated by relating DVS data to the relative humidity inside the chamber used for storing the samples. The relative humidity inside the storage chamber was calculated from relative humidity measurements outside the storage chamber by using the Antoine equation and the ideal gas law (Eq. (2) and Eq. (3) respectively).

$$p_{w.sat} = 10^{A-\frac{B}{C+T}} \tag{2}$$

$$p_w = \frac{x_w \cdot \rho \cdot R \cdot (T+273.15)}{M_w} \tag{3}$$

Where $p_{w.sat}$ is the saturated water vapor pressure, A, B, and C are the Antoine constants of water (10.20, 1730.63, and 233.43, respectively [24], T is the temperature (°C), p_w is the partial water vapor pressure (Pa), x_w is the specific humidity (defined as the mass of water divided by the mass of dry air), ρ is the density of air, R is the gas constant, and M_w is the molecular mass of water. The two equations enable the calculation of the relative humidity (%) (defined as $p_w / p_{w.sat} \cdot 100$). When the relative humidity outside the storage chamber is measured, the specific humidity of the ambient air can be calculated by multiplying by $p_{w.sat} / 100$ at ambient temperature, and subsequently dividing by ρ·R·(T+273.15) / Mw. Because the storage chamber is well ventilated with the ambient air, the humidity inside the storage chamber is equal to the humidity of the ambient air. However, since the air is heated inside the storage chamber, the relative humidity decreases. This can be calculated by first calculating p_w at 60 °C, and subsequently the relative humidity by dividing $100 \cdot p_w$ by $p_{w.sat}$ at 60 °C. For example, since the average relative humidity of the ambient air during one of the experiments was 53 % at 20 °C, the relative humidity inside the storage chamber was approximately 6%. Knowing the relative

humidity inside the storage chamber, the moisture content of the samples could be determined using the water vapor isotherm obtained with DVS. To take into account any possible influence of protein on water vapor sorption, DVS was performed with protein containing samples. The moisture content of the samples was found to be between 1.2 - 2.5 % by mass. Subsequently, the glass transition temperature of both inulin-ammediol-water and trehalose-ammediol-water mixtures was calculated with the Gordon-Taylor equation (Eq. (1)). The glass transition temperatures as calculated with the Gordon-Taylor equation were used to estimate the different glass transition temperatures of the prepared protein samples. These results are presented in **Table 2**.

Table 2. Estimated glass transition temperatures for stabilized protein samples.
[a] *The glass transition temperature was determined for samples without protein.*

	Glass transition temperature (°C)							
Protein/ sugar mass ratio[a]	**1/1**	**1/3**	**1/5**	**1/7**	**1/10**	**1/12**	**1/15**	**1/20**
Inulin	-16	28	51	64	76	81	86	89
Trehalose	-20	18	37	49	59	64	69	73

3. 2. Varying glass transition temperature

To investigate the contribution of both the vitrification and water replacement mechanism on the stability of the protein at different glass transition temperatures, samples with varying glass transition temperatures (and thus varying translational molecular mobility) were prepared. The enzymatic activity of the protein was measured after storing these samples in a desiccator overnight and after storing them for 12 days at 60 °C.

Samples with the lowest glass transition temperature, below 0 °C, already lost most of their activity during spray drying (**Figure 1**). However, when the glass transition temperature is raised above 30 - 40 °C (close to the spray dryer outlet temperature of around 60 °C), the loss of activity was strongly reduced and became negligible at values over 45 °C. After storing the spray dried samples for 12 days at 60 °C, the stability curve shifted to the right, indicating that a higher glass transition temperature is required for protein stabilization during storage. Complete activity loss was observed for samples with a glass transition temperature below 50 - 60 °C, while at higher glass transition temperatures the activity loss quickly decreased.

Although the shift is observed for both sugars, trehalose appeared to stabilize the protein already at somewhat lower glass transition temperatures than inulin.

To investigate the shift of the stability curve in time, inulin samples with varying glass transition temperatures were stored for various periods of time. After the first 5 days of storage at 60 °C a similar shift of the stability curve was observed (**Figure 2**) as after 12 days of storage (**Figure 1**). Prolonged storage time intervals did not reveal any further shift of the curve, but rather an overall decrease of activity independent from the glass transition temperature. This overall activity loss was most apparent for samples with a higher glass transition temperature. These higher glass transition temperatures, in the range of 76 to 89 °C, appear to increase stability only slightly. This range will further be referred to as the "plateau phase".

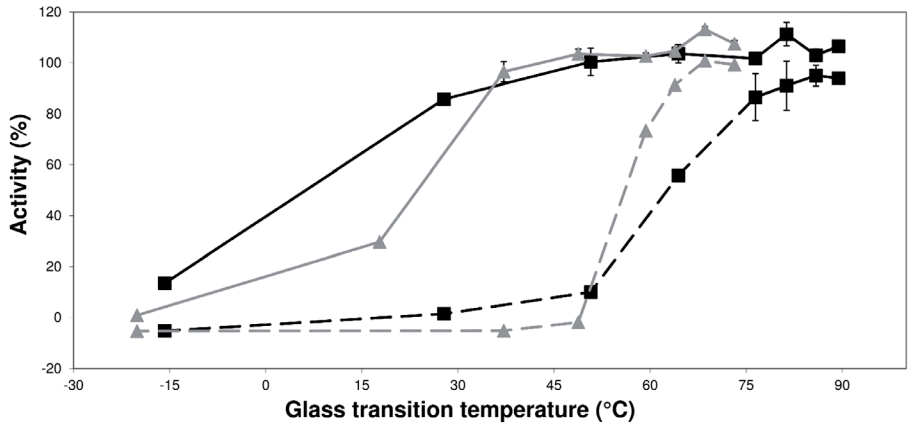

Figure 1. Activity of alkaline phosphatase incorporated in inulin (■) and trehalose (▲) after spray drying (──) and after 12 days storage at 60 °C (- - -), (n = 3 ± SD).

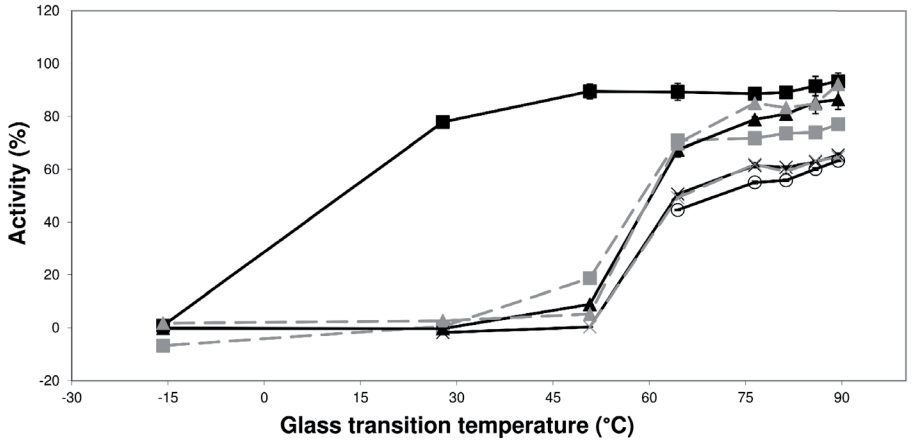

Figure 2. Inulin stabilized protein activity after spray drying (■) and upon storage at 60 °C for 5 (■), 12 (▲), 19 (▲), 26 (X), 33 (X), and 40 (O) days, (n = 3 ± SD).

3. 3. Constant glass transition temperature

The decrease of activity within the plateau phase was investigated in more detail. This was done by measuring the activity loss of samples with varying protein content and a constant glass transition temperature during storage at 60 °C. The protein content was varied by preparing samples with varying protein/sugar mass ratios of 1/1, 1/10, and 1/20 (**Table 3**). A fixed buffer/sugar mass ratio of 0.20 and 0.13 was chosen for inulin samples to obtain a glass transition temperature of around 80 °C and 93 °C, respectively. Trehalose samples with similar glass transition temperatures were obtained by choosing a buffer/sugar mass ratio of 0.06 and 0.02, respectively.

Table 3. Stabilized protein sample compositions with constant glass transition temperature.

	Trehalose (T_g = 81 °C)			**Trehalose (T_g = 91 °C)**		
Protein sugar mass ratio	**1/1**	**1/10**	**1/20**	**1/1**	**1/10**	**1/20**
Trehalose	0.48	0.86	0.90	0.50	0.89	0.93
Protein	0.48	0.09	0.04	0.50	0.09	0.05
Buffer	0.03	0.05	0.06	0.01	0.02	0.02
	Inulin (T_g = 78 °C)			**Inulin (T_g = 95 °C)**		
Protein sugar mass ratio	**1/1**	**1/10**	**1/20**	**1/1**	**1/10**	**1/20**
Inulin	0.45	0.77	0.80	0.47	0.81	0.85
Protein	0.45	0.08	0.04	0.47	0.08	0.04
Buffer	0.09	0.15	0.16	0.06	0.11	0.11

Since the stability data of the samples with varying protein/sugar ratios (for both inulin and trehalose samples) were not significantly different, the results were combined (**Figure 3**), which is justified by the overall small standard deviation (<5 %). This shows that, within the range tested, the stability of the protein is independent from the protein content of the sample. Furthermore, **Figure 3** shows that the activity loss for inulin samples was larger than for the trehalose samples. After 19 days the activity loss for trehalose samples was almost 10 %, while for inulin samples almost 30 % was lost. Finally, there is no difference in the activity loss between samples with a glass transition temperature of 80 °C and 93 °C when the same sugar was used.

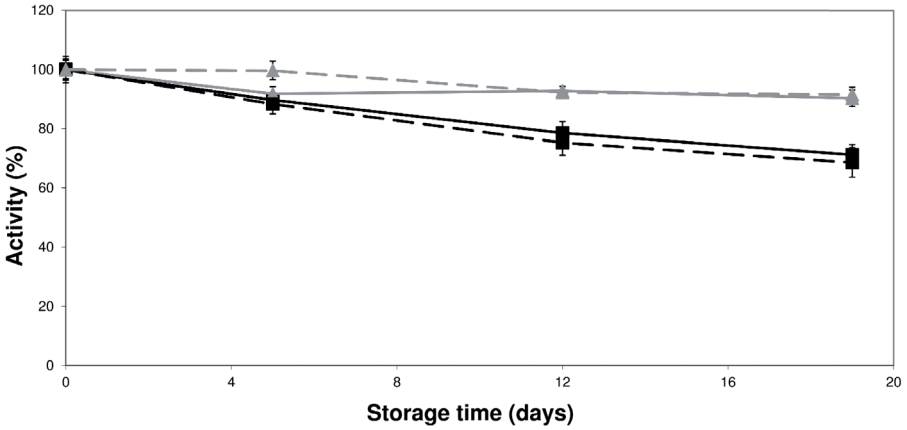

Figure 3. Alkaline phosphatase activity upon storage at 60 °C of trehalose (▲) and inulin (■) samples with a glass transition temperature of around 80 °C (——) and 93 °C (- - -). Data points are an average of three samples with varying protein/sugar mass ratios of 1/1, 1/10, and 1/20 and scaled to 100% at 0 days (original values were between 100 – 107 %), (n = 9 ± SD).

4. Discussion

In this study the Gordon-Taylor equation was used to calculate the glass transition temperature of the samples containing protein, based on DSC data from samples without protein. It could be argued that the presented glass transition temperatures are not a reflection of the actual values due to the fact that they are based on a mixture of the sugar, ammediol, and water, thereby neglecting the presence of the protein. Especially for the high protein containing samples (having the lowest glass transition temperatures) this raises questions. However, there are indications in literature that even a high mass fraction of protein influences the glass transition temperature to only a limited extent. Imamura et al. showed that the glass transition temperature of a trehalose glass was increased by 9 °C when the mass content of bovine serum albumin was increased from 20 % to 50 %, while the change in glass transition temperature at lower protein contents was only marginal [25]. Bellavia et al. also investigated the effect of the protein content on the glass transition temperature and the applicability of the Gordon-Taylor equation [26]. It was shown that, at high protein contents, the glass transition temperature of ternary sugar-protein-water mixtures was higher than predicted by a binary Gordon-Taylor curve for a sugar-water mixture, without protein. Up to 40 % the effect of protein mass content on the glass transition temperature was only minor. Only at a mass content of 76 % a substantial effect was found. Therefore, it can be assumed that the addition of protein will only moderately influence the glass transition temperature of the samples with

a protein/sugar mass ratio of 1/1, corresponding to a protein mass content of 24 %. Moreover, the protein activity in the 24 % samples was completely lost after storage at 60 °C and conclusions of our study were not based on these samples but on samples with much lower protein content. Therefore, it is justified to consider the glass transition temperatures as obtained from the Gordon-Taylor equation for ternary systems representative for the glass transition temperatures of the protein containing samples (**Table 2**).

The employed three-component system for protein stabilization clearly shows two different phases. In the first phase, protein stability increases rapidly with increasing glass transition temperature. In the second phase (the plateau phase) the stability is independent from the glass transition temperature of the sugar matrix but varies depending on the sugar applied.

In general, the results show an increased stability with an increasing glass transition temperature up to 10 - 20 °C above the storage temperature. This observation is in line with the vitrification mechanism. When the glass transition temperature of the sugar is below the storage temperature, the material will be in a mobile rubbery state, which will enable the protein to degrade and lose its activity. This effect is observed both during spray drying and storage. Of course, considering the variation in sugar content of the samples, one could argue that a minimum amount of sugar is required to saturate the surface of the protein, thereby maximizing protein stability by forming as many hydrogen bonds as possible. Although it is tempting to explain the lack of stability of samples with a low glass transition temperature, having a protein/sugar mass ratio of 1/1 and 1/3, by the low amount of sugar, the low standard deviation shown in **Figure 3** shows that the protein stability is independent of the protein/sugar mass ratio within the range tested. Therefore, the relatively low sugar content of the samples with a protein/sugar mass ratio of 1/1 and 1/3 cannot account for the activity loss of these samples, clearly pointing to the glass transition temperature as the major determinant of the stability in this phase.

During the relatively short spray drying process, the maximum temperature of the dried samples is assumed to be that of the outlet temperature, which was 58 - 64 °C. However, even samples with a glass transition temperature as low as 30 °C exhibited quite good process stability, whereas during storage at 60 °C for 12 days, the protein activity of the samples with a glass transition temperature below storage temperature was completely lost. Apparently, within the short spray drying time, even the samples with a glass transition temperature up to 30 °C below the outlet temperature did not degrade fast enough to show any loss of activity.

When the glass transition temperature is raised above storage temperature, the amorphous sugar will change to the less mobile glassy state, which should

keep the protein vitrified in its original conformation. Although the onset of protein stabilization appeared to occur when the glass transition temperature was around the storage temperature, it was also shown that the protein was still not fully stabilized immediately after the glass transition temperature was raised above the storage temperature. The stability appeared to increase exponentially over a 20 - 30 °C temperature range around the storage temperature before reaching a plateau phase, showing maximum stabilization. This finding is in agreement with the result of a study by Hancock et al. [6], who showed that in the range between the glass transition temperature and 50 °C below glass transition temperature, the translational molecular mobility strongly decreases. In addition, from the data presented in the same study, one can conclude that the change of the translational molecular mobility was much faster for the relatively small sucrose than for the much larger PVP K90. This resulted in a difference in mobility of several orders of magnitude when the storage temperature was 50 °C below the glass transition temperature. A similar difference was found in this study. The stability of the protein when incorporated in trehalose increased faster with increasing glass transition temperature than when the protein was incorporated in the larger inulin. The same phenomenon was also observed in more detail by others. Wolkers et al. [27] and [28] applied FT-IR to show that the wavenumber temperature coefficient of the OH-stretching mode band position increased with increasing molecular mass, indicating that the average hydrogen bond length increases with increasing molecular mass. This results in a more loosely packed glass and a higher mobility of the sugar matrix.

It should, however, be kept in mind that in order to obtain a glass transition temperature similar to the trehalose samples, the inulin samples contained higher amounts of buffer. Therefore, one could argue that the stability difference found to exist between both sugars was caused by the replacement of sugar with buffer, which could result in a less favorable interaction with the protein. However, under that assumption, this could be said for the entire stability curve and this would imply that the loss of activity at lower glass transition temperatures is not due to increased translational molecular mobility but due to replacement of sugar by the buffer. In other words, the protein stability would under such assumption be dominated entirely by water replacement and never by vitrification. However, it seems unlikely that this is the case. Although the same difference in stability between inulin and trehalose is observed at high glass transition temperatures where mobility is expected to be negligible, at lower glass transition temperatures the rate of activity loss is much faster and is more likely to be caused by a higher mobility. In addition, the facts that the sudden exponential increase of stability occurs only at glass transition temperatures around the storage temperature and it shows a good correlation with results from literature, strongly suggests that protein stability is dominated by mobility and thus vitrification at lower glass transition temperatures.

Therefore, the fact that for maximum protein stability a slightly higher glass transition temperature is required with inulin than with trehalose, should be attributed to their difference in molecular size. While trehalose has a molecular mass of 342 g/mol, the type of inulin used in this study is a long chained oligosaccharide with a molecular mass of 3908 g/mol. Therefore, the translational molecular mobility of a trehalose glass is likely to be smaller than the translational molecular mobility of an inulin glass, resulting in the slight difference in protein stability with trehalose and inulin at glass transition temperatures where the mobility dictates the protein stability. These findings confirm that the protein stability at temperatures near the glass transition temperature is largely dependent on the translational molecular mobility of the sugar.

It is important to be aware of a study by Francia et al., which reported an increased rigidity of a trehalose matrix when its water content is decreased [29]. The increased rigidity of the matrix was attributed to the anchorage hypothesis, which links the water replacement theory with the water entrapment theory, stating that at low hydration levels the water and trehalose molecules form a continuous matrix with a high rigidity. Therefore, the increased stability of samples when their glass transition temperature is increased to above storage temperature could have simply been coinciding with a decrease of the water content in the mixtures. However, the water content in the trehalose and inulin mixtures used in this study showed only little variation for samples with a glass transition temperature between 40 °C below and 40 °C above the storage temperature. With an average water/sugar mass ratio of approximately 0.02, the standard deviation was only around 10 %. Therefore the anchorage mechanism does not appear to play a role in this system, which further reinforces the statement that the stability increase can be attributed to the vitrification theory.

At high glass transition temperatures, where the protein stability reaches the plateau phase, the activity loss was found to be independent from the glass transition temperature. Instead, a steady decline in activity was observed over time, both for inulin and trehalose samples. As is shown in the previous section, the protein can be considered immobile at these glass transition temperatures. At these high glass transition temperatures degradation can only take place when there is either enough free volume or insufficient interaction between the protein and the sugar (i.e. hydrogen bonding). Therefore, the primary stabilization mechanism in this region is most likely water replacement. This hypothesis is supported by the fact that the activity loss with inulin is higher than with trehalose. If stabilization is indeed realized by water replacement, then the difference in activity loss between inulin and trehalose must be caused by a difference in the ability of the sugar to form hydrogen bonds with the protein. Since it is likely that the small trehalose molecule

will be able to fit the irregular protein surface more closely than the larger inulin molecules, thereby forming more hydrogen bonds with the protein, the resulting stability will be higher. Although the question whether the interaction between the protein and the sugar is direct or facilitated by a hydration layer is not relevant for the current discussion, an interesting observation can be made regarding this subject. If water molecules would be entrapped at the interface between protein and sugar, an influence of the water/protein molar ratio on the stability would be expected. However, no difference in stability was observed between samples with a protein/sugar ratio of 1, 10, and 20 (**Figure 3**), while the water/protein molar ratio differed substantially (3200, 1600, and 160, respectively). Although it is unknown whether there is a minimum water/protein ratio above which the stability is no longer affected, the results seem to correspond more with the water replacement mechanism rather than the water entrapment mechanism.

In addition to the water entrapment theory, another interesting perspective on the topic is given by the slaving model as proposed by Frauenfelder et al. [30]. In their study, a clear physical explanation is given for the mobility of a protein incorporated in a sugar glass. Instead of considering the mobility of the protein as being autonomous, it is considered to be governed by the surrounding matrix ("slaved"). Large scale motions of the protein are caused by mobility of the matrix bulk, while internal protein motions are caused by local movements in the, several molecules thick, layer surrounding the protein. These motions are designated as primary (α) and secondary (β) fluctuations, respectively. When the viscosity of the matrix bulk is low (rubbery state), α fluctuations cause large scale motions of the protein, which then mainly determine the protein stability. This is in agreement with the vitrification theory. However, at high viscosity (glassy state), α fluctuations in the bulk are absent and the protein stability is mainly determined by local β fluctuations. Although this is a different explanation than given by the water replacement theory, both theories appear to be in agreement with the results of our study. Instead of attributing the stability of the protein at high glass transition temperature to the hydrogen bond formation with the matrix, it is instead attributed to the local mobility of the surrounding, closely confined, molecules. Therefore, the difference in stability at high glass transition temperature between trehalose and inulin should then be explained by their difference in mobility, comparable to the vitrification theory.

If the stability at the high glass transition temperatures is indeed dominated by the water replacement mechanism, it is important to consider the following. As mentioned before, in order to compare the activity of trehalose and inulin samples at the same glass transition temperatures, a relatively higher ammediol/sugar ratio was required for inulin due to its inherently higher glass transition temperature of 155 °C. It could be argued whether or not the protein stability with inulin was different

from trehalose due to a more prevalent interaction of the buffer with the protein and not just a difference in molecular mass of the sugar. Studies have already shown that plasticizers can even have a stabilizing effect when added in small amounts, despite the decrease of the glass transition temperature [31] and [32]. It is hypothesized that the buffer can form hydrogen bonds with the protein at sites that have not been occupied by the sugar, further stabilizing the protein until the reduced glass transition temperature increases the mobility of the sugar matrix to an extent that enables the protein to degrade. Although in our case ammediol did not seem to have a stabilizing effect on the protein, it is more than likely that higher ammediol content will cause replacement of part of the sugar, interacting with the protein, by ammediol instead. If ammediol is not as good a stabilizer as the sugar, this could decrease the stability of the protein. However, although it remains unclear whether the difference in stability with inulin and trehalose is mainly caused by the difference in ammediol content or molecular mass, both causes are consistent with the water replacement theory.

5. Conclusion

In conclusion, it is shown that at glass transition temperatures below the storage temperature protein stabilization is dominated by the vitrification mechanism. However, when the glass transition temperature is raised significantly above the storage temperature, the protein becomes immobile and the water replacement mechanism becomes the dominant mechanism for protein stabilization.

Acknowledgments

This research forms part of the Project P3.02 DESIRE of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. This project is part-financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta.

References

[1] L.L. Chang, M.J. Pikal, Mechanisms of Protein Stabilization in the Solid State, J. Pharm. Sci. 98 (2009) 2886–2908.

[2] L.L. Chang, D. Shepherd, J. Sun, D. Ouellette, K.L. Grant, X.C. Tang, et al., Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix?, J. Pharm. Sci. 94 (2005) 1427–1444.

[3] J.G. Sampedro, S. Uribe, Trehalose-enzyme interactions result in structure stabilization and activity inhibition. The role of viscosity., Mol. Cell. Biochem. 256-257 (n.d.) 319–327.

[4] J.F. Carpenter, J.H. Crowe, An infrared spectroscopic study of the interactions of carbohydrates with dried proteins, Biochemistry. 28 (1989) 3916–3922.

[5] P.S. Belton, a M. Gil, IR and Raman spectroscopic studies of the interaction of trehalose with hen egg white lysozyme., Biopolymers. 34 (1994) 957–961.

[6] B.C. Hancock, S.L. Shamblin, G. Zografi, Molecular mobility of amorphous pharmaceutical solids below their glass transition temperatures., Pharm. Res. 12 (1995) 799–806.

[7] L.X. Yu, Pharmaceutical quality by design: product and process development, understanding, and control., Pharm. Res. 25 (2008) 781–791.

[8] N.K. Jain, I. Roy, Effect of trehalose on protein structure., Protein Sci. 18 (2009) 24–36.

[9] W.L.J. Hinrichs, M.G. Prinsen, H.W. Frijlink, Inulin glasses for the stabilization of therapeutic proteins, Int. J. Pharm. 215 (2001) 163–174.

[10] K. Kawai, T. Suzuki, Stabilizing effect of four types of disaccharide on the enzymatic activity of freeze-dried lactate dehydrogenase: step by step evaluation from freezing to storage., Pharm. Res. 24 (2007) 1883–1890.

[11] J.-P. Amorij, J. Meulenaar, W.L.J. Hinrichs, T. Stegmann, A. Huckriede, F. Coenen, et al., Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying., Vaccine. 25 (2007) 6447–6457.

[12] G.S. Zijlstra, B.J. Ponsioen, S. a Hummel, N. Sanders, W.L.J. Hinrichs, A.H. de Boer, et al., Formulation and process development of (recombinant human) deoxyribonuclease I as a powder for inhalation., Pharm. Dev. Technol. 14 (2009) 358–368.

[13] L.T. Rodríguez Furlán, A.P. Padilla, M.E. Campderrós, Inulin like lyoprotectant of bovine plasma proteins concentrated by ultrafiltration, Food Res. Int. 43 (2010) 788–796.

[14] J.H.C. Eriksson, W.L.J. Hinrichs, G.J. de Jong, G.W. Somsen, H.W. Frijlink, Investigations into the stabilization of drugs by sugar glasses: III. The influence of various high-pH buffers., Pharm. Res. 20 (2003) 1437–1443.

[15] B.C. Hancock, G. Zografi, The relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids., Pharm. Res. 11 (1994) 471–477.

[16] M. Gordon, J.S. Taylor, Ideal copolymers and the second-order transitions of synthetic rubbers. I. Non-crystalline copolymers, J. Appl. Chem. 2 (1952) 493–500.

[17] A.A. Miller, Glass-transition temperature of water., Science. 163 (1969) 1325–1326.

[18] N. Giovambattista, C. Angell, F. Sciortino, H. Stanley, Glass-Transition Temperature of Water: A Simulation Study, Phys. Rev. Lett. 93 (2004) 047801.

[19] V. Velikov, S. Borick, C.A. Angell, The glass transition of water, based on hyperquenching experiments., Science (80-.). 294 (2001) 2335–2338.

[20] M. Barrio, J. Font, J. Muntasell, J.L. Tamarlt, Polymorphism in 2-Amino-2-Methyl-1,3 Propanediol Plastic Crystal, J. Phase Equilibria. 12 (1991) 409–415.

[21] L.M. Crowe, D.S. Reid, J.H. Crowe, Is trehalose special for preserving dry biomaterials?, Biophys. J. 71 (1996) 2087–2093.

[22] T. Chen, A. Fowler, M. Toner, Literature review: supplemented phase diagram of the trehalose-water binary mixture., Cryobiology. 40 (2000) 277–282.

[23] Y. Roos, Melting and glass transitions of low molecular weight carbohydrates, Carbohydr. Res. 238 (1993) 39–48.

[24] C.L. Yaws, H.C. (Lamar U.. B.T. (USA)) Yang, To estimate vapor pressure easily, Hydrocarb. Process. 68:10 (1989).

[25] K. Imamura, K.-I. Ohyama, T. Yokoyama, Y. Maruyama, N. Kazuhiro, Temperature scanning FTIR analysis of secondary structures of proteins embedded in amorphous sugar matrix., J. Pharm. Sci. 98 (2009) 3088–3098.

[26] G. Bellavia, S. Giuffrida, G. Cottone, A. Cupane, L. Cordone, Protein thermal denaturation and matrix glass transition in different protein-trehalose-water systems., J. Phys. Chem. B. 115 (2011) 6340–6346.

[27] W.F. Wolkers, A.E. Oliver, F. Tablin, J.H. Crowe, A Fourier-transform infrared spectroscopy study of sugar glasses., Carbohydr. Res. 339 (2004) 1077–1085.

[28] S. Shirke, R.D. Ludescher, Molecular mobility and the glass transition in amorphous glucose, maltose, and maltotriose., Carbohydr. Res. 340 (2005) 2654–2660.

[29] F. Francia, M. Dezi, A. Mallardi, G. Palazzo, L. Cordone, G. Venturoli, Protein-matrix coupling/uncoupling in “dry” systems of photosynthetic reaction center embedded in trehalose/sucrose: the origin of trehalose peculiarity., J. Am. Chem. Soc. 130 (2008) 10240–10246.

[30] H. Frauenfelder, G. Chen, J. Berendzen, P.W. Fenimore, H. Jansson, B.H. McMahon, et al., A unified model of protein dynamics., Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 5129–5134.

[31] L.L. Chang, D. Shepherd, J. Sun, X.C. Tang, M.J. Pikal, Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state., J. Pharm. Sci. 94 (2005) 1445–1455.

[32] M.T. Cicerone, C.L. Soles, Fast dynamics and stabilization of proteins: binary glasses of trehalose and glycerol., Biophys. J. 86 (2004) 3836–3845.

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Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery

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European Journal of Pharmaceutical Sciences 2013, 49(4): 578-587

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 deterimental 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_{1500}-b-poly(ε-caprolactone) [PCL-PEG_{1500}] 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_{1500}]-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_{1500}]-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 *Mw*=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-butanediisocyanate was confirmed by ^1H 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 9.72×10^{-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 1.92×10^{-4} mol/mol monomer. The mixture was magnetically stirred for 164 h at 130 ˚C and subsequently cooled down to room temperature.

PCL and PCL-PEG_{1500} prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare 30[poly(ε-caprolactone)-PEG_{1500}-poly(ε-caprolactone)]$_{2000}$-*b*-70[poly(ε-caprolactone)]$_{4000}$ multiblock copolymer (30[PCL-PEG_{1500}]-70[PCL]) (**Figure 1**). 18.91 g (4.74 mmol) of PCL and 8.00 g (4.00 mmol) of PCL-PEG_{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.

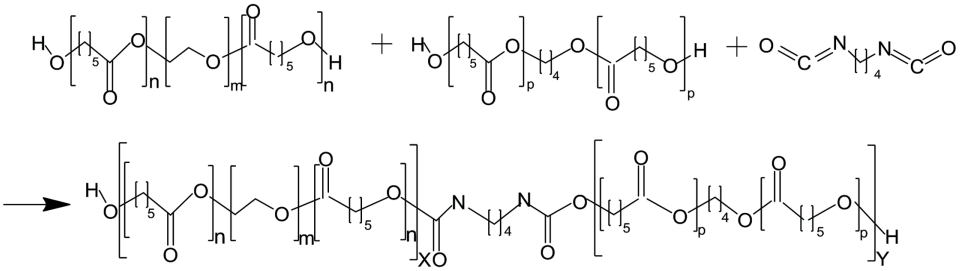

Figure 1. Schematic drawing of the chain-extension reaction of PCL and PCL-PEG_{1500} with 1,4-butanediisocyanate and formation of multiblock copolymer with randomly distributed PCL-PEG and PCL blocks, linked by chain-extender, and OH end groups.

2. 3. Polymer and implant characterization

^{1}H NMR was used to determine monomer conversion, monomer ratios, number average molecular weight and overall chemical composition of the polymer. ^{1}H NMR was performed on a VXR Unity Plus NMR Machine (Varian, California, USA) operating at 300 MHz. The d_1 waiting time was set to 20 s, and the number of scans was 16 - 32. ^{1}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 $CHCl_3$ present as trace element in $CDCl_3$ as reference.

Monomer conversion, i.e. residual ε-caprolactone content, of the prepolymers was calculated from the peaks of the O-$CH_2CH_2CH_2CH_2$**CH_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 ^{1}H NMR using the peaks of the methylene end groups of PCL

at δ 3.6 - 3.7 and the O-$CH_2CH_2CH_2CH_2$**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_2CH_2**-O of PEG at δ 3.6 - 3.7 and the O-$CH_2CH_2CH_2CH_2$**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} prepolymers 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-$CH_2CH_2CH_2CH_2$**CH_2**C(O)O methylene group of PCL at δ 2.2 - 2.4 and the –**CH_2CH_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 prepolymers 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-PEG_{1500}]-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.

*Table 1. The Lys/Inulin ratios used in preparation of implants. The lysozyme loading was 10 wt% in each formulation; * Spray dried.*

Formulation	Lys/Inulin weight ratio	Inulin loading (wt%)	30[PCL-PEG$_{1500}$]-70[PCL] loading (wt%)
Lys as received	1/0	0	90
Lys/Inulin 1/0 *	1/0	0	90
Lys/ Inulin 1/1 *	1/1	10	80
Lys/Inulin 1/2 *	1/2	20	70
Lys/Inulin 1/3 *	1/3	30	60

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 NaN_3 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

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):

$$Q_t = Q_0 + K_0 \times t \quad (1)$$

where *Qt* is the amount of drug released in time t, *Qo* is the initial amount of drug in the solution and *Ku* is the zero order release constant.

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

$$\log Q_t = \log Q_0 + \frac{K_1 t}{2.303} \quad (2)$$

where Q_t is the amount of drug released in time t, *Qo* 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]:

$$Q_t = K_H \times t^{1/2} \quad (3)$$

where Q_t is a cumulative amount of drug released at time t, and K_H is 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 1H 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 prepolymers 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

30[PCL-PEG_{1500}]-70[PCL] multiblock copolymer was almost identical to that of the PCL prepolymer, while the peaks from PEG_{1500} of the prepolymer were absent in the multiblock copolymer, indicating that the crystalline structure of PEG_{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_{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].

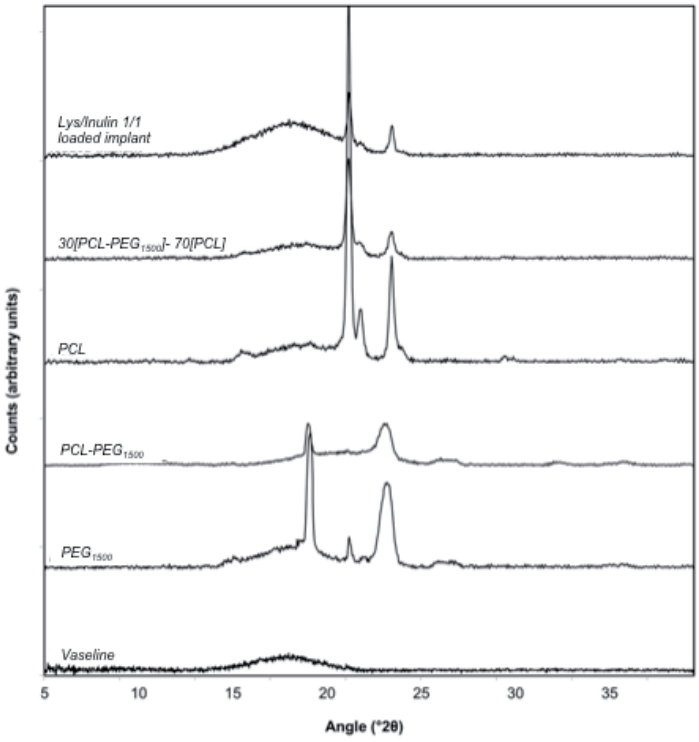

Figure 2. X-ray powder diffraction patterns of PEG_{1500}, PCL-PEG_{1500} and PCL prepolymers, 30[PCL-PEG_{1500}]-70[PCL] multiblock copolymer and Lys/Inulin 1/1 loaded implants.

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_{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.

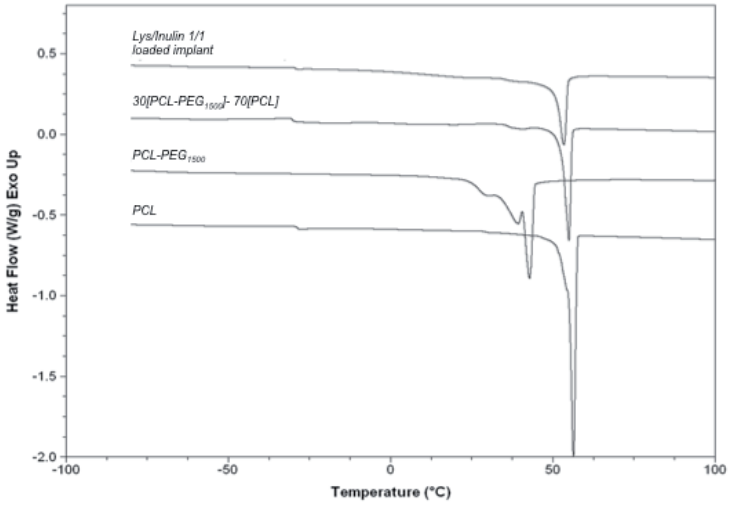

Figure 3. Thermograms of prepolymers PCL, PCL-PEG_{1500}, multiblock copolymer 30[PCL-PEG_{1500}]-70[PCL] and Lys/Inulin 1/1 loaded implant.

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 equitation: 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).

	(protein +sugar)/ polymer weight ratio	**Thermal properties**				Particle size measurements (μm)			
		Tg **(°C)**	Tm **(°C)**	**ΔH (J/g)**	crystallinity of PCL **(% ± SD)**	**X_{10}**	**X_{50}**	**X_{90}**	Span+SD
30[PCL-PEG$_{1500}$]-70[PCL] before extrusion	0/100	-60.9	52.4	58.5	60±0.1				
Lys as received	10/90	-62.6	53.7	37.0	42±1.8	5.06	20.82	46.08	1.97±0.035
Lys/Inulin 1/0	10/90	-61.8	53.7	34.6	39±2.7	0.61	1.09	2.22	1.48±0.002
Lys/Inulin 1/1	20/80	-62.7	53.4	31.7	40±3.5	0.67	1.50	3.07	1.60±0.002
Lys/Inulin 1/2	30/70	-63.0	53.2	31.7	46±1.1	0.64	1.29	2.74	1.63±0.001
Lys/Inulin 1/3	40/60	-62.1	53.1	30.2	51±5.3	0.64	1.32	2.81	1.65±0.002

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_{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.

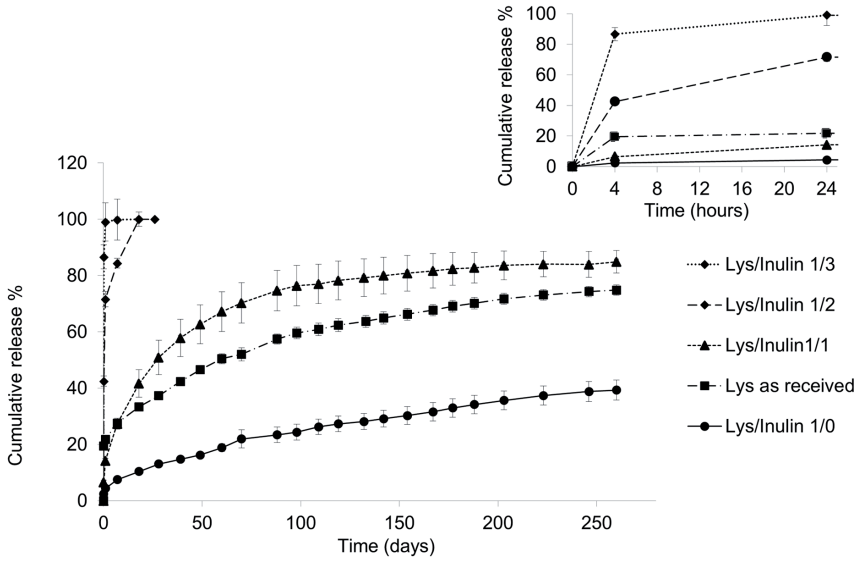

Figure 4. The in-vitro release of lysozyme in 100 mM phosphate buffer, pH 7.4 at 37 ˚C for 260 days, (n=3). The initial 24 h release (upper figure) is shown in hours.

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

Formulation	Zero order	First order	Higuchi
Lys as received	0.812	0.931	0.957
Lys/Inulin 1/0	0.933	0.963	0.997
Lys/Inulin 1/1	0.669	0.844	0.890
Lys/Inulin 1/2	0.550	0.895	0.742
Lys/Inulin 1/3	0.197	0.677	0.352

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

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**).

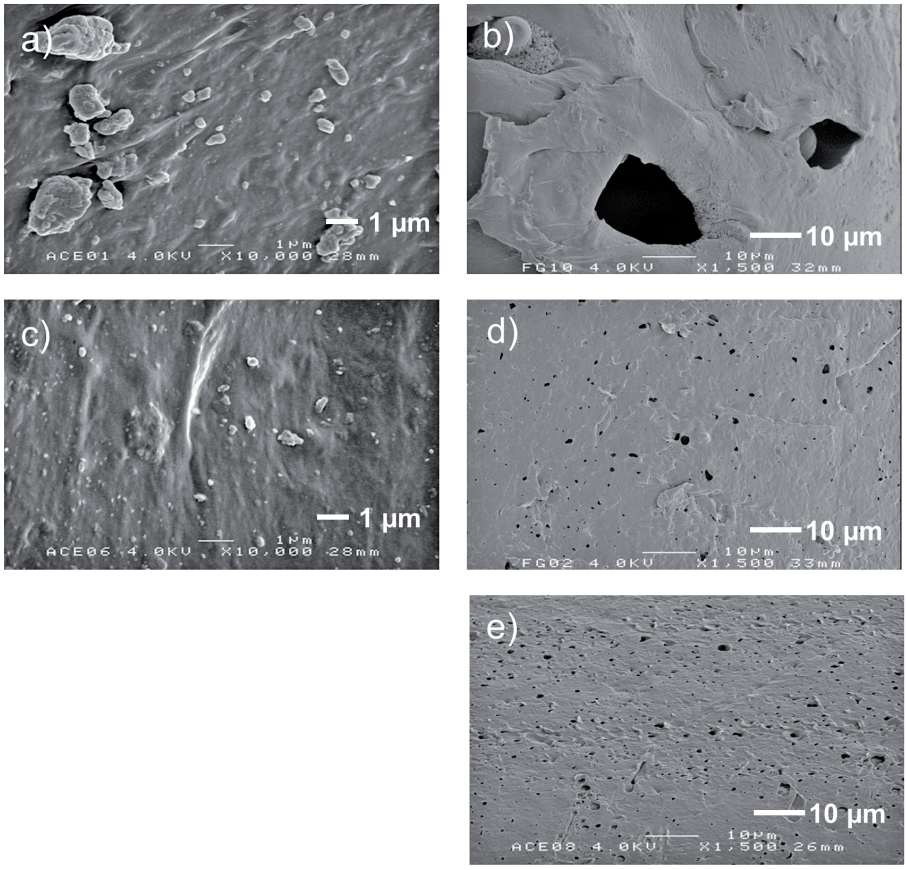

Figure 5. SEM images of 30[PCL-PEG$_{1500}$]-70[PCL] implants containing:
a) Lys as received before release (note higher magnification);
b) Lys as received after 1 day of release;
c) Lys/Inulin 1/0 before release (note higher magnification);
d) Lys/Inulin 1/0 after 1 day of release;
e) Lys/Inulin 1/3 after 1 day of release.

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 prepolymers PCL and PCL-PEG_{1500} were synthesized by ring opening polymerization of ε-caprolactone initiated by 1,4-butanediol and polyethylene glycol (PEG M_W=1500), respectively, catalyzed by stannous octoate. For both prepolymers, the Mn as determined by ^{1}H NMR agreed well with the theoretical values, showing that prepolymers with well-defined composition were obtained.

30[PCL-PEG_{1500}]-70[PCL] multiblock copolymer was prepared by chain-extension of PCL-PEG_{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_{1500} of the PCL-PEG_{1500} prepolymer lost most of its crystalline structure during synthesis of 30[PCL-PEG_{1500}]-70[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[PCL-PEG_{1500}]-70[PCL] multiblock copolymer was shown by the presence of a *Tm* for crystalline PCL at 57 ˚C and a *Tg* at -60 ˚C representing an amorphous phase. The amorphous phase of the 30[PCL-PEG_{1500}]-70[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[PCL-PEG_{1500}]-70[PCL] under physiological conditions, i.e. in an aqueous environment at 37 ˚C. The *Tm* values of both PEG and PCL in 30[PCL-PEG_{1500}]-70[PCL] were lower than in the prepolymers, which is most likely due to formation of less dense crystals, caused by partial phase-mixing of the PCL and PCL-PEG_{1500} blocks and possibly also by hampered crystallization when the chain ends of the prepolymers are embedded in the multiblock copolymer. Lys(/Inulin) loaded implants showed similar *Tm* and *Tg* as the non-extruded 30[PCL-PEG_{1500}]-70[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(ε-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

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_{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.

Acknowledgements

This research was performed within the framework of the Northern Drug Targeting and Delivery Cluster (EFRO grant)**.**

References

[1] M.C. Manning, K. Patel, R.T. Borchardt, Stability of Protein Pharmaceuticals, Pharm. Res. 6 (1989) 903–918.

[2] M.A. Repka, S. Majumdar, S.K. Battu, R. Srirangam, S.B. Upadhye, Applications of hot-melt extrusion in drug delivery, Expert Opin. Drug Deliv. 5 (2008) 1357–1376.

[3] A.A. Ignatius, L.E. Claes, In vitro biocompatibility of bioresorbable polymers: poly(L, DL-lactide) and poly(L-lactide-co-glycolide)., Biomaterials. 17 (1996) 831–839.

[4] J.M. and M.S.S. Anderson, Biodegradation and biocompatibility of PLA and PLGA microspheres., Adv. Drug Deliv. Rev. 28(1) (1997) 5–24.

[5] Z. Ghalanbor, M. Körber, R. Bodmeier, Improved lysozyme stability and release properties of poly(lactide-co-glycolide) implants prepared by hot-melt extrusion., Pharm. Res. 27 (2010) 371–379.

[6] J.J. Gray, The interaction of proteins with solid surfaces., Curr. Opin. Struct. Biol. 14 (2004) 110–115.

[7] M. Malmsten, Ellipsometry studies of the effects of surface hydrophobicity on protein adsorption, Colloids Surfaces B Biointerfaces. 3 (1995) 297–308.

[8] K. Fu, D.W. Pack, A.M. Klibanov, R. Langer, Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres, Pharm. Res. 17 (2000) 100–106.

[9] A. Shenderova, T.G. Burke, S.P. Schwendeman, The Acidic Microclimate in Poly(lactide-co-glycolide) Microspheres Stabilizes Camptothecins, Pharm. Res. 16 (1999) 241–248.

[10] A. Giteau, M.C. Venier-Julienne, S. Marchal, J.L. Courthaudon, M. Sergent, C. Montero-Menei, et al., Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres, Eur. J. Pharm. Biopharm. 70 (2008) 127–136.

[11] M. van de Weert, W.E. Hennink, W. Jiskoot, Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles, Pharm. Res. 17 (2000) 1159–1167.

[12] B.S. Zolnik, D.J. Burgess, Effect of acidic pH on PLGA microsphere degradation and release., J. Control. Release. 122 (2007) 338–344.

[13] M. Sandor, D. Enscore, P. Weston, E. Mathiowitz, Effect of protein molecular weight on release from micron-sized PLGA microspheres, J. Control. Release. 76 (2001) 297–311.

[14] R.E. Eliaz, J. Kost, Characterization of a polymeric PLGA-injectable implant delivery system for the controlled release of proteins, J. Biomed. Mater. Res. 50 (2000) 388–396.

[15] S. Freiberg, X.X. Zhu, Polymer microspheres for controlled drug release, Int. J. Pharm. 282 (2004) 1–18.

[16] J.-E. Kim, S.-R. Kim, S.-H. Lee, C.-H. Lee, D.-D. Kim, The effect of pore formers on the controlled release of cefadroxil from a polyurethane matrix, Int. J. Pharm. 201 (2000) 29–36.

[17] R.L. Cleek, K.C. Ting, S. G. Eskin, A.G. Mikos, Microparticles of poly (dl-lactic-co-glycolic acid)/poly (ethylene glycol) blends for controlled drug delivery, J. Control. Release. 48 (1997) 259–268.

[18] S. Herrmann, G. Winter, S. Mohl, F. Siepmann, J. Siepmann, Mechanisms controlling protein release from lipidic implants: effects of PEG addition., J. Control. Release. 118 (2007) 161–168.

[19] G. Sax, G. Winter, Mechanistic studies on the release of lysozyme from twin-screw extruded lipid implants, J. Control. Release. 163 (2012) 187–194.

[20] W. Gunder, B.H. Lippold, B.C. Lippold, Release of drugs from ethyl cellulose microcapsules (diffusion pellets) with pore formers and pore fusion, Eur. J. Pharm. Sci. 3 (1995) 203–214.

[21] C.. Hissink, R. Meyboom, T.A.. Flipsen, Biodegradable phase-separated segmented multi block co-polymer, PCT Patent No. WO2004007588 (A1), 2004.

[22] M.D. Dhanaraju, D. Gopinath, M.R. Ahmed, R. Jayakumar, C. Vamsadhara, Characterization of polymeric poly(epsilon-caprolactone) injectable implant delivery system for the controlled delivery of contraceptive steroids., J. Biomed. Mater. Res. A. 76 (2006) 63–72.

[23] V.R. Sinha, K. Bansal, R. Kaushik, R. Kumria, A. Trehan, Poly-e-caprolactone microspheres and nanospheres: an overview, Int. J. Pharm. 278 (2004) 1–23.

[24] M.A. Woodruff, D.W. Hutmacher, The return of a forgotten polymer—Polycaprolactone in the 21st century, Prog. Polym. Sci. 35 (2010) 1217–1256.

[25] K. Al-Tahami, A. Meyer, J. Singh, Poly lactic acid based injectable delivery systems for controlled release of a model protein, lysozyme., Pharm. Dev. Technol. 11 (2006) 79–86.

[26] G. Jiang, B.H. Woo, F. Kang, J. Singh, P.P. DeLuca, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D, L-lactide-co-glycolide) microspheres., J. Control. Release. 79 (2002) 137–145.

[27] A. Paillard-Giteau, V.T. Tran, O. Thomas, X. Garric, J. Coudane, S. Marchal, et al., Effect of various additives and polymers on lysozyme release from PLGA microspheres prepared by an s / o / w emulsion technique, Eur. J. Pharm. Biopharm. 75 (2010) 128–136.

[28] A.H. Ghassemi, M.J. van Steenbergen, H. Talsma, C.F. van Nostrum, W. Jiskoot, D.J.A. Crommelin, et al., Preparation and characterization of protein loaded microspheres based on a hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid), J. Control. Release. 138 (2009) 57–63.

[29] C. Guse, S. Koennings, F. Kreye, F. Siepmann, A. Goepferich, J. Siepmann, Drug release from lipid-based implants: elucidation of the underlying mass transport mechanisms, Int J Pharm. 314 (2006) 137–144.

[30] T. Barclay, M. Ginic-Markovic, P. Cooper, N. Petrovsky, Inulin - a versatile polysaccharide with multiple pharmaceutical and food chemical uses ., J. Excipients Food Chem. 1 (2010) 27–50.

[31] M. Körber, R. Bodmeier, Development of an in situ forming PLGA drug delivery system: I. Characterization of a non-aqueous protein precipitation, Eur. J. Pharm. Sci. 35 (2008) 283–292.

[32] P. Costa, J.M. Sousa Lobo, Modeling and comparison of dissolution profiles., Eur. J. Pharm. Sci. 13 (2001) 123–133.

[33] T. Higuchi, Mechanism of Sustained- Action Medication, J. Pharm. Sci. (1963) 1145–1149.

[34] G. Gorin, S. Wang, L. Papapavlou, Assay of Lysozyme by Its Lytic Action on M. lysodeikticus Cells, Anal. Biochem. 39 (1971) 113–127.

[35] N. Grasmeijer, M. Stankovic, H. de Waard, H.W. Frijlink, W.L.J. Hinrichs, Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system., Biochim. Biophys. Acta. (2013).

[36] S. Verheyen, P. Augustijns, R. Kinget, G. Van den Mooter, Melting behavior of pure polyethylene glycol 6000 and polyethylene glycol 6000 in solid dispersions containing diazepam or temazepam: a DSC study, Thermochim. Acta. 380 (2001) 153–164.

[37] H. Larhrib, J.I. Wells, M.H. Rubinstein, G. Ricart, Characterization of PEGs using matrix-assisted laser desorption / ionisation mass spectrometry and other related techniques, Int. J. Pharm. 147 (1997) 187–198.

[38] V. Crescenzi, G. Manzini, G. Calzolari, C. Borri, Thermodynamics of fusion of poly-β-propiolactone and poly-ε-caprolactone. Comparative analysis of the melting of aliphatic polylactone and polyester chains, Eur. Polym. J. 8 (1972) 449–463.

[39] W. Pfeil, P.L. Privalov, Thermodynamic investigations of proteins, Biophys. Chem. 4 (1976) 23–32.

[40] D. Blanco, M.J. Alonso, Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants, Eur. J. Pharm. Biopharm. 45 (1998) 285–294.

[41] H.F. Naguib, M.S. Abdel Aziz, S.M. Sherif, G.R. Saad, Synthesis and thermal characterization of poly(ester-ether urethane)s based on PHB and PCL-PEG-PCL blocks, J. Polym. Res. 18 (2010) 1217–1227.

[42] J. Yang, L. Jia, L. Yin, J. Yu, Z. Shi, Q. Fang, et al., A novel approach to biodegradable block copolymers of epsilon-caprolactone and delta-valerolactone catalyzed by new aluminum metal complexes., Macromol. Biosci. 4 (2004) 1092–1104.

[43] S.A.M. Ali, S.-P. Zhong, P.J. Doherty, D.F. Williams, Mechanisms of polymer degradation in implantable devices. I. Poly(caprolactone)., Biomaterials. 14 (1993) 648–656.

[44] H. Sun, L. Mei, C. Song, X. Cui, P. Wang, The in vivo degradation, absorption and excretion of PCL-based implant., Biomaterials. 27 (2006) 1735–1740.

[45] F.T. Meng, G.H. Ma, W. Qiu, Z.G. Su, W/O/W double emulsion technique using ethyl acetate as organic solvent: effects of its diffusion rate on the characteristics of microparticles., J. Control. Release. 91 (2003) 407–416.

[46] H. Sah, A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres, J Pharm Sci. 86 (1997) 1315–1318.

[47] M. van de Weert, J. Hoechstetter, W.E. Hennink, D.J. Crommelin, The effect of a water/organic solvent interface on the structural stability of lysozyme., J. Control. Release. 68 (2000) 351–359.

[48] W.L.J. Hinrichs, M.G. Prinsen, H.W. Frijlink, Inulin glasses for the stabilization of therapeutic proteins, Int. J. Pharm. 215 (2001) 163–174.

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Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly(ε-caprolactone) multiblock-copolymer implants

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European Journal of Pharmaceutics and Biopharmaceutics 2014, (87): 329-337

Abstract

In this study, the *in-vitro* release of proteins from novel, biodegradable phase-separated poly(ε-caprolactone)-PEG)-*b*-poly(ε-caprolactone), [PCL-PEG]-*b*-[PCL]) multiblock copolymers with different block ratios and with a low melting temperature (49 - 55 ˚C) was studied. The effect of block ratio and PEG content of the polymers (i.e. 22.5, 37.5 and 52.5 wt%) as well as the effect of protein molecular weight (1.2, 5.8, 14, 29 and 66 kDa being goserelin, insulin, lysozyme, carbonic anhydrase and albumin, respectively) on protein release was investigated. Proteins were spray-dried with inulin as stabilizer to obtain a powder of uniform particle size. Spray-dried inulin-stabilized proteins were incorporated into polymeric implants by hot melt extrusion. All incorporated proteins fully preserved their structural integrity as determined after extraction of these proteins from the polymeric implants. In general, it was found that the release rate of the protein increased with decreasing molecular weight of the protein and with increasing the PEG content of the polymer. Swelling and degradation rate of the copolymer increased with increasing PEG content. Hence, release of proteins of various molecular weights from [PCL-PEG]-*b*-[PCL] multi-block copolymers can be tailored by varying the PEG content of the polymer.

1. Introduction

The use of proteins and peptides as therapeutic agents has significantly increased in the last few decades. Since the 1980's more than 200 biopharmaceuticals have been registered [1]. However, despite extensive research, the development of patient-friendly dosage forms capable of maintaining the concentration of the proteins in plasma at pharmacologically effective levels for extended periods of time is still a major challenge [2]. Oral delivery of proteins and peptides has so far been unsuccessful, since it results in a low bioavailability due to degradation caused by gastrointestinal enzymes and low permeability through the gastrointestinal membrane. Therapeutic proteins are preferably administered parenterally as an aqueous solution, but due to their short half-life most proteins require frequent injection. For these reasons, research on the development of parenteral sustained release (or depot) formulations has expanded enormously during the last few decades. Biodegradable polymers such as poly(DL-lactide-co-glycolide) (PLGA) or poly(DL-lactide) (PDLA) have been widely applied as release controlling polymers in microparticle and implant-based depot formulations for peptides and proteins [2–4]. These polymers degrade in the body due to hydrolysis and are finally metabolized into water and carbon dioxide [5]. Sustained release depot formulations are typically administered parenterally via subcutaneous or intra-muscular injection, with the use of customized applicators [6,7]. Due to incompatibility of both the polymers and the drug molecules with standard sterilization procedures such as heat, autoclavation or radiation, these depot formulations are usually manufactured aseptically.

Microparticle-based sustained release depot formulations are typically manufactured via process routes that require the use of organic solvents to dissolve the polymers and aqueous media to extract the organic solvents from the microparticles. It is well known that the resulting organic solvent - water interfaces can have a devastating effect on the structural integrity and bioactivity of protein therapeutics. Processes such as molding, melt compression and hot melt extrusion offer the possibility for solvent free processing, which make them attractive alternatives for the manufacturing of sustained release depot formulations for proteins. However, these production processes often expose the proteins to heat and shear stresses. Hot melt extrusion of polymers like PLGA and PDLA requires high processing temperatures, which in combination with the shear stresses may lead to protein degradation [8].

In a previous study, we have synthesized novel hydrophilic multiblock co-polymers composed of semi-crystalline poly(ε-caprolactone) (PCL) blocks and amorphous blocks consisting of PCL and poly(ethylene glycol) (PEG). We have shown that the combination of this type of hydrophilic [PCL-PEG]-*b*-[PCL] polymers

and low temperature HME allows incorporation of proteins into the implants without protein degradation and controlled release of fully intact protein [9].

When formulating proteins and peptides into polymeric implants, one needs to take into account the various factors that affect the release kinetics. Protein release from polymeric matrices is governed by the physico-chemical properties of both polymer and drug, as well as by the conditions at the site of release. Among others, protein release is affected by protein charge [10], protein loading [9] and protein molecular weight [11]. Essential polymer properties include the degradation rate and swellability [12], which depend on composition and molecular weight of the polymer [13]. Although several studies have already been conducted on the importance of protein molecular weight on the release from polymeric depots [14–16], the aim of this study was to investigate in more detail the correlation between protein molecular weight and protein release from this new type of hydrophilic [PCL-PEG]-*b*-[PCL] polymers with different PEG contents. We synthesized [PCL-PEG]-*b*-[PCL] multiblock co-polymers with different [PCL-PEG] / [PCL] ratios and prepared protein-loaded implants thereof by HME. The release of five proteins with different molecular weight, i.e. goserelin (1.2 kDa), recombinant human insulin (5.8 kDa), lysozyme (14 kDa), carbonic anhydrase (29 kDa) and bovine serum albumin (66 kDa) from [PCL-PEG]-*b*-[PCL] polymers with varying PEG content was evaluated. Additionally, the degradation behavior of the polymers was studied to obtain more insight into the release mechanism of the proteins from these polymeric implants.

2. Materials and methods

2. 1. Materials

Goserelin acetate (Gos) was purchased from BCN (Barcelona, Spain). Lyophilized human recombinant insulin (Ins) (26.9 units/mg, ~ 5.8 kDa), lyophilized Lys (Lys) from chicken egg white (70,000 units/mg, ~14 kDa), lyophilized carbonic anhydrase (CA) from bovine erythrocytes (protein > 3500 W-A units/mg, ~29 kDa) and lyophilized albumin from bovine serum (BSA), (protein >96 %, ~66 kDa), Micrococcus Lysodeikticus, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride and trifluoroacetic acid (TFA) were all purchased from Sigma (St.Louis, Missouri, USA). Inulin 4000 g/mol was a gift from Sensus (Rosendaal, The Netherlands). Sodium azide, ε-caprolactone, 1,4-butanediol and 1,4-dioxane were purchased from Acros Organics (Geel, Belgium). Acetonitrile (HPLC gradient grade) was purchased from Biosolve® (Valkenswaard, The Netherlands). Hydrochloric acid

37 % (reagent grade) was purchased from VWR International Ltd. (Leicestershire, UK). Tri (hydroxymethyl) aminomethane was purchased from Merck (Darmstadt, Germany). 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, Buchs, Switzerland) were purchased from Sigma.

2. 2. Polymer synthesis

Low molecular weight poly(ε-caprolactone) (PCL) (Mw 4000 g/mol) and poly(ε-caprolactone)-PEG_{1500}-poly(ε-caprolactone) (PCL-PEG_{1500}) (Mw 2000 g/mol) prepolymers were synthesized by standard stannous octoate catalyzed ring-opening polymerization, as described previously [9]. Shortly, ε-caprolactone was dried over CaH_2 and distilled under reduced pressure in a nitrogen atmosphere prior to polymerization. PEG 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. The purity of distilled ε-caprolactone, 1,4-butanediol and 1,4-butanediisocyanate was confirmed by ^1H NMR ($CDCl_3$). PCL and PCL-PEG_{1500} prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare [poly(ε-caprolactone)-PEG_{1500}-poly(ε-caprolactone)]$_{2000}$-*b*-[poly(ε-caprolactone)]$_{4000}$ [PCL-PEG_{1500}]-*b*-[PCL] multiblock co-polymers with different [PCL-PEG_{1500}]/[PCL] block ratios (30/70, 50/50 and 70/30 w/w abbreviated as 30[PCL-PEG_{1500}]-70[PCL], 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL]) as described in [9]. Shortly, the prepolymers PCL and PCL-PEG_{1500} were introduced into a three-necked bottle under nitrogen atmosphere. 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. 1,4-Butanediisocyanate (BDI) was added and the reaction mixture was stirred mechanically overnight for 20h. 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 for all three multiblock co-polymers, showing effective removal of 1,4-dioxane by vacuum drying.

2. 3. Polymer characterization

^{1}H NMR was used to verify the overall ε-caprolactate / PEG (CL/PEG) monomer ratio of the multiblock co-polymers after synthesis and during degradation. ^{1}H NMR was performed on a VXR Unity Plus NMR spectrometer (Varian, California, USA) operating at 300 MHz. The d_1 waiting time was set to 20 s, and the number of scans was 16-32. ^{1}H NMR samples were prepared by dissolving 10 mg of multiblock co-polymer into 1 mL of deuterated chloroform ($CDCl_3$), and the spectrum was determined from 0 - 8 ppm using $CHCl_3$ present as trace element in $CDCl_3$ as reference. The CL/PEG molar ratio was calculated from the O-$CH_2CH_2CH_2CH_2\mathbf{CH_2}$C (O)- methylene group of PCL at δ 2.2-2.4 and the –$\mathbf{CH_2CH_2}$-O methylene groups of PEG at δ 3.6-3.7.

The intrinsic viscosity of multiblock co-polymers 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 copolymers 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.

The molecular weight of the multiblock co-polymers after synthesis and during degradation was determined using size exclusion chromatography coupled to refractive index detector (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 (50μL) 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 the multiblock co-polymers were measured by modulated 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, with 0.318 °C amplitude over a 60 s period. During the measurement, the sample cell was purged with nitrogen gas. The reversed heat flow was used to determine the glass transition temperature (midpoint of the transition, determined via inflection), and the total heat flow was used to determine the melting temperature (maximum of endothermic peak). The heat of fusion was calculated from the surface area of the melting endotherm.

2. 4. Spray-drying

Spray-drying was performed using a B-290 mini spray drier (Büchi Labortechnik AG, Flawil, Switzerland). Proteins (2.5 mg/ml) were separately dissolved in demineralized water and an aqueous solution of inulin (25 mg/ml) was added, to obtain a protein/inulin weight ratio of 1/10. 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 58 °C ± 3 °C. The obtained spray-dried powder was stored in a desiccator overnight until further use.

2. 5. Particle size measurements

The particle size distribution of the spray-dried samples was determined by laser diffraction. Small amounts of material (20 – 30 mg) were dispersed using a RODOS dispersing system at 3.0 bars. 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 (HME)

HME 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 10 - 20 rpm, developing a torque of 10 - 20 Nm. In total, ten formulations were prepared (**Table 1**). Powder blends (5.5 g) containing protein / inulin spray-dried particles and polymer at a weight ratio of 11/89 were manually mixed using a mortar and pestle and then fed into the preheated barrel of the extruder. The temperature used for extrusion was 55 °C for 30[PCL-PEG_{1500}]-70[PCL] and 49 °C for 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL]. A cylindrical die of 0.5 mm was used resulting in strands with a diameter of 0.35 mm (± 0.05), as measured with an in-line laser. Strands were cut into 1 cm x 0.35 mm pieces and stored in a freezer at -20°C until further evaluation. Polymer only implants (used for the degradation study) were prepared similarly by feeding the preheated extruder with polymer without any further additives.

Table 1. Implant formulations prepared by HME. Protein/inulin weight ratio is 1/10. Protein/inulin loading is 11 wt%.

Polymers / **Molecular weight of proteins**	**30[PCL-PEG_{1500}]-70[PCL] (22.5 wt% PEG)**	**50[PCL-PEG_{1500}]-50[PCL] (37.5 wt% PEG)**	**70[PCL-PEG_{1500}]-30[PCL] (52.5 wt% PEG)**
1.2 kDa	Gos/Inulin_30[PCL-PEG_{1500}]-70[PCL]	Gos/Inulin_50[PCL-PEG_{1500}]-50[PCL]	
5.8 kDa	Ins/Inulin_30[PCL-PEG_{1500}]-70[PCL]	Ins/Inulin_50[PCL-PEG_{1500}]-50[PCL]	
14 kDa	Lys/Inulin_30[PCL-PEG_{1500}]-70[PCL]	Lys/Inulin_50[PCL-PEG_{1500}]-50[PCL]	Lys/Inulin_70[PCL-PEG_{1500}]-30[PCL]
29 kDa		CA/Inulin_50[PCL-PEG_{1500}]-50[PCL]	
66 kDa		BSA/Inulin_50[PCL-PEG_{1500}]-50[PCL]	BSA/Inulin_70[PCL-PEG_{1500}]-30[PCL]

2. 7. Polymer mass loss and water content during degradation

The degradation of polymeric implants without incorporated proteins (prepared as described in section 2.6) was studied to obtain more insight into the role of polymer degradation in the release mechanism. About 130 mg of polymeric implants (n = 3) was placed in plastic vials and 25 mL of 100 mM phosphate buffer (pH 7.4, 100 mM, 9.1 mM NaCl, 0.02 wt% NaN_3) was added to each vial. The vials were then incubated in an oven at 37 ºC. At various time intervals, samples were removed from the buffer and washed with ultra pure water over 0.45 μm filters to remove the buffer salts. Adherent water was removed from the implants with a tissue where after their wet mass ($m_{wet,t}$) was determined. Samples were then dried in a desiccator and additionally in a vacuum oven for 24 h until constant mass ($m_{dry,t}$). Water content was measured after the implants had reached an equilibrium value (after 1 day); water content and remaining mass were determined using Eq. (1) and (2), respectively.

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

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

Where $m_{dry,0}$ and $m_{dry,t}$ are the masses of the dry sample at day 0 and dry sample at day t.

In-vitro degradation of the 30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL] polymers was studied for 140 days. However, the 70[PCL-PEG_{1500}]-30[PCL] polymer was assessed for its degradation characteristics for only 7 days, since partial dissolution of the polymer occurred after that period of time.

2. 8. Analysis of protein content

To determine the actual protein content and structural integrity of protein after extrusion, proteins were extracted from the implants. Samples randomly taken from the extrusion run were weighed and to each of the samples 1.5 ml of acetonitrile was added to dissolve the polymer. After the polymer was fully dissolved, the sample was centrifuged (Microcentrifuge SIGMA 1-14, Shropshire, United Kingdom) and the supernatant consisting of dissolved polymer was removed. The procedure was repeated three times and the remaining protein pellet was dried in a desiccator overnight to remove residual solvent. The protein pellet was dissolved in 100 mM phosphate buffer (pH 7.4, 9.1 mM NaCl, 0.02 wt% NaN_3) and analyzed as described in section 2.9. 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 the protein could be fully recovered and remained structurally intact by the extraction procedure.

2. 9. In-vitro release testing

Protein containing implants (70 - 90 mg, n = 3) were weighed into glass tubes, and 1.5 mL of 100 mM phosphate buffer (pH 7.4, 9.1 mM NaCl, 0.02 wt% NaN_3) was added. Thereafter, the tubes were capped and placed in a shaking water bath (GFL, model 1083, Burgwerdel, Germany) thermostated at 37°C, and incubated for a total of 160 days. After various time intervals, 1.25 mL aliquots were removed for HPLC analysis, as described in section 2.10, and refreshed. The cumulative amount of released protein within 4h was considered as a burst release.

To elucidate the mechanisms of release of the proteins from the three different polymers, release data were plotted into Korsmeyer-Peppas' model (Eq. (3)).

$$Q_t / Q_0 = K_p \times t^n \quad (3)$$

where Q_t is amount of drug released in time t, Q_0 is initial amount of the drug in the implant, t is time, n is diffusional exponent indicative of the transport mechanism and K_p is Korsmeyer-Peppas constant incorporating structural and geometric characteristics of the dosage form.

Using Peppas' model, the diffusional exponent n was calculated from the fitted linear regression lines of log percent of drug released versus log time (n was equal to the slope taken for the first 60 % of drug released). When $n \leq 0.45$ for a cylindrically shaped release system, then the drug is released by Fickian diffusion. For n values between 0.45 and 0.89, non-Fickian or anomalous transport is the predominant mechanism of release, where both drug diffusion and polymer swelling and erosion

play a role. For n = 0.89 (Case II transport) release does not depend on time and it corresponds to zero order kinetics. Finally, values of n > 0.89 are indicative for a release mechanism known as super case II transport where the release is governed by high polymer swelling and thus diffusion of the drug [17–19]. Coefficients of determination (R^2) were calculated for all formulations as a measure of linearity of data sets.

Further, using a mathematical model that describes the similarity factor f_2, two similar data sets can be compared (Eq.(4)). The similarity factor f_2 equals 100 when two data sets are equivalent, while 10 % of difference between sets would give an f_2 value of 50. Therefore, it is accepted that two data sets are highly similar or equivalent if the f_2 value is between 50 and 100, while different if the $f_2 < 50$ [18].

$$f_2 = \log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{t=1}^{n}(R_t - T_t)^2\right]^{-0.5} x\ 100\right\} \quad (4)$$

2. 10. Protein quantification measurements

The concentration of the proteins Lys, CA and BSA in aqueous solutions was determined by RP-HPLC using a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, California), protein detection at 280 nm and a C18 ProZap LC/MS reversed phase column (Grace Davidson, Deerfield, IL, USA) (20 mm x 4. 6 mm, 1. 5 μm). The composition of the mobile phase was 0.1 vol% TFA in acetonitrile (eluent A) and 0.1 vol% TFA in millipore water (eluent B). The quantification of the proteins was carried out in gradient elution mode using the following scheme: 0 min - 1 min 30 vol% A, 70 vol% B; 1 – 3 min 60 vol% A – 40 vol% B; 3.01 - 6 min 30 vol% A, 70 vol% B. The retention time of Lys, BSA and CA was 1.19 min, 1.29 min and 1.57 min, respectively. The concentration of Gos was determined by RP-HPLC using a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA). Waters C18 reverse phase column (4.6 mm x 150 mm, 5 μm) (Milford, MA, USA) was used in isocratic mode using 0.1 vol% TFA in 76/24 v/v water / acetonitrile as eluent. The concentration Gos was determined spectrophotometrically at a wavelength of 214 nm. Ins was quantified using a UV-VIS method, with Unicam UV500 spectrophotometer (Madison, USA). Absorbance of Ins was recorded at wavelength of 226 nm using a quartz cuvette (L=10 mm).

2. 11. Structural integrity of the proteins

Since the various proteins have a different sensitivity for temperature (and shear stress), it is important to determine their structural integrity after spray-drying and HME. The integrity of Gos was evaluated using the RP-HPLC method as described

in section 2.10 [3]. The structural integrity of Ins was assessed by RP-HPLC [20] using a mobile phase of 10/70/20 v/v/v acetonitrile / Millipore water / Na_2SO_4 (Eluent A, 2M, pH 2.3) and 40/40/20 acetonitrile / Millipore water / Na_2SO_4 (Eluent B). Ins was measured using an isocratic mode using 35 vol% of eluent A and 65 vol% of eluent B. The concentration of Ins was determined spectrophotometrically at a wavelength of 214 nm. The biological activity of Lys was measured with a turbidimetric assay [21] modified for a plate-reader as described previously [9]. The activity of CA was measured by a method as described by Wilbur and Anderson [22]. The assay is based on the time taken by saturated CO_2 solution to drop the pH of a 0.02 M Tris-HCl buffer from 8.3 to 6.3 at 0 ºC. The total activity (A_{tot}) of CA was calculated using Eq. (5). A calibration curve was established using aqueous solutions of CA of various concentrations. The retained activity (A_{ret}) of CA after spray-drying and HME was determined using Eq. (6).

$$A_{tot} = \frac{t_0 - t}{t} \quad (5)$$

Where t and t_0 are the times recorded with and without enzyme, respectively.

$$A_{ret} = \frac{C_A}{C_0} \times 100\% \quad (6)$$

Where C_A is the active concentration of extracted CA from samples after HME and C_0 is the concentration of the CA control.

Since no biological assay for BSA is available, its integrity after processing was studied using the RP-HPLC method as described in section 2.10 (determination of additional peaks) and SDS-PAGE as described by Laemmli [23] to evaluate whether aggregation occurred. Electrophoresis was performed at a constant current mode of 100 volts and gels were stained with Coomassie brilliant blue.

3. Results

3. 1. Polymer characterization

^{1}H NMR analysis confirmed that the CL/PEG molar ratios of the [PCL-PEG]-*b*-[PCL] multi-block copolymers were in line with the in weight values (**Table 2**) indicating that the polymer compositions were according to the targets. DSC thermograms confirmed the phase-separated nature of the multiblock copolymers, showing a glass transition temperature (*Tg*) between -60 and -56 °C (**Figure 1b**), which can be attributed to phase-mixed amorphous PEG and PCL. 30[PCL-PEG_{1500}]-70[PCL]

and 50[PCL-PEG_{1500}]-50[PCL] exhibited two melting endotherms, which can be attributed to melting of PEG crystals at 13 °C and 40 °C and to melting of PCL crystals at 50 – 56 °C (**Figure 1a**). For 70[PCL-PEG_{1500}]-30[PCL] polymer, however, three instead of two melting endotherms were observed. The melting peaks at 25 °C and 40 °C can be attributed to melting of two crystalline modifications of PEG as described previously [24]. The third melting endotherm at 60 °C can be ascribed to melting of PCL crystals. The crystallinity of the PCL block was calculated to be 58 %, 70 % and 50 % for 30[PCL-PEG_{1500}]-70[PCL], 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL], respectively, assuming a heat of fusion *(*ΔH) of 139.9 J/g for 100 % crystalline PCL [25]. The crystallinity of the PEG domains was 16 %, 23 %, 41 % for 30[PCL-PEG_{1500}]-70[PCL], 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL], respectively, assuming a ΔH of 165.5 J/g for 100 % crystalline PEG [26]. The melting temperature of PEG (*Tm*) increased with increase in [PCL-PEG_{1500}] / [PCL] block ratio. We propose that this increase in *Tm* is due to the formation of larger and more perfect PEG crystals at higher PEG content [24], [25]. This is supported by the increasing melting enthalpy of the PEG crystals from 14 J/g for 30[PCL-PEG_{1500}]-70[PCL] to 53 J/g for 70[PCL-PEG_{1500}]-30[PCL]. The intrinsic viscosity and the molecular weight of the multiblock copolymers, as determined by SEC, are shown in **Table 2**. SEC was performed relative to PEG standards and thus apparent molecular weight values were obtained. Number averaged molecular weight of the multiblock copolymers was in the range of 26 x 10^3; 28 x 10^3 and 13 x 10^3 g/mol for 30[PCL-PEG_{1500}]-70[PCL], 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL], respectively, while PCL-PEG_{1500} and PCL prepolymers have molecular weight of 2000 and 4000 g/mol, respectively, again confirming the successful chain-extension of the prepolymers.

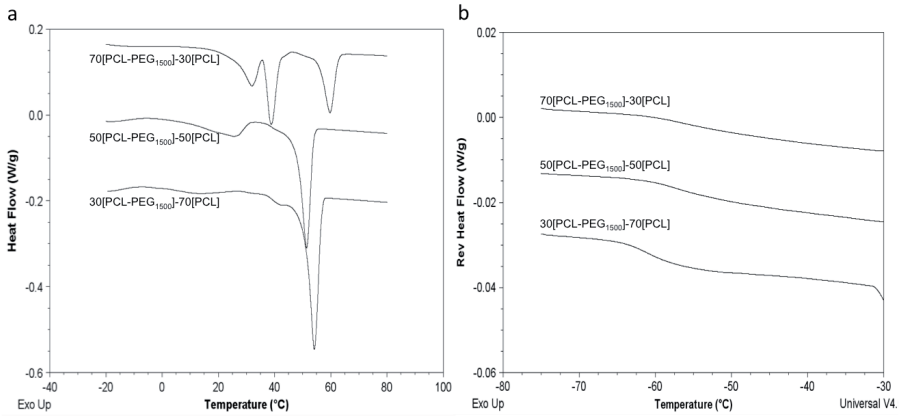

Figure 1. Thermograms of multiblock copolymers;
a) total heat flow -40 to 100 °C showing the melting endotherm of PEG and PCL;
b) reversing heat flow showing the glass transition of PCL

*Table 2. Characterization of the multiblock-copolymers. *Determined after HME.*

Polymers	IV (dL/g)	CL/PEG molar ratio (-)		Tg (°C)	Tm (°C)		H (J/g)		Cryst. PCL (%)	Molecular weight * (g/mol)		PDI*
		H NMR	In-weights		PEG	PCL	PEG	PCL		Mn	Mw	
30[PCL-PEG_{1500}]-70[PCL]	0.84	45	44.2	-60	13	54	14	54	58	25.664	48.287	1.88
50[PCL-PEG_{1500}]-50[PCL]	0.75	19.6	21.3	-56	21	50	33	68	70	28.464	54.233	1.79
70[PCL-PEG_{1500}]-30[PCL]	0.41	10.1	11.1	-59	30	56	53	19	50	12.965	22.316	1.80

3. 2. Polymer degradation

The mass loss after 140 days of polymer-only implants with low (30[PCL-PEG_{1500}]-70[PCL], 22.5 %) and intermediate PEG content (50[PCL-PEG_{1500}]-50[PCL], 37.5 %) was 8 % and 15 %, respectively (**Figure 2**). The polymer with the highest PEG content (70[PCL-PEG_{1500}]-30[PCL], 52.5 % PEG) lost around 52 % of its mass already within one day and only marginal additional mass loss occurred within the next 6 days. Unfortunately, handling of this polymer was difficult due to its high swellability. Therefore, and because all incorporated protein was released from this polymer already within 7 days, the degradation study of this polymer was terminated after 7 days.

With increasing amounts of PEG incorporated in the polymer, the water content slightly increased, i.e. 56 % for 30[PCL-PEG_{1500}]-70[PCL], 60 % for 50[PCL-PEG_{1500}]-50[PCL], to 78 % for 70[PCL-PEG_{1500}]-30[PCL], after 1 day of incubation. Water content remained constant during 140 days.

SEC was done to determine molecular weight decrease of polymer during degradation. It was observed that polymer 30[PCL-PEG_{1500}]-70[PCL] showed only minor molecular weight decrease, while for 50[PCL-PEG_{1500}]-50[PCL] molecular weight decrease started gradually and proceeded to 58 % of its original value in 140 days. Polymer 70[PCL-PEG_{1500}]-30[PCL] showed a small increase in average molecular weight during 7 days. The polydispersity index (M_w / M_n) remained around 1.8 for all samples during degradation as shown in **Table 2**.

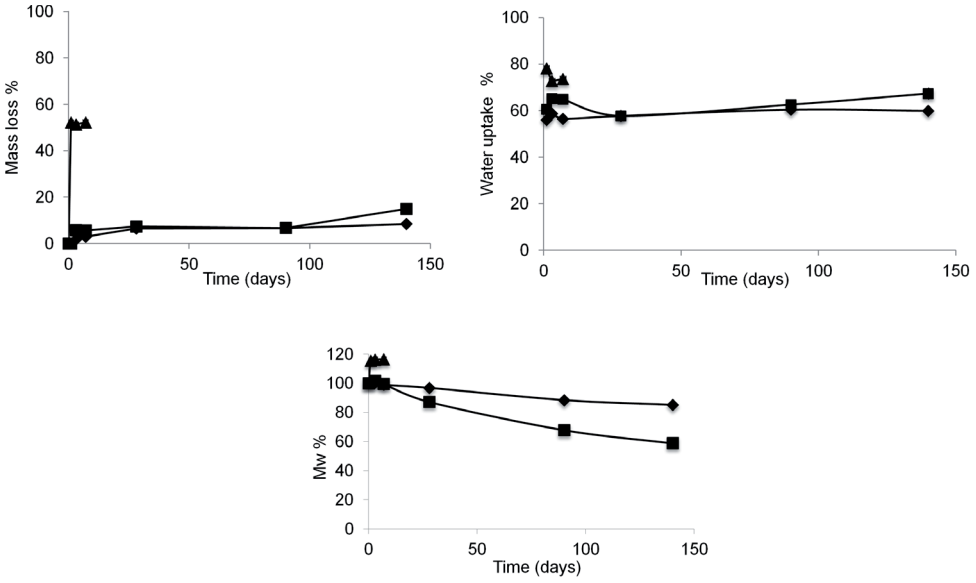

Figure 2. Mass loss (%), water uptake (%) and molecular weight loss (%) of multiblock co-polymers 30[PCL-PEG_{1500}]-70[PCL] (◆), 50[PCL-PEG_{1500}]-50[PCL] (■) and 70[PCL-PEG_{1500}]-30[PCL] (▲) during degradation.

3. 3. Spray-drying, HME and determination of protein content

Spray-drying of protein / inulin solutions resulted in all cases in a powder with an average particle size of around 2.5 μm, while span values indicated narrow size distributions within the batch (results not shown). Further, as expected, HME of [PCL-PEG]-*b*-[PCL] polymers could be performed at temperatures between 49 and 55 °C. Extraction of the proteins from different parts of the polymeric implants showed uniform distribution of the protein / inulin powders within the polymeric matrices as indicated by the small standard deviation, with actual loading varying only slightly from the theoretical loading (1 %) as shown in **Table S1** (supporting information). The slightly lower loading of insulin found after extraction was attributed to less effective extraction of insulin from the implants.

3. 4. Protein stability

The integrity of the proteins was studied after spray-drying and HME (n = 3). Gos and Ins were analyzed by RP-HPLC, because degradation of both substances can be observed by the presence of additional peaks in chromatograms. For both Gos and Ins extracted from different polymers, 30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL], no additional peaks were observed as compared to the control (data not shown). The stability of Lys and CA was studied by both RP-HPLC and bioactivity assays. Also in these cases no additional peaks in RP-HPLC chromatograms were observed (data not shown). The activity of Lys was found to be 100 ± 4.7 and 104 ± 8.1 % for Lys extracted from the 30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL] polymer, respectively. Regarding CA, measured activity of protein extracted from the 50[PCL-PEG_{1500}]-50[PCL] polymer was found to be 99 ± 1.4 %, indicating no loss in activity after spray-drying and HME. The integrity of extracted BSA was investigated using SDS-PAGE. As shown in **Figure S1** (supporting information), a single band corresponding to the molecular weight of BSA, i.e. 66 kDa, was found, which indicates preservation of the integrity of BSA after spray-drying and HME with both 50[PCL-PEG_{1500}]-50[PCL] and 70[PCL-PEG_{1500}]-30[PCL].

3. 5. In-vitro protein release

In-vitro release of the proteins from the [PCL-PEG]-*b*-[PCL] implants was studied during 160 days. Polymer 30[PCL-PEG_{1500}]-70[PCL] (with 22.5 wt% PEG) released 59 % of Gos in the first 4h, after which a slightly slower release of Gos was noticed which accumulated to 100 % within 8 days. Release of Ins from 30[PCL-PEG_{1500}]-70[PCL] was slower and more continuous with 8 % of burst and release completed

within 80 days. Finally, release of Lys from this polymer was slowest, with 13 % burst and continuous release of 90 % of protein within 160 days (**Figure 3a**).

Polymer 50[PCL-PEG$_{1500}$]-50[PCL] (with intermediate PEG content, 37.5 wt%) also showed an increased release rate when the molecular weight of the protein was decreased (**Figure 3b**). The fastest release was observed for Gos (burst release 53 %, complete release in 8 days), followed by biphasic release of Ins (burst release 12 %, complete release in 80 days), continuous release of Lys (4 % burst, 100 % released in 160 days) and biphasic release of CA (the lag phase followed by the release of 80 % of protein in 160 days). Additionally, only around 3 % of release was noticed for the largest protein, i.e. BSA. The polymer with the highest PEG content (52.5 wt%), 70[PCL-PEG$_{1500}$]-30[PCL], released more than 60 % of Lys in the first 4 hours. The remaining protein was released within the next 7 days. Also, rapid release was observed for BSA, i.e. 40 % of protein was released after 4h and all protein was released within 7 days (**Figure 3c**). Because the release profiles of Gos as well as Ins from implants prepared from either 30[PCL-PEG$_{1500}$]-70[PCL] or 50[PCL-PEG$_{1500}$]-50[PCL] looked quite similar, the release data were inserted into the f_2 equation. It was found that, for Gos, f_2 was 72, indicating high similarity or equivalence between release of Gos from both polymers. f_2 value for Ins was 55, showing similarity as well (but to a lower extent) between the two release profiles.

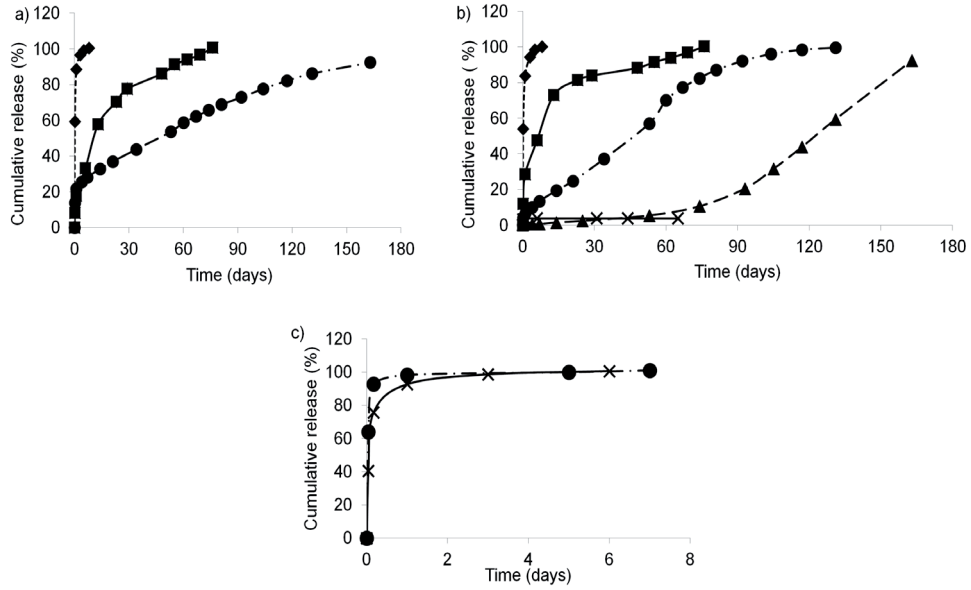

Figure 3. Release profiles of proteins from a) 30[PCL-PEG$_{1500}$]-70[PCL], b) 50[PCL-PEG$_{1500}$]-50[PCL] and c) 70[PCL-PEG$_{1500}$]-30[PCL], Gos (◆),Ins (■), Lys (●),CA (▲), BSA (×).

To obtain a better understanding of the mechanisms of release from the different formulations, all release data were fitted into Korsmeyer-Peppas model (**Table 3**). Except for the release date of Gos from 30[PCL-PEG_{1500}]-70[PCL], the relatively high R^2 of 0.934 – 0.957 for Gos for 50[PCL-PEG_{1500}]-50[PCL] and Ins from both 30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL] indicate a good fit into this model. The n value was lower than 0.45, which implies that the release was diffusion controlled. The release of Lys from both polymers showed lower values for R^2 of 0.913 and 0.861, while an n value of 0.239 for 30[PCL-PEG_{1500}]-70[PCL] and 0.474 for 50[PCL-PEG_{1500}]-50[PCL], suggested diffusion based release and anomalous transport, respectively. CA, having a lag phase of 45 days, showed a poor fit into the model. The diffusional exponent n = 0.597 indicated anomalous transport of this protein through the polymer matrix. From 70[PCL-PEG_{1500}]-30[PCL], all proteins were released very fast, therefore fitting only 60 % of drug release into model could not provide enough sampling points for meaningful conclusion.

Table 3. Coeficients of determination (R^2) and diffusional exponent (n) for the Korsmeyer-Peppas model. For highly swellable 70[PCL-PEG_{1500}]-30[PCL], not enough sampling points were available (n.a)

Formulation	**R^2**	**n**
Gos/Inulin_30[PCL-PEG_{1500}]-70[PCL]	0.904	0.176
Gos/Inulin_50[PCL-PEG_{1500}]-50[PCL]	0.934	0.200
Ins/Inulin_30[PCL-PEG_{1500}]-70[PCL]	0.976	0.430
Ins/Inulin_50[PCL-PEG_{1500}]-50[PCL]	0.957	0.385
Lys/Inulin_30[PCL-PEG_{1500}]-70[PCL]	0.913	0.239
Lys/Inulin_50[PCL-PEG_{1500}]-50[PCL]	0.861	0.474
Lys/Inulin_70[PCL-PEG_{1500}]-30[PCL]	n. a.	n. a.
CA/Inulin_50[PCL-PEG_{1500}]-50[PCL]	0.555	0.597
BSA/Inulin_70[PCL-PEG_{1500}]-30[PCL]	n. a.	n. a.

4. Discussion

In this study, we synthesized novel phase-separated [PCL-PEG]-*b*-[PCL] multiblock co-polymers with varying PEG content and we showed that these polymers can be extruded at relatively low temperatures of 49 - 55 °C. Furthermore, we have shown that various proteins, spray-dried in the presence of inulin, can be incorporated in these polymers by HME without losing structural integrity. Moreover, it was found

that the *in-vitro* release rate of the proteins from [PCL-PEG]-*b*-[PCL] implants increased with decreasing molecular weight of the protein and with increasing PEG content of the polymer.

DSC measurements showed that the multiblock co-polymers were phase separated with crystalline PCL domains, amorphous mixed PCL / PEG domains and crystalline PEG domains. However, in an aqueous environment, the crystallinity of PEG will be lost due to water uptake. The crystalline PCL domains, being insoluble in water, will act as physical cross-links and ensure controlled swelling. Water uptake experiments on polymer implants during degradation under *in-vitro* conditions showed that the swelling and degradation rate of the polymer was controlled by the [PCL-PEG_{1500}] / [PCL] block ratio and thus by the PEG content. Swelling and degradation rate was limited for two polymers containing lower PEG amount (30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL]), while for the polymer with the highest PEG content (70[PCL-PEG_{1500}]-30[PCL]), and thus with increased percentage of the amorphous block, the swelling and degradation rate of the polymer was much higher. Increased diffusion of water into the polymer resulted in a faster degradation of ester bonds, and thereby a faster decrease in mass and molecular weight of the polymer. It was observed that both 30[PCL-PEG_{1500}]-70[PCL] and 50[PCL-PEG_{1500}]-50[PCL] exhibited a low degradation rate. This is not surprising when keeping in mind that caprolactone based polymers slowly degrade *in-vitro* and *in-vivo* [27,28]. In contrast, 70[PCL-PEG_{1500}]-30[PCL] lost 50 % of its mass already after 1 day. However, as explained before, it is unlikely that this is caused by degradation. Most likely, due to the small fraction of crystalline PCL block, the hydrophilic amorphous blocks exhibit unlimited swelling behavior and simply dissolve completely, leaving semi-crystalline PCL attached to remaining amorphous blocks. During degradation of this polymer, a small increase in average molecular weight of 70[PCL-PEG_{1500}]-30[PCL] might be explained by dissolution of smaller molecular weight chains of this polymer.

The preservation of integrity of all proteins during both spray-drying and HME can be attributed to the stabilizing effect of inulin [29–31] in combination with the relatively low extrusion temperature [9] .

Drug release from polymeric matrices depends on many factors. For bulk degrading polymers, the release is governed by drug solubility, drug diffusion, polymer swelling and / or polymer degradation, or a combination of these factors [32]. Furthermore, the rate of drug diffusion is dependent on the size of the drug compared to the polymer mesh size. Additionally, the particle size of the encapsulated drug is equally important for the release rate, as shown previously [9]. Therefore, in the present study, to avoid this additional influence of the particle size on the protein release, all protein / sugar samples were spray-dried to obtain powder particles with a

uniform and narrow size distribution. The polymer mesh size is highly dependent on the degree of swelling and the extent of degradation of the polymer matrix. Further, the rate of drug diffusion depends on the diffusion constant of the drug in the water-filled pores. For smaller molecules, diffusion is faster providing faster release.

To obtain more information on the mechanisms of the protein release from the multiblock co-polymers, the release data were fitted into the Korsmeyer-Peppas model. This model is suitable to analyze the release of drugs from pharmaceutical products of which the release mechanism is not well known or of which the release is governed by more than one mechanism [18]. In this model the value of n (diffusional exponent) is considered to give an indication of the release mechanisms for different geometry of matrices for moderately swelling polymers.

If the polymer mesh size is equal or larger than the size of the drug it seems quite obvious that the drug can rapidly diffuse out of the polymer matrix, which was observed for the smallest peptide Gos from 30[PCL-PEG_{1500}]-70[PCL]. For this small molecule, the amount of PEG incorporated in the polymer matrix, and therefore polymer swelling, did not play a role in release kinetics. The slightly higher molecular weight protein Ins showed more continuous release from this polymer. Lys, as a higher molecular weight protein, showed slower and continuous release during the 160 days, which was the total time the release was monitored.

The Peppas model showed an n value of 0.45 for the release of Gos, Ins as well as Lys from 30[PCL-PEG_{1500}]-70[PCL], suggesting a Fickian diffusion-based release mechanism. This suggests that these proteins were sufficiently small to pass through the polymer meshes [33]. The decreased release rate with increased drug size can be explained by a combination of inherent slower diffusion in water for larger proteins, but also by increased diffusion resistance through the polymer meshes, as the polymer mesh size is a distribution. The diffusion controlled mechanism is supported by the fact that 60 % of the protein was already released before any molecular weight decrease occurred for 30[PCL-PEG_{1500}]-70[PCL] and that water content was constant during the release experiment. This observation rules out the occurrence of degradation or swelling controlled release mechanisms.

Similarly, the release of Gos and Ins from 50[PCL-PEG_{1500}]-50[PCL] implants showed instantaneous release and equal kinetic behavior as 30[PCL-PEG_{1500}]-70[PCL] based implants, which was confirmed by the similarity factor f_2. This was also perceived for Lys, where the release from 50[PCL-PEG_{1500}]-50[PCL] was slower than that found for the smaller proteins, but more rapid than the release of Lys from the less swellable 30[PCL-PEG_{1500}]-70[PCL], demonstrating that polymer swelling is important for the release of larger proteins. When increasing the protein size to 29 kDa i.e. CA, a lag phase occurred, followed by continuous protein

release. In addition, the molecular weight loss of this polymer was faster than that of 30[PCL-PEG_{1500}]-70[PCL] indicating a faster degradation rate. Apparently, a certain degradation (or surface erosion) of the polymer matrix was needed before the protein could be released from the implants after which it was released by dissolution, which has also been previously shown by others [30].

Fitting the data into the Korsmeyer-Peppas model showed that Gos and Ins showed release from 50[PCL-PEG_{1500}]-50[PCL] based on Fickian diffusion ($n < 0.45$), similar to 30[PCL-PEG_{1500}]-70[PCL]. The Korsmeyer-Peppas model showed an n value of 0.474 for release of Lys from 50[PCL-PEG_{1500}]-50[PCL], suggesting anomalous transport, while for 30[PCL-PEG_{1500}]-70[PCL] it was found that $n < 0.45$. In contrast to 30[PCL-PEG_{1500}]-70[PCL], 50[PCL-PEG_{1500}]-50[PCL] did show certain degradation during the period that 60 % of Lys was released. Water uptake and thus swelling remained constant during the release experiment. Therefore, besides diffusion, probably also degradation has contributed to the release of Lys from 50[PCL-PEG_{1500}]-50[PCL]. The n values for the release of CA from the same polymer ($n = 0.597$) suggested anomalous transport similar to Lys, where besides diffusion the release also depended on polymer degradation.

Obviously, when the polymer mesh size was smaller than the drug, the diffusion constant of the drug in the polymer matrix and thus the rate of release of the drug depended on the rate of degradation. Only when polymer chain scission continued causing the molecular weight of polymer to decrease, the protein started to diffuse out of the polymer matrix. Therefore, bulk degradation of the polymer and formation of interconnecting channels in the polymer matrix were necessary for larger molecular weight proteins to be released. However, BSA being the largest protein used in this study (66 kDa) showed no release at all from this polymer during 160 days. Most likely, degradation needs to proceed further causing more significant molecular weight and mass loss and therefore larger polymer mesh size for BSA to be released [33].

Our findings correlate well with studies done before [34], [35], where it was shown that the initial release of proteins from hydrogels depended on the hydrodynamic diameter of the encapsulated proteins, compared to the polymer mesh size, while subsequent release depended on polymer degradation. In a recent study by Lavin et al. the release of Ins, Lys, and BSA from wet spun microfibers was investigated. They confirmed that the release behavior was primarily driven by diffusion and depended on molecular weight, with smaller proteins releasing faster in the initial phase, and achieving a higher cumulative released fraction [16].

Evaluating the polymer with highest PEG content, 70[PCL-PEG_{1500}]-30[PCL], both Lys and BSA were released within 8 days, whereas the larger molecular weight

protein BSA showed a slightly slower release rate than Lys. More than 50 % of mass loss indicated dissolution of the amorphous part of this polymer and consequently dissolution of the protein, which was assumed to be preferentially incorporated in the amorphous phase of the polymer matrix.

In conclusion, in this study we demonstrated that phase separated [PCL-PEG]-*b*-[PCL] multiblock co-polymers can successfully be used as a matrix for the incorporation of proteins by HME at temperatures as low as 49 - 55 °C, which could be beneficial for thermolabile proteins. As expected, all proteins retained their structural integrity, which could be ascribed to incorporation into the glassy matrix of inulin [30]. Hence, we were able to demonstrate that indeed molecular weight of the protein and PEG content of the polymer are factors of utmost importance in regulating protein release from polymeric matrix implants. Careful matching of both aspects is essential in designing the optimal delivery depot with tailored protein release.

Acknowledgments

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

Supporting information:

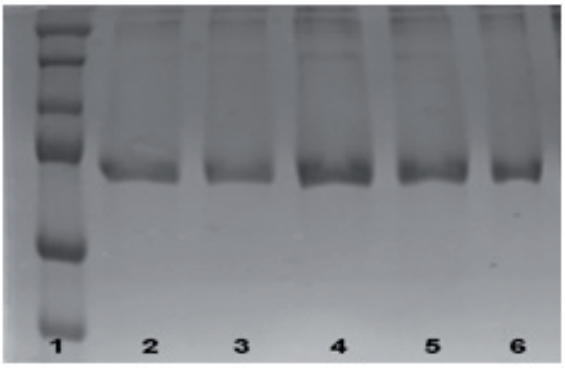

Figure S1. SDS-PAGE results of BSA samples. Lanes: 1 Molecular weight marker; 2 and 3 extracted BSA from 50[PCL-PEG1500]-50[PCL]; 4 and 5 extracted BSA from 70[PCL-PEG1500]-30[PCL]; 6 native BSA.

Table S1. Actual protein loading of implants (%) as determined by extraction (n = 6).

	Formulation	**Average loading (% ± SD)**
1	Gos/Inulin_30[PCL-PEG$_{1500}$]-70[PCL]	1.10 ± 0.09
2	Gos/Inulin_50[PCL-PEG$_{1500}$]-50[PCL]	1.10 ± 0.08
3	Ins/Inulin_30[PCL-PEG$_{1500}$]-70[PCL]	0.76 ± 0.09
4	Ins/Inulin_50[PCL-PEG$_{1500}$]-50[PCL]	0.77 ± 0.02
5	Lys/Inulin_30[PCL-PEG1500]-70[PCL]	0.98 ± 0.03
6	Lys/Inulin_50[PCL-PEG$_{1500}$]-50[PCL]	1.03 ± 0.03
7	Lys/Inulin_70[PCL-PEG$_{1500}$]-30[PCL]	1.06 ± 0.01
8	CA/Inulin_50[PCL-PEG$_{1500}$]-50[PCL]	0.85 ± 0.02
9	BSA/Inulin_50[PCL-PEG$_{1500}$]-50[PCL]	0.81 ± 0.02
10	BSA/Inulin_70[PCL-PEG$_{1500}$]-30[PCL]	0.89 ± 0.05

References:

[1] G. Walsh, Biopharmaceutical benchmarks 2010, Nat. Biotechn. 28 (2010) 917–924.

[2] J.L. Cleland, R. Langer, Formulation and Delivery of Proteins and Peptides, in: J.L. Cleland, R. Langer (Eds.), Formul. Deliv. Proteins Pept., 1994: pp. 1–19.

[3] A. Kovalchuk, W. Fischer, M. Epple, Controlled release of goserelin from microporous polyglycolide and polylactide., Macromol. Biosci. 5 (2005) 289–298.

[4] H. Okada, One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate., Adv. Drug Deliv. Rev. 28 (1997) 43–70.

[5] L.S. Nair, C.T. Laurencin, Biodegradable polymers as biomaterials, Prog. Polym. Sci. 32 (2007) 762–798.

[6] J.C. Wright, A.S. Hoffman, Long Acting Injections and Implants, in: J.C. Wright, D.J. Burgess (Eds.), Adv. Deliv. Sci. Technol., Springer US, Boston, MA, 2012: pp. 11–25.

[7] http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/019726s054,020578s032lbl.pdf NDA 019726/S-054 Zoladex (goserelin acetate implant), (n.d.).

[8] M.A. Repka, S. Majumdar, S.K. Battu, R. Srirangam, S.B. Upadhye, Applications of hot-melt extrusion in drug delivery, Expert Opin. Drug Deliv. 5 (2008) 1357–1376.

[9] M. Stanković, H. de Waard, R. Steendam, C. Hiemstra, J. Zuidema, H.. H.W. Frijlink, W.L.J.Hinrichs, Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery, Eur. J. Pharm. Sci. 49 (2013) 578–587.

[10] D. Blanco, M.J. Alonso, Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants, Eur. J. Pharm. Biopharm. 45 (1998) 285–294.

[11] D. Bodmer, T. Kissel, E. Traechslin, Factors influencing the release of peptides and proteins from biodegradable parenteral depot systems, J. Control. Release. 21 (1992) 129–137.

[12] L. Youxin, C. Volland, T. Kissel, In-vitro degradation and bovine serum albumin release of the ABA triblock copolymers consisting of poly (L(+) lactic acid), or poly(L(+) lactic acid-co-glycolic acid) A-blocks attached to central polyoxyethylene B-blocks, J. Control. Release. 32 (1994) 121–128.

[13] M. Lück, K.F. Pistel, Y.X. Li, T. Blunk, R.H. Müller, T. Kissel, Plasma protein adsorption on biodegradable microspheres consisting of poly(D,L-lactide-co-glycolide), poly(L-lactide) or ABA triblock copolymers containing poly(oxyethylene). Influence of production method and polymer composition., J. Control. Release. 55 (1998) 107–120.

[14] E.A. Appel, X.J. Loh, S.T. Jones, C. A. Dreiss, O. A. Scherman, Sustained release of proteins from high water content supramolecular polymer hydrogels., Biomaterials. 33 (2012) 4646–4652.

[15] M. Sandor, D. Enscore, P. Weston, E. Mathiowitz, Effect of protein molecular weight on release from micron-sized PLGA microspheres, J. Control. Release. 76 (2001) 297–311.

[16] D.M. Lavin, L. Zhang, S. Furtado, R. A. Hopkins, E. Mathiowitz, Effects of protein molecular weight on the intrinsic material properties and release kinetics of wet spun polymeric microfiber delivery systems., Acta Biomater. 9 (2013) 4569–4578.

[17] P.L. Ritger, N.A. Peppas, A simple equation for description of soulte release from swellable devices, J. Control. Release. 5 (1987) 37–42.

[18] P. Costa, J.M. Sousa Lobo, Modeling and comparison of dissolution profiles., Eur. J. Pharm. Sci. 13 (2001) 123–133.

[19] N.. Thomas, A.H. Windle, A theory of Case II diffusion, Polymer (Guildf). 23 (1982) 529–542.

[20] B.K. Nyambura, I.W. Kellaway, K.M.G. Taylor, Insulin nanoparticles: stability and aerosolization from pressurized metered dose inhalers, Int. J. Pharm. 375 (2009) 114–122.

[21] G. Gorin, S. Wang, L. Papapavlou, Assay of Lysozyme by Its Lytic Action on M. lysodeikticus Cells, Anal. Biochem. 39 (1971) 113–127.

[22] K.M. Wilbur, N.G. Anderson, Electrometric and colorimetric determination of Carbonic Anhidrase, J.Biol.Chem. (1948) 147–154.

[23] U.K. Laemmli, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature. (1970) 680–685.

[24] S. Verheyen, N. Blaton, R. Kinget, G. Van den Mooter, Mechanism of increased dissolution of diazepam and temazepam from polyethylene glycol 6000 solid dispersions, Int. J. Pharm. 249 (2002) 45–58.

[25] V. Crescenzi, G. Manzini, G. Calzolari, C. Borri, Thermodynamics of fusion of poly-β-propiolactone and poly-ε-caprolactone. Comparative analysis of the melting of aliphatic polylactone and polyester chains, Eur. Polym. J. 8 (1972) 449–463.

[26] H. Larhrib, J.I. Wells, M.H. Rubinstein, G. Ricart, Characterization of PEGs using matrix-assisted laser desorption / ionisation mass spectrometry and other related techniques, Int. J. Pharm. 147 (1997) 187–198.

[27] H. Sun, L. Mei, C. Song, X. Cui, P. Wang, The in vivo degradation, absorption and excretion of PCL-based implant., Biomaterials. 27 (2006) 1735–1740.

[28] M.A. Woodruff, D.W. Hutmacher, The return of a forgotten polymer—Polycaprolactone in the 21st century, Progr. Polym. Sci. 35 (2010) 1217–1256.

[29] J.-P. Amorij, J. Meulenaar, W.L.J. Hinrichs, T. Stegmann, A. Huckriede, F. Coenen, et al., Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying., Vaccine. 25 (2007) 6447–6457.

[30] W.L.J. Hinrichs, M.G. Prinsen, H.W. Frijlink, Inulin glasses for the stabilization of therapeutic proteins, Int. J. Pharm. 215 (2001) 163–174.

[31] N. Grasmeijer, M. Stankovic, H. de Waard, H.W. Frijlink, W.L.J. Hinrichs, Unraveling protein stabilization mechanisms: vitrification and water replacement in a glass transition temperature controlled system., Biochim. Biophys. Acta. 1834 (2013) 763–769.

[32] F. Yao, W.J. Kao, Drug Release Kinetics and Transport Mechanisms of Nondegradable and Degradable Polymeric Delivery Systems, Expert Opin Drug Deliv. 7 (2011) 429–444.

[33] R. van Dijkhuizen-Radersma, S. Métairie, J.R. Roosma, K. de Groot, J.M. Bezemer, Controlled release of proteins from degradable poly(ether-ester) multiblock copolymers, J. Control. Release. 101 (2005) 175–186.

[34] A. Bertz, S. Wöhl-Bruhn, S. Miethe, B. Tiersch, J. Koetz, M. Hust, et al., Encapsulation of proteins in hydrogel carrier systems for controlled drug delivery: influence of network structure and drug size on release rate., J. Biotechnol. 163 (2013) 243–249.

[35] J.M. Bezemer, D.W. Grijpma, P.J. Dijkstra, C. a van Blitterswijk, J. Feijen, A controlled release system for proteins based on poly(ether ester) block-copolymers: polymer network characterization., J. Control. Release. 62 (1999) 393–405.

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Incomplete protein release from hydrophilic poly(D,L-lactide-PEG)-b-poly(ε-caprolactone) implants

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Abstract

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

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

1. Introduction

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

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

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

Block copolymers composed of hydrophobic poly(DL-Lactide) or poly(ε-caprolactone) (PCL) in combination with hydrophilic PEG [15–17] offer the

possibility of varying the hydrophilicity and swelling degree of the polymer and allow modulation of drug release and degradation kinetics of the copolymer. More hydrophilic polymers generally provide better compatibility with protein drugs. Furthermore, an increased swelling degree allows for more continuous diffusion-based release, preventing the biphasic release profile that is typically encountered for poly(lactide-co-glycolide)-based protein delivery matrices.

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

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

2. Materials and methods

2. 1. Materials

Lyophilized lysozyme (Lys) (from chicken egg white ~14 kDa), lyophilized albumin from bovine serum (BSA), (protein >96 %, ~66 kDa), stannous octoate, dimethylformamide (DMF), *Micrococcus lysodeikticus,* acetonitrile (HPLC gradient grade), ethyl acetate, dimethylsulfoxide (DMSO), dichloromethane, sodium azide, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, trifluoroacetic acid, hydroxylamine hydrochloride, sodium dodecyl sulphate (SDS), urea and dithiotretiol (DTT), were purchased from Sigma. ε-Caprolactone,

D,L-lactide, PEG (Mw 1000 g/mol), 1,4-butanediol and 1,4-dioxane were obtained from Acros, Geel, Belgium. 1,4-Butanediisocyanate was purchased from Bayer. Deuterated chloroform and lithium bromide were obtained from Fisher and PEG standards were purchased from Fluka.

2. 2. Polymer synthesis

PCL and poly(D,L-lactide)-PEG_{1000}-*b*-poly(D,L-lactide) [PDLA-PEG_{1000}] prepolymers were synthesized by standard stannous octoate catalyzed ring-opening polymerization, as described previously [18]. ε-Caprolactone was dried over CaH_2 and distilled under reduced pressure in an atmosphere of dry nitrogen. D,L-lactide was dried overnight at 50 ˚C under vacuum. PEG with a molecular weight of 1000 g/mol (PEG_{1000}) was dried overnight at 90 ˚C under vacuum. 1,4-Butanediol and 1,4-butanediisocianate were distilled under reduced pressure. The purity of the distilled ε-caprolactone, 1,4-butanediol and 1,4-butanediisocyanate was confirmed by ^1H NMR ($CDCl_3$). PCL prepolymer with a target molecular weight of 4000 g/mol was prepared by introducing 241 g (2.11 mol) of anhydrous ε-caprolactone into a three-necked bottle under an atmosphere of dry nitrogen and adding of 5.6 g (62.03 mmol) of anhydrous 1,4-butanediol to initiate the ring-opening polymerization. Stannous octoate was used as a catalyst at a catalyst / monomer molar ratio of $8.40 \times 10^{-5}/1$. The mixture was magnetically stirred for 70 h at 140 ˚C and subsequently cooled to room temperature.

[PDLA-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} /1. The mixture was magnetically stirred for 10 days at 140 ˚C and subsequently cooled to room temperature.

[PCL] and [PDLA-PEG_{1000}] prepolymers were then chain-extended with 1,4-butanediisocyanate to prepare x[PDLA-PEG_{1000}]-y[PCL] multiblock copolymer where *x/y* is the [PDLA-PEG_{1000}] / [PCL] weight ratio, being 10/90, 20/80, 30/70 or 50/50 (**Table 1).** [PCL] and [PDLA-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] (previously abbreviated as 30CP15C20-C40) has been described elsewhere [18].

Table 1. In weights of pre-polymers used in synthesis of x[PDLA-PEG$_{1000}$]-y[PCL] multiblock copolymers.

	PDLA-PEG$_{1000}$ prepolymer	**PCL prepolymer**	**PDLA-PEG$_{1000}$ prepolymer**	**PCL prepolymer**	**BDI**
	wt%	**wt%**	**In weights**	**In weights**	**In weights**
10[PDLA-PEG$_{1000}$]-90[PCL]	10	90	10.28 g (5.09 mmol)	88.01g (21.68 mmol)	3.22 g (22.95 mmol)
20[PDLA-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[PDLA-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[PDLA-PEG$_{1000}$]-50[PCL]	50	50	48.42 g (23.97 mmol)	47.16 g (11.85 mmol)	5.08 g (36.24 mmol)

2. 3. Polymer characterization

^{1}H NMR was used to determine monomer conversion, number average molecular weight (Mn) and overall chemical composition of the polymer after synthesis and during degradation. ^{1}H NMR was performed on a VXR Unity Plus NMR Machine (Varian, California, USA) operating at 300 MHz. The d_1 waiting time was set to 20 s, and the number of scans was 16-32. ^{1}H NMR samples were prepared by dissolving 10 mg of polymer in 1 mL of deuterated chloroform ($CDCl_3$), and the spectrum was determined from 0 - 8 ppm using $CHCl_3$ present as trace element in $CDCl_3$ as reference.

Monomer conversion was calculated from peaks originating from the polymer and the monomer. For [PCL], monomer conversion was calculated from the peaks of the -O-$CH_2CH_2CH_2CH_2\mathbf{C}\mathbf{H}_2$C(O)- methylene groups of [PCL] and monomer ε-caprolactone at δ 2.2-2.5 and δ 2.65, respectively. For [PDLA-PEG$_{1000}$], monomer conversion was calculated from the peaks of the -O-C**H**(CH_3)C(O)- methylene 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 methylene end groups of PCL at δ 3.6-3.7 and the -O-$CH_2CH_2CH_2CH_2\mathbf{C}\mathbf{H}_2$C(O)- methylene group of PCL at δ 2.2-2.5. The experimental Mn of the [PDLA-PEG$_{1000}$] prepolymer was determined by the peak of the PDLA methylene groups -O-C**H**(CH_3)C(O)- at δ 5.1-5.4 and the peaks of the PEG methylene groups –$\mathbf{C}\mathbf{H}_2\mathbf{C}\mathbf{H}_2$-O at δ 3.6-3.7.

^{1}H NMR was also 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-$CH_2CH_2CH_2CH_2\mathbf{C}\mathbf{H}_2$C(O)- methylene

group of PCL and ε-caprolactone at δ 2.2 - 2.5 and δ 2.65, respectively, and the –**CH_2CH_2**-O methylene groups of PEG at δ 3.6 - 3.7. PDLA/PEG molar ratio was calculated from the -O-C**H**(CH_3)C(O)- methyne groups of PDLA and D,L-lactide monomer at δ 5.1 - 5.4 and δ 5.0 - 5.1, respectively, and the –**CH_2CH_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 the dynamic viscosity at three different polymer concentrations at a temperature of 25 ˚C using an Ubbelohde viscometer (DIN, type 0C Schott Geräte supplied with a Schott AVS-450 Viscometer equipped with a water bath).

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

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

2. 4. Hot melt extrusion

For the preparation of implants HME was performed using a HAAKE MiniLab Rheomex CTW5 co-rotating twin-screw extruder (Thermo-Electron). Implant formulations were prepared from x[PDL-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 wt% Lys, as listed in a **Table 2**. Since protein particle size may influence the release kinetics from polymer matrices [18,19], BSA particles were milled in a stainless steel container with an aid of stainless steel beads, using a tumbling mixer (Turbula T2X, WA Bachofen AG, Switzerland) until the volume-averaged particle size was approximately 18 μm, which was similar to the volume-averaged particle size of Lys, as determined by laser diffraction (Sympatec GMbH, Clausthal-Zellerfeld, Germany). Milled BSA or Lys powder were then physically mixed with polymer powder and fed into the preheated barrel of the extruder. Extrusion was performed at 50 - 55 °C using a screw speed of 10 - 20 rpm and a torque of 4 - 7 Nm. A cylindrical die of 0.5 mm was used, resulting in strands with a diameter of 0.35 mm, as measured with an *in-line* laser. Polymer-only implants, used for the degradation study were extruded similarly, without further additives. Polymer strands were cut into pieces of 2 cm x 0.35 mm (for the degradation study) and 1 cm x 0.35 mm (for the *in-vitro* release study) and stored at -20 °C prior to use.

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

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

2. 5. In-vitro polymer degradation

The degradation of polymer-only implants was evaluated during 180 days. About 130 ± 5 mg (n=3) of polymeric implants were placed in plastic vials and 25 ml of phosphate buffer (PBS) (100 mM, pH 7.4 ± 0.2, 9.1 mM NaCl, 0.02 wt% NaN_3) was added to each vial. The vials were then incubated in an oven at 37˚C. pH was regularly measured and adjusted to pH 7.4 ± 0.2 using 1 M NaOH. At various time intervals, samples were removed from the buffer and washed with ultra-pure water over a 0.45 μm filter to remove the buffer salts. Adherent water from the implants was removed with a tissue. Wet mass was determined ($m_{wet,t}$) after which the samples were dried in a desiccator for 15 h and then in a vacuum oven (30 °C, pressure < 0.01 mbar) for 24 h and weighted again ($m_{dry,t}$). Water content and mass loss were calculated using Eq. (1) and Eq. (2).

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

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

Where $m_{dry,0}$ 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.

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

2. 6. In-vitro protein release and quantification

The *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 (n = 3) were incubated in 1.3 ml of the release buffer and test tubes were placed vertically in a shaking water bath, under mild agitation, thermostated at 37 ˚C for a total of 180 days. At different time intervals, 1.1 ml of aliquots were removed for HPLC analysis and refreshed. The cumulative amount of released protein within 4h was considered as burst release.

Lys concentrations were measured with Dionex Ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with C18 ProZap LC/MS reversed phase column (Grace Davidson, Deerfield, IL, USA) (20 x 4.6 mm, 1.5 μm). Chromatographs were obtained using an UV detector at 280 nm. Gradient system consisted of 0.1 vol% trifluoroacetic acid in acetonitrile (A) and 0.1 vol% trifluoroacetic acid in ultrapure water (B). The solvent flow rate was 1 mL/min

and the gradient was applied for 6 min, using the following scheme: 0 min-1 min: A/B = 3/7 (v/v); 1–3 min: A/B = 6/4 (v/v); 3.01-6 min: A/B = 3/7 (v/v). The retention time of Lys was 1.19 min. Data were analyzed with Chromeleon software.

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

2. 7. Content uniformity and structural integrity of proteins

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

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

2. 8. Protein-polymer interaction

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

2. 9. Scanning Electron Microscopy

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

3. Results

3. 1. Polymer synthesis and characterization

The results of the characterization of x[PDLA-PEG_{1000}]-y[PCL] multiblock copolymers by IV, ^{1}H NMR, SEC and DSC are summarized in **Table 3**. Similar IV values were obtained for the various multiblock copolymers, ranging from 0.61 - 0.73 dL/g. As expected from the similar IV values, SEC indicated that the apparent molecular weights of the various multiblock copolymers were also similar as the *Mn* and *Mw* values ranged from 15.4 - 21.3 kg/mol and 31 - 40 kg/mol, respectively. The polydispersity index, defined as the ratio *Mw / Mn*, 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 Mn of 3490 g/mol, which

was reasonably close to the theoretical value of 3980 g/mol, as calculated from in weight values and monomer conversion. The Mn of [PDLA-PEG_{1000}] was 1950 g/mol, which was also close to the theoretical value of 1950 g/mol, as determined from in weights and monomer conversion.

Furthermore, ^{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[PDLA-PEG_{1000}]-y[PCL] multiblock copolymers ranged from 14.5 - 17.4 mol / mol, which was close to the theoretical LA / PEG molar ratio from in-weights (13.9 - 14.2 mol / mol). CL/PEG molar ratio of the x[PDLA-PEG_{1000}]-y[PCL] multiblock copolymers varied with varying PCL block content. CL/PEG molar ratios ranged from 16.8 - 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**).

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

1,4- Dioxane was well removed from all x[PDLA-PEG_{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 [18].

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

Multiblock copolymers composition	IV (dL/g)	LA / PEG molar ratio		CL / PEG molar ratio		SEC (kg/mol)			Tg[a] (C)	Tm[a] (°C)	ΔH [a,b] (J/g)
		in-weights	^{1}H NMR	in-weights	^{1}H NMR	Mn	Mw	Mw/Mn			
10[PDLA-PEG$_{1000}$]- 90[PCL]	0.67	14.2	16.2	148.2	134.9	15.4	30.9	2.01	-54	53	72
20[PDLA-PEG$_{1000}$]- 80[PCL]	0.71	14.2	17.4	64.4	68.0	21.3	37.5	1.76	-49	53	74
30[PDLA-PEG$_{1000}$]- 70[PCL]	0.73	13.9	15.8	38.1	38.5	18.0	39.6	2.20	-40	53	74
50[PDLA-PEG$_{1000}$]- 50[PCL]	0.61	14.2	14.5	15.1	16.8	21.3	40.0	1.88	-39	52	61

a) Determined after HME

b) Total of the PCL fraction of the multiblock copolymers, calculated by ΔH(J/g PCL) = (ΔHm$_{PCL}$/ wt% PCL), where Hm$_{PCL}$ is the melting enthalpy of the PCL block per gram of multiblock copolymer, wt%$_{PCL}$ is calculated by ^{1}H-NMR

3. 2. Protein content uniformity and integrity

The amount of extracted protein of randomly collected samples of the various implants only exhibited small variations, suggesting a homogeneous distribution of the protein within the polymer matrix with an average loading of 9.08 ± 0.66 % (Lys) and 9.42 ± 0.44 % (BSA) in the x[PDLA-PEG_{1000}]-y[PCL] polymers and 10.05 ± 0.01 % (Lys) in 30[PCL-PEG_{1500}]-70[PCL] polymer. Both proteins preserved their structural integrity during extrusion as evidenced by the absence of additional peaks in the HPLC chromatograms. In addition, the biological assay indicated that the enzymatic activity of Lys was fully preserved after extrusion.

3. 3. In-vitro polymer degradation

3. 3. 1. Water content

The *in-vitro* degradation study showed substantial water uptake by the polymer implants during the first day of incubation. The water content increased with increased [PDLA-PEG_{1000}] / [PCL] block ratio and thus increased amorphous block content of the multiblock copolymer. After the first day of incubation, equilibrium was reached and water content remained more or less constant throughout the duration of the study and was 58 ± 2.1 %, 59 ± 1.7 %, 63 ± 0.7 %, 68 ± 4.9 % for 10[PDLA-PEG_{1000}]-90[PCL], 20[PDLA-PEG_{1000}]-80[PCL], 30[PDLA-PEG_{1000}]-70[PCL], and 50[PDLA-PEG_{1000}]-50[PCL], respectively (**Figure 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. The difference in water content between copolymers 10[PDLA-PEG_{1000}]-90[PCL], 30[PDLA-PEG_{1000}]-70[PCL] and 50[PDLA-PEG_{1000}]-50[PCL] was significant. Furthermore, the difference in water content between 20[PDLA-PEG_{1000}]-80[PCL] and 50[PDLA-PEG_{1000}]-50[PCL] as well as between 30[PCL-PEG_{1500}]-70[PCL] and 50[PDLA-PEG_{1000}]-50[PCL] was significant.

3. 3. 2. Mass loss

During incubation the mass loss of x[PDLA-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[PDLA-PEG_{1000}]-90[PCL] was only 5.7 ± 0.1 % after 180 days of incubation (**Figure 1b**). With increased [PDLA-PEG_{1000}] / [PCL] block

ratio, and thus with an increased percentage of the amorphous block, the percentage of mass loss increased amounting to 14.7 ± 0.7 % for 20[PDLA-PEG_{1000}]-80[PCL], 28.9 ± 0.9 % for 30[PDLA-PEG_{1000}]-70[PCL] and 50.6 ± 0.9 % for 50[PDLA-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 differences in the rate of mass loss among the all polymers were significant.

3. 3. 3. Decrease of molecular weight

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

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

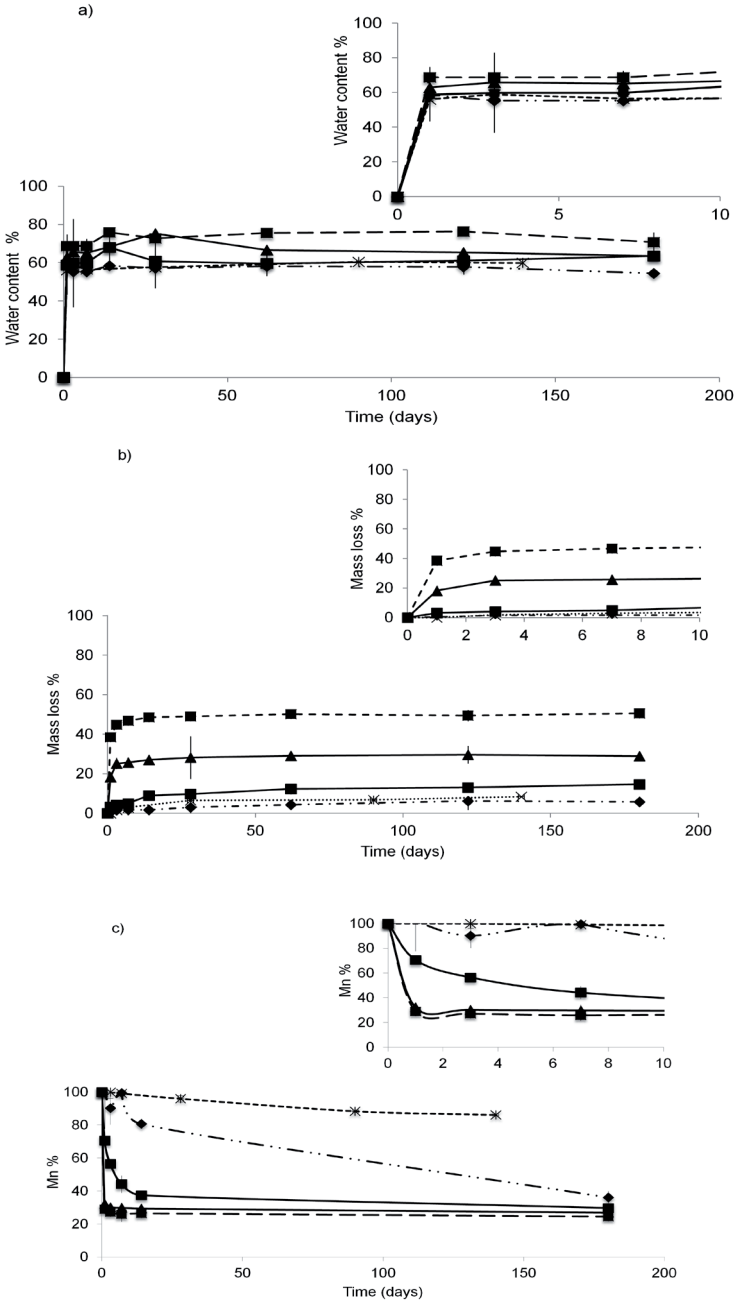

Figure 1. Percentage of water content (a), mass loss (b) and (c) Mn loss for multiblock copolymers during degradation: 10[PDLA-PEG$_{1000}$]-90[PCL] (dashed line, ♦), 20[PDLA-PEG$_{1000}$]-80[PCL] (solid line,■), 30[PDLA-PEG$_{1000}$]-70[PCL] (solid line,▲), 50[PDLA-PEG$_{1000}$]-50[PCL] (dashed line,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

3. 3. 4. Crystallinity of the ε -caprolactone block during degradation

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

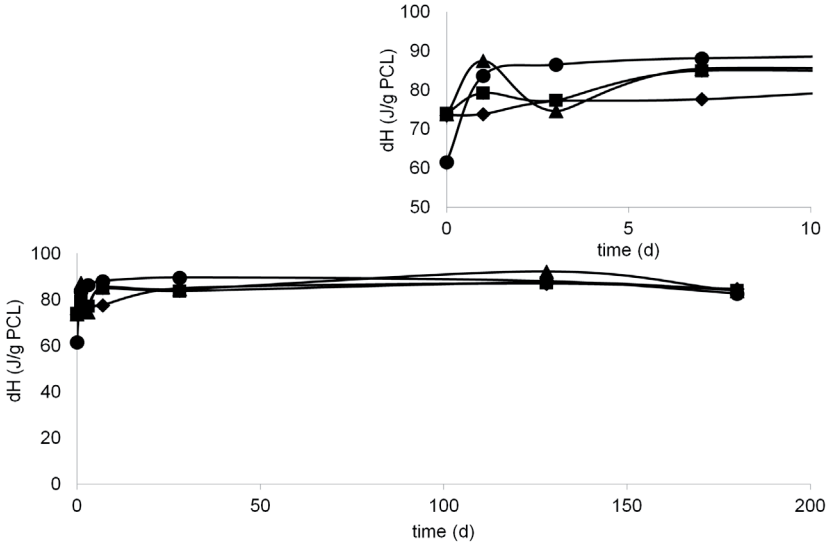

Figure 2. The melting enthalpy of the crystalline [PCL] of the multiblock copolymers during degradation, corrected for the total PCL content, 10[PDLA-PEG_{1000}]-90[PCL] (♦), 20[PDLA-PEG_{1000}]-80[PCL](■), 30[PDLA-PEG_{1000}]-70[PCL] (▲), 50[PDLA-PEG_{1000}]-50[PCL] (●).

3. 3. 5. Polymer composition

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

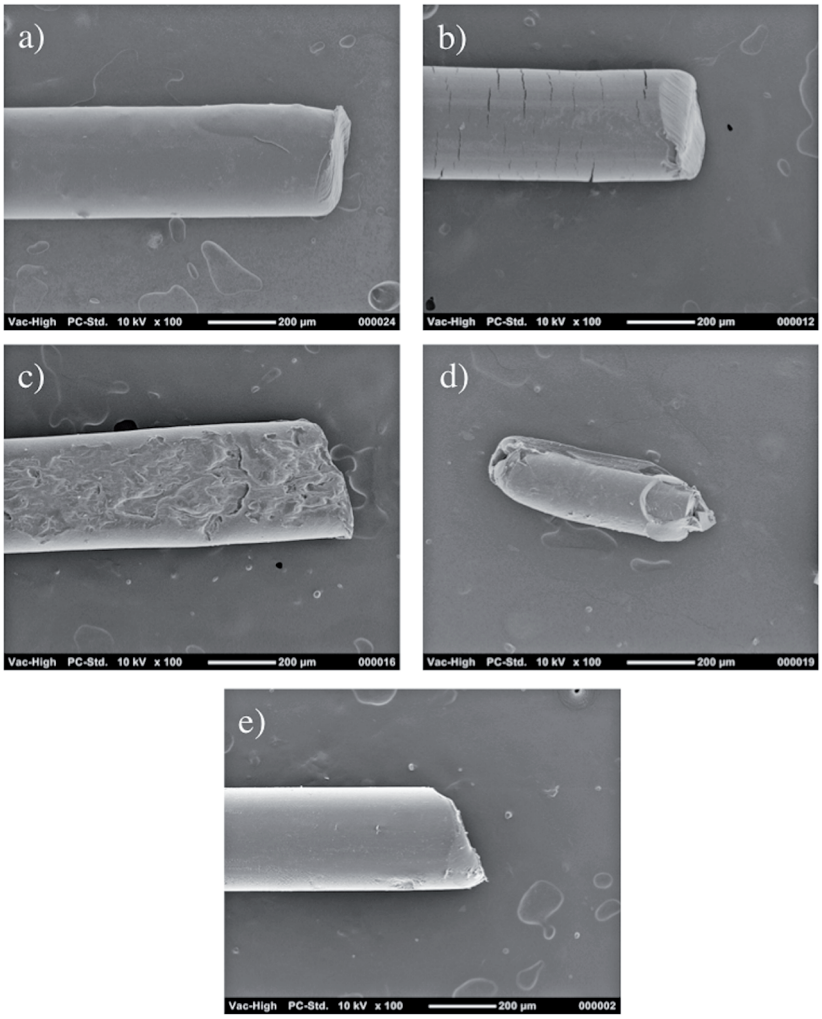

Figure 4. Surface of the polymer-only implants after 180 days of incubation in PBS. (a) 10[PDLA-PEG$_{1000}$]-90[PCL]; (b) 20[PDLA-PEG$_{1000}$]-80[PCL]; (c) 30[PDLA-PEG$_{1000}$]-70[PCL], (d) 50[PDLA-PEG$_{1000}$]-50[PCL], (e) 30[PCL-PEG$_{1500}$]-70[PCL] (140 days of incubation).

3. 4. In-vitro protein release

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

content, i.e. 50[PDLA-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 formulations. In contrast, 30[PCL-PEG$_{1500}$]-70[PCL], containing PCL instead of PDLA in the hydrophilic block showed an initial burst of 20 % followed by a slow and continuous Lys release until 70 % of the protein was released after 180 days (after which the release was not further followed).

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

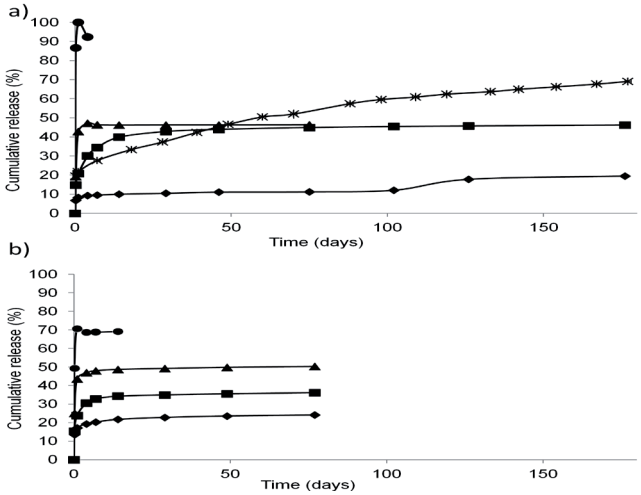

Figure 5. In-vitro release of Lys (a) and BSA (b) from multiblock copolymers (n=3): 10[PDLA-PEG$_{1000}$]-90[PCL] (♦), 20[PDLA-PEG$_{1000}$]-80[PCL] (■), 30[PDLA-PEG$_{1000}$]-70[PCL] (▲), 50[PDLA-PEG$_{1000}$]-50[PCL] (●) and 30[PCL-PEG$_{1500}$]-70[PCL] (∗).

3. 5. Interaction of protein and polymer

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

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

	% 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		UREA 6M		DTT		SDS		Hydroxyl-amine HCl	
t =24h	93.60	93.63	46.57	47.40	39.57	45.94	45.51	50.21	41.21	45.94

4. Discussion

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

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

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

For the x[PDLA-PEG_{1000}]-y[PCL] multiblock copolymers with higher [PDLA-PEG_{1000}] / [PCL] block ratio mass loss occurred immediately after 1 day of incubation, while for the copolymers with lower [PDLA-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 [PDLA-PEG_{1000}] / [PCL] block ratio). Mass loss of the multiblock copolymer with [PCL-PEG_{1500}] as the amorphous block (8.4 % in 140 days) was slow as compared to copolymer with [PDLA-PEG_{1000}] as the amorphous block and with similar PEG content and degree of swelling (29 % in 140 days), which can be ascribed to the relatively slow degradation of [PCL-PEG_{1500}] as compared to [PDLA-PEG_{1000}] [31]. From the DSC data, it was clear that the crystallinity of the PCL block increases during degradation.

It has previously been described that burst release of protein is most likely due to dissolution of particles from the polymer surface followed by dissolution and liberation of the neighboring particles [18]. In this study, we observed that the burst release increased with increasing [PDLA-PEG_{1000}] / [PCL] block ratio, which implies that for this type of copolymers, besides dissolution of the particles from or close to the surface also polymer swelling and subsequent degradation played a role in the initial release. Polymers with a [PDLA-PEG_{1000}] / [PCL] block ratio of 30/70 and 50/50 exhibited a certain mass loss already during the burst phase, supporting that the increased burst release with increased [PDLA-PEG_{1000}] / [PCL] block ratio may also be ascribed to liberation of the proteins together with parts of the [PDLA-PEG_{1000}] blocks of the polymer.

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

Incomplete protein release from biodegradable polymer matrices has been observed before but little is known about the exact mechanism. Protein release can be compromised by protein adsorption to the polymer or by protein aggregation [26,32,33]. Other authors reported that incomplete peptide or protein release can be due to the chemical modification of proteins during incubation, e.g. by acylation [21,34–37]. In order to assess the nature of the interaction between protein and polymer, the implants were exposed to aqueous solution of urea, SDS, DTT, hydroxylamine HCL and NaOH after 17 days of incubation. It was shown that only

sodium hydroxide could liberate the protein most likely due to its ability to hydrolyze the ester bonds and completely degrade the polymer, which suggests irreversible interaction or covalent bonding between the protein and [PDLA-PEG_{1000}] related degradation products as a cause for incomplete protein release. However, it has also been observed that after a few days of incubation, the crystallinity of PCL was substantially increased indicating molecular rearrangements of PCL of the semi-crystalline PCL blocks. Therefore, it cannot be ruled out that during these molecular rearrangements of the PCL blocks the protein was physically entrapped in these structures instead of irreversibly interacted with the polymer degradation products in the implant, which has also been shown before [38]. However, if the protein would remain physically entrapped, it should have been recovered upon the protein extraction using ethyl acetate.

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

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

Acknowledgements

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

References

[1] M. Stanković, J. Tomar, C. Hiemstra, R. Steendam, H.W. Frijlink, W.L.J. Hinrichs, Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly (ε-caprolactone) multiblock-copolymer implants, Eur. J. Pharm. Biopharm. 87 (2014) 329–337.

[2] S. Freiberg, X.X. Zhu, Polymer microspheres for controlled drug release, Int. J. Pharm. 282 (2004) 1–18.

[3] J.H. Park, M. Ye, K. Park, Biodegradable polymers for microencapsulation of drugs., Molecules. 10 (2005) 146–161.

[4] M. Vert, Aliphatic Polyesters : Great Degradable Polymers That Cannot Do Everything, Biomacromolecules. 6 (2005) 538–546.

[5] G. Vilar, J. Tulla-Puche, F. Albericio, Polymers and drug delivery systems., Curr. Drug Deliv. 9 (2012) 367–394.

[6] A. Lucke, J. Teßmar, E. Schnell, G. Schmeer, A. Göpferich, 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 (2000) 2361–2370.

[7] H. Seyednejad, A.H. Ghassemi, C.F. Van Nostrum, T. Vermonden, W.E. Hennink, Functional aliphatic polyesters for biomedical and pharmaceutical applications, J. Control. Release. 152 (2011) 168–176.

[8] R.A. Jain, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices., Biomaterials. 21 (2000) 2475–2490.

[9] A. Frank, S.K. Rath, S.S. Venkatraman, Controlled release from bioerodible polymers: effect of drug type and polymer composition., J. Control. Release. 102 (2005) 333–344.

[10] M.-H. Huang, S. Li, D.W. Hutmacher, J. Coudane, M. Vert, Degradation characteristics of poly(epsilon-caprolactone)-based copolymers and blends, J. Appl. Polym. Sci. 102 (2006) 1681–1687.

[11] M. Vert, S. Li, H. Garreau, More about the degradation of LA / GA-derived aqueous media matrices in aqueous media, J. Control. Release Release. 16 (1991) 15–26.

[12] C.G. Pitt, A.R. Jeffcoat, A. Ruth, Sustained Drug Delivery Systems. I. The Permeability of Poly(e-Caprolactone), Poly(DL-Lactic Acid), and Their Copolymers, J. Biomed. Mater. Res. 13 (1979) 497–507.

[13] H. Sun, L. Mei, C. Song, X. Cui, P. Wang, The in vivo degradation, absorption and excretion of PCL-based implant., Biomaterials. 27 (2006) 1735–1740.

[14] C.G. Pitt, F.I. Chasalow, Y.M. Hibionada, D.M. Klimas, Aliphatic Polyesters I. The Degradation of poly(ε-caprolactone) In Vivo, J. Appl. Polym. Sci. 26 (1981) 3779–3787.

[15] I. Rashkov, N. Manolova, S.M. Li, J.L. Espartero, M. Vert, Synthesis, Characterization, and Hydrolytic Degradation of PLA / PEO / PLA Triblock Copolymers with Short Poly (L -lactic acid) Chains, Macromolecules. 29 (1996) 50–56.

[16] S. Li, H. Garreau, M. Vert, T. Petrova, N. Manolova, I. Rashkov, Hydrolytic Degradation of Poly(oxyethylene)-Poly-(ε-caprolactone) Multiblock Copolymers, J. Appl. Polym. Sci. 68 (1997) 989–998.

[17] W. Chen, W. Luo, S. Wang, J. Bei, Synthesis and properties of poly(L-lactide)-Poly(Ethylene glycol) multiblock copolymers by coupling triblock copolymers, Polym. Adv. Technol. 14 (2003) 245–253.

[18] M. Stanković, H. de Waard, R. Steendam, C. Hiemstra, J. Zuidema, H.W. Frijlink, W.L.J. Hinrichs, Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery, Eur. J. Pharm. Sci. 49 (2013) 578–587.

[19] Z. Ghalanbor, M. Körber, R. Bodmeier, Improved Lysozyme Stability and Release Properties of Poly (lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion, Pharm. Res. 27 (2010) 371–379.

[20] G. Gorin, S. Wang, L. Papapavlou, Assay of Lysozyme by Its Lytic Action on M. lysodeikticus Cells, Anal. Biochem. 39 (1971) 113–127.

[21] Z. Ghalanbor, M. Körber, R. Bodmeier, 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 (2012) 302–306.

[22] S.S. Fenton, R.C. Fahey, Analysis of biological thiols: determination of thiol components of disulfides and thioesters., Anal. Biochem. 154 (1986) 34–42.

[23] U. Bilati, E. Allémann, E. Doelker, Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles., Eur. J. Pharm. Biopharm. 59 (2005) 375–388.

[24] B. Bogdanov, A. Vidts, A. Van Den Bulcke, R. Verbeeck, E. Schacht, Synthesis and thermal properties of poly(ethylene glycol)-poly(ε-caprolactone) copolymers, Polymer (Guildf). 39 (1998) 1631–1636.

[25] H. Sah, A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres, J Pharm Sci. 86 (1997) 1315–1318.

[26] G. Zhu, S.P. Schwendeman, Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives, Pharm. Res. 17 (2000) 351–357.

[27] Y. Hu, L. Zhang, Y. Cao, H. Ge, X. Jiang, C. Yang, Degradation behavior of poly(epsilon-caprolactone)-b-poly(ethyleneglycol)-b-poly(epsilon-caprolactone) micelles in aqueous solution., Biomacromolecules. 5 (2004) 1756–1762.

[28] R.-W. Alexandra, K. Besseghir, R. Gurny, Analysis of the influence of polymer characteristics and core loading on the in vivo release of a somatostatin analogue, Eur. J. Pharm. Sci. 5 (1997) 303–313.

[29] C. Engineer, J. Parikh, A. Raval, Hydrolytic Degradation Behavior of 50 / 50 Poly Lactide-co-Glycolide from Drug Eluting Stents, Trends Biomater. Artifitial Organs. 24 (2010) 131–138.

[30] A. Göpferich, Mechanisms of polymer degradation and erosion., Biomaterials. 17 (1996) 103–114.

[31] C.G. Pitt, M.M. Gratzl, G.L. Kimmel, J. Surles, A. Schindler, Aliphatic polyesters II. The degradation of poly (DL-lactide), poly (epsilon-caprolactone), and their copolymers in vivo., Biomaterials. 2 (1981) 215–220.

[32] G. Jiang, B.H. Woo, F. Kang, J. Singh, P.P. DeLuca, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres., J. Control. Release. 79 (2002) 137–145.

[33] Y.S. Nam, S.H. Song, J.Y. Choi, T.G. Park, Lysozyme Microencapsualtion Within Biodegradable PLGA Microspheres: Urea Effect on Protein Release and Stability, Biotechnol. Bioeng. 70 (2000) 270–277.

[34] M.L. Houchin, K. Heppert, E.M. Topp, Deamidation, acylation and proteolysis of a model peptide in PLGA films., J. Control. Release. 112 (2006) 111–119.

[35] D.H. Na, Y.S. Youn, S.D. Lee, M.-W. Son, W.-B. Kim, P.P. DeLuca, et al., Monitoring of peptide acylation inside degrading PLGA microspheres by capillary electrophoresis and MALDI-TOF mass spectrometry, J. Control. Release. 92 (2003) 291–299.

[36] T.G. Park, W. Lu, G. Crotts, 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 (1995) 211–222.

[37] G. Crotts, T.G. Park, Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues., J. Microencapsul. 15 (1998) 699–713.

[38] F. Quaglia, L. Ostacolo, G. Nese, G. De Rosa, M.I. La Rotonda, R. Palumbo, et al., Microspheres made of poly(epsilon-caprolactone)-based amphiphilic copolymers: potential in sustained delivery of proteins., Macromol. Biosci. 5 (2005) 945–954.

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Summary and concluding remarks

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Summary

Recent developments in the area of biotechnology and molecular biology have resulted in an increased interest in the use of peptides and proteins for treatment of a variety of diseases and disorders. However, due to their instability in GI tract and poor permeability over the intestinal wall these macromolecules require parenteral administration and often frequent administrations because generally proteins exhibit a short half-life in the circulation. Polymer based injectable depot preparations for the sustained release of proteins over prolonged periods of time offer an attractive alternative to frequent injections, because amongst other reasons they increase patient compliance and reduce therapy costs.

Hot melt extrusion (HME) is a technology extensively used to prepare polymer based implants for the controlled release of drugs. This method offers numerous advantages over the traditional emulsification based microencapsulation methods. Nevertheless, the encapsulation of proteins into polymer matrices using this technique still presents a challenge due to highly sensitive nature of these macromolecules. Therefore, stabilization of proteins prior to, during and after encapsulation is of a high interest. Further, the use of biodegradable polymers, which are metabolized in the body into non-toxic products and subsequently excreted, is preferred, as they do not require surgical removal after the drug is released.

The aim of the studies described in this thesis was to investigate the mechanisms of stabilization of proteins by sugar glass technology, to encapsulate sugar glass stabilized proteins into innovative biodegradable polymer matrices by means of HME, and to study the release of the proteins from these matrices. The path this research followed consisted of two major steps:

1. Evaluation of the spray-drying technology for the stabilization of proteins using sugar glasses, followed by the incorporation of the stabilized proteins in biodegradable polymers.

2. Evaluation of novel multiblock copolymers as matrices for the controlled release of proteins. HME was chosen as a method for manufacturing of depot formulation with encapsulated proteins.

In **chapter 2**, a literature review is given on the HME technique and the polymers used. The application of HME for the preparation of different formulations intended for oral, sublingual, transdermal, intraocular and parenteral administration is evaluated. Biodegradable and non-biodegradable polymers that are widely used in this technique are described and the most relevant techniques to characterize polymer based HME products are summarized.

In **chapter 3,** the role of the vitrification and the water replacement mechanism in protein stabilization using sugar glasses has been studied. Alkaline phosphatase was used as a model protein. The protein was incorporated into a matrix of inulin and trehalose in the glassy state using spray-drying. Due to the glass forming ability of ammediol, it was not only used as a buffer but also as a plasticizer. Variation of the sugar/buffer mass ratio resulted in spray-dried products with different glass transition temperatures. The protein storage stability at different glass transition temperatures has been studied. It was shown that when the glass transition was below storage temperature, protein stabilization was controlled by the vitrification mechanism. However, when the glass transition temperature was above storage temperature, the water replacement mechanism was dominant.

In **chapter 4** the synthesis and characterization of a novel hydrophilic multiblock copolymer composed of the two phase-separated blocks is described. One block consisted of semi-crystalline poly(ε-caprolactone) (PCL) (30 wt% of the copolymer) and the other consisted of the amorphous poly(ethylene glycol)–poly(ε-caprolactone) block [PCL-PEG] (70 wt% of the copolymer). A major advantage of this copolymer was that it could be extruded at a much lower temperature (55 °C) than the widely used poly(lactide-co-glycolide) (>85 °C). Furthermore, it was studied whether the model protein lysozyme could be encapsulated into the polymer by HME, without compromising its biological activity.

An aqueous solution of lysozyme was first spray dried with and without inulin to obtain powder with a lysozyme/inulin weight ratio of 1/0, 1/1, 1/2, 1/3. The spray-dried powders had a similar particle size distribution (X_{50} was 1.09 - 1.50 μm). Subsequently, the powders were encapsulated into the polymer by HME resulting in 10 wt%, 20 wt%, 30 wt% and 40 wt% of lysozyme/inulin load, while keeping the lysozyme load the same. Furthermore, to evaluate the effect of particle size, lysozyme as received (X_{50} was 20.82 μm) was also encapsulated into the polymer. In all cases the enzymatic activity was fully maintained during HME. The *in-vitro* lysozyme release kinetics were evaluated, together with the enzymatic activity of the protein during 260 days of incubation in phosphate buffer (pH 7.4) at 37°C. It was observed that co-incorporation of different amounts of inulin resulted in an increased lysozyme release rate, indicating that inulin acted as a pore-forming excipient. The samples with a 10 and 20 wt% protein/sugar load released, after an initial burst, the protein continuously during 260 days with kinetics close to first order kinetics suggesting that after the burst release, at low loadings the release is governed by the protein diffusion through the polymer matrix. At higher loading, lysozyme release was very fast indicating that the lysozyme/inulin particles were connected via a percolating network, forming channels through which the protein diffused out. It was also observed that the initial release of lysozyme was highly dependent on the

protein particle size. The formulation consisting of 10 wt% spray dried lysozyme exhibited a smaller burst release than the formulation containing the same amount of lysozyme with larger particle size ("as received" material). After 70 days of the release, the release rate of protein from both formulations was similar, indicating that in this later stage, the release is not dependent on the particle size. Lysozyme remained 80 % active after the 180 days of release. It was also observed by scanning electron microscopy that the polymer morphology remained intact after 180 days of release, indicating slow degradation of the polymer. In conclusion, in this study it was shown that not only the protein/sugar load, but also the particle size of the protein should be considered when designing a controlled release device.

In **chapter 5**, the degradation kinetics of the multiblock copolymer described in chapter 4 was evaluated. It was further studied whether the degradation rate of this copolymer could be enhanced by increasing the amorphous (PCL- PEG) content of the polymer. Therefore, polymers with different x[PCL-PEG_{1500}]/y[PCL] block ratios (x/y being 30/70, 50/50, 70/30 (w/w)) were synthesized and characterized and the in-vitro degradation behavior of polymer-only implants was evaluated during 140 days of incubation in phosphate buffer (pH 7.4) at 37 °C. It was observed that the degradation rate of these copolymers increased upon increasing the [PCL-PEG]/[PCL] ratio. Additionally, the effect of molecular weight of the protein and the block ratio of the polymer on the release kinetics was investigated. Because the results of the studies described in chapter 4 clearly indicated that both protein particle size and protein loading influenced the release kinetics, we spray-dried proteins of different molecular weight (goserelin (1.2 kDa), insulin (5.8 kDa), lysozyme (14 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa)) with inulin in the constant weight ratio (1/10) to obtain uniform particle size distribution after which these powders were encapsulated in the polymers at constant powder (protein+inulin) load (11 wt%). All formulations were extruded at low temperatures (50 – 55 °C) and it was found that the structural integrity of proteins was fully preserved during extrusion. Furthermore, it was shown that the protein release rate increased with decreasing molecular weight of the protein and increasing [PCL- PEG]/[PCL] ratio of the polymer. It was concluded that small molecular weight proteins were released mainly by diffusion, but when the protein was larger than the polymer mesh size both diffusion of protein and degradation of the polymer played a role in protein release. Therefore, this study demonstrated that knowledge of both protein molecular weight and [PCL- PEG]/[PCL] ratio of the polymer are essential in designing the optimal delivery depot with tailored protein release.

In **chapter 6,** novel multiblock copolymers composed of semi-crystalline poly(ε-caprolactone) (PCL) blocks and amorphous blocks consisting of poly(D,L-lactide) (PDLA) and poly(ethylene glycol) (PEG) [PDLA-PEG] were synthesized.

The block ratio of these [PDLA-PEG]-*b*-[PCL] multi block copolymers was varied and the degradation of implants prepared thereof by HME was compared with implants prepared of 30[PCL-PEG_{1500}]-70[PCL], a copolymer which has been described in previous chapters. Furthermore, the release of the model proteins lysozyme and bovine serum albumin incorporated into polymer implants was studied.

The copolymers composed of [PDLA-PEG]-*b*-[PCL] showed increased degradation rate in the initial phase, which could be controlled by varying [PDLA-PEG]/[PCL] block ratio. As expected, it was found that with increased polymer degradation rate the protein release rate also increased. However, protein release from [PDLA-PEG]-*b*-[PCL] copolymers was incomplete, in contrast to [PCL-PEG]-*b*-[PCL] copolymers that showed slow and continuous release. The incomplete protein release was attributed to either irreversible interaction between the proteins and the polymer degradation products or to the physical entrapment of the protein into the semi-crystalline PCL matrix. In contrast, protein release from the slowly degrading [PCL-PEG]-*b*-[PCL] polymer was continuous and complete during the entire study, suggesting the absence of protein-polymer interactions or irreversible protein entrapment for this type of copolymer.

Concluding remarks and perspectives

There are many emulsification based microencapsulation methods, which are used to entrap molecules in polymer matrices. HME is a solvent-free process gaining a lot of attention in development of a large variety of dosage forms. Next to the many advantages this method may offer (discussed in the **chapter 2**), it often requires high temperatures and shear stresses that can be detrimental for unstable drug substances like proteins. Therefore, in this thesis a combination of two strategies was evaluated to overcome protein degradation during encapsulation in polymer matrices by HME: 1) pre-stabilization of the protein using sugar glass technology and 2) application of novel polymers that can be extruded at relatively low temperatures.

In the literature, the disaccharide trehalose is often referred to as the gold standard for protein stabilization during freeze- or spray-drying processes and subsequent storage. The oligosaccharide inulin is also known to be an excellent protein stabilizer [1]. We have, therefore, evaluated and compared the stabilizing capacities of trehalose and inulin (**chapter 3**). Although protein stabilization has often been ascribed to occur either through vitrification or through water replacement, we have shown that the difference between glass transition temperature (T_g) and storage temperature determines the mechanism of protein stabilization. When the T_g of the spray-dried products was adjusted to the same value by using ammediol

as a plasticizer, trehalose performed slightly better as stabilizer than inulin did. However, because pure inulin has a higher T_g than pure trehalose, inulin could be an attractive alternative to trehalose, especially when samples are stored at high relative humidities and temperatures. Additionally, the higher T_g of inulin would provide better stabilization of proteins during high temperature processes like HME.

The novel, hydrophilic multiblock copolymer based on PCL and PEG described in **chapter 4** showed favorable properties to be used as matrix for the encapsulation of proteins by HME. This polymer allowed for successful extrusion at 55°C, which is 30°C lower than the extrusion temperature required for PLGA. This low temperature could be beneficial for the encapsulation of thermolabile drug substances like proteins and peptides. Furthermore, the model protein lysozyme showed continuous release kinetics as compared to the biphasic release usually observed with PLGA. The lysozyme release was driven mainly by diffusion, while the polymer showed very limited degradation during the period the release occurred (260 days), which was further described in **chapter 5.** The slow degrading polymers could be favorable when molecules with a required release longer than 1 year are to be encapsulated [2–4]. However, for molecules below a certain hydrodynamic diameter, the release occurs faster than the polymer degradation rate [5]. Therefore, it might seem to be beneficial to develop a polymer with enhanced degradation kinetics, which will not exceed extensively the duration of protein release.

Faster degradation behavior could be obtained by replacing the [PCL-PEG] amorphous block in [PCL-PEG]-[PCL] by [PDLA-PEG]. However, we have shown that proteins irreversibly interact with degradation products of the [PDLA-PEG]-[PCL] polymer (**chapter 6**). Protein-polymer interactions have been previously described as an important cause of *in vitro* incomplete protein release from PLGA based depot systems. They have been ascribed to protein aggregation, protein adsorption to hydrophobic PLGA [6–10], electrostatic interactions between protein and polymer [7,11] and chemical modifications of protein, e.g. acylation [12–14]. We speculated whether the formation of polymer degradation products could be responsible for the protein-polymer interaction or whether the protein remained entrapped in the semi-crystalline block upon the dissolution/degradation of the amorphous block. However, the exact mechanism of this incomplete release should further be investigated in more detail. One of the possibilities to further evaluate the nature of the covalent protein-polymer bonding could be the analysis of samples by LC-MS after incubation with an aqueous NaOH solution. These methods would allow determination of additional peaks and identification of the molecular weights of the obtained compounds. Additionally, protein labeling and subsequent visualization after extrusion and during the *in-vitro* release could provide more information on the protein distribution inside the implants.

Experiencing incomplete release of protein as a possible consequence of polymer degradation, one can thus argue whether the polymer degradation should occur in parallel to protein release or whether massive polymer degradation should occur only after the entire protein has already been released to avoid the risks of these irreversible interactions. In the study described in **chapter 5** it was observed that certain degradation (or surface erosion) of polymer and formation of interconnecting channels was necessary for release of larger proteins. However, to avoid the risk of the interaction of proteins with the degrading polymer, ideally protein should be released from the polymer depot by diffusion. The diffusion of the protein from the polymer bulk could be enhanced by co-incorporation of pore formers into polymer matrix. This can be for example achieved by incorporation of PEG into the polymer matrix, thereby increasing the swellability of polymers (**chapter 5 and 6**) or by co-encapsulation of inulin as a pore-forming excipient (**chapter 4**).

Although we have shown that inulin sufficiently stabilized the model proteins lysozyme and albumin during the extrusion process, these proteins may be inherently much more stable than many other therapeutically relevant proteins e.g. interleukins, human growth hormone or TNF-Related Apoptosis Inducing Ligand. Additionally, other model proteins used in our study, i.e. goserelin, insulin and carbonic anhydrase, were extruded only in presence of inulin, hence their stability during the extrusion process without stabilizer remains questionable. Therefore, future investigations should be directed towards the evaluation of the therapeutically relevant thermolabile proteins and the capability of inulin to protect them during this process. Alternatively, when polymers with fusion temperatures much higher than 55 °C are required for the preparation of protein-loaded polymer depots, inulin could offer sufficient protection during the HME process. Future studies on this issue are therefore also recommended.

Since protein stability is of great importance during the encapsulation and controlled release from drug delivery systems, the use of *in-line* techniques for protein characterization (FTIR, CD, Raman) during the extrusion process or during the protein release and characterization would be of high interest to address the protein's structural integrity. Additionally, even though we have shown in this thesis that inulin preserved the proteins integrity during extrusion, it remains questionable to which extent the activity of inulin-stabilized proteins would be preserved under *in-vivo* conditions. Upon the administration of the implants and upon contact with the aqueous medium, the inulin particles will be wetted and will (partially) be dissolved before all the incorporated protein is released. Therefore, rational *in-vivo* studies using appropriate animal models would be required in order to give an answer to these questions. Moreover, the *in-vivo* pharmacokinetics studies should be performed to confirm the predictive value of *in-vitro* release profiles.

For the rational development of the protein loaded polymer matrices and optimization of protein stability, one must first determine the stage at which protein degradation occurs and identify the stress factors which compromise protein stability. Afterwards a rational stabilization approach can be followed to ensure the safety and efficacy of the protein-loaded polymer depots. These developments could result in the clinical availability of sustained protein release systems.

References

[1] W.L.J. Hinrichs, N.N. Sanders, S.C. De Smedt, J. Demeester, H.W. Frijlink, Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes., J. Control. Release. 103 (2005) 465–479.

[2] L. Cheng, S. Guo, W. Wu, Characterization and in vitro release of praziquantel from poly(epsilon-caprolactone) implants., Int. J. Pharm. 377 (2009) 112–119.

[3] C.G. Pitt, M.M. Gratzl, G.L. Kimmel, J. Surles, A. Schindler, Aliphatic polyesters II. The degradation of poly (DL-lactide), poly (epsilon-caprolactone), and their copolymers in vivo., Biomaterials. 2 (1981) 215–220.

[4] H. Sun, L. Mei, C. Song, X. Cui, P. Wang, The in vivo degradation, absorption and excretion of PCL-based implant., Biomaterials. 27 (2006) 1735–1740.

[5] M. Stanković, J. Tomar, C. Hiemstra, R. Steendam, H.W. Frijlink, W.L.J. Hinrichs, Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly(ε-caprolactone) multiblock-copolymer implants, Eur. J. Pharm. Biopharm. 87 (2014) 329–337.

[6] G. Crotts, T.G. Park, Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues., J. Microencapsul. 15 (1998) 699–713.

[7] T.G. Park, H. Yong Lee, Y. Sung Nam, A new preparation method for protein loaded poly(D, L-lactic-co-glycolic acid) microspheres and protein release mechanism study, J Control Release. 55 (1998) 181–191.

[8] T. Tsai, R.C. Mehta, P.P. Deluca, Adsorption of peptides to poly(D,L-lactide-co-glycolide): 1. Effect of physical factors on the adsorption, Int. J. Pharm. 127 (1996) 31–42.

[9] S.M. Butler, M.A. Tracy, R.D. Tilton, Adsorption of serum albumin to thin films of poly(lactide-co-glycolide), J Control Release. 58 (1999) 335–347.

[10] G. Jiang, B.H. Woo, F. Kang, J. Singh, P.P. DeLuca, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres., J. Control. Release. 79 (2002) 137–145.

[11] J. Chesko, J. Kazzaz, M. Ugozzoli, T. O'Hagan D, M. Singh, An investigation of the factors controlling the adsorption of protein antigens to anionic PLG microparticles, J Pharm Sci. 94 (2005) 2510–2519.

[12] Z. Ghalanbor, M. Körber, R. Bodmeier, 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 (2012) 302–306.

[13] M.L. Houchin, K. Heppert, E.M. Topp, Deamidation, acylation and proteolysis of a model peptide in PLGA films., J. Control. Release. 112 (2006) 111–119.

[14] Y. Zhang, S.P. Schwendeman, Minimizing acylation of peptides in PLGA microspheres., J. Control. Release. 162 (2012) 119–126.

—Appendix I—

Samenvatting en conclusies

Nederlandse samenvatting

Recente ontwikkelingen op het gebied van de biotechnologie en de moleculaire biologie hebben geleid tot een groeiende belangstelling voor het gebruik van peptiden en eiwitten voor de behandeling van een verscheidenheid aan ziekten en aandoeningen. Peptiden en eiwitten worden echter gekenmerkt door instabiliteit in het maagdarmkanaal en slechte absorptie over de darmwand. Daarom worden deze macromoleculen doorgaans per injectie toegediend. Vaak is frequente toediening noodzakelijk omdat eiwitten in het algemeen een korte eliminatiehalfwaardetijd bezitten. Op polymeren gebaseerde, injecteerbare depotformuleringen voor vertraagde afgifte van eiwitten over een langere periode bieden een aantrekkelijk alternatief voor deze frequente injecties, onder andere vanwege hun potentie om de therapietrouw te verhogen en therapiekosten te verlagen.

Smelt-extrusie (hot melt extrusion, HME) is een technologie die veelvuldig wordt toegepast voor de bereiding van polymeerimplantaten met gereguleerde afgifte van geneesmiddelen. Deze methode biedt tal van voordelen ten opzichte van de meer traditionele micro-incorporatiemethoden die zijn gebaseerd op emulsies. Maar men moet zich realiseren dat de incorporatie van eiwitten in polymeermatrices met behulp van HME een complex proces is, vanwege de zeer gevoelige aard van deze macromoleculen. Stabilisatie van eiwitten vóór, tijdens en na de incorporatie is daarom van groot belang. Als matrix verdienen biologisch afbreekbare polymeren de voorkeur, aangezien deze in het lichaam worden gemetaboliseerd tot onschadelijke producten en vervolgens worden uitgescheiden. Ze behoeven dan geen chirurgische verwijdering wanneer de werkzame stof is afgegeven.

De studies die in dit proefschrift beschreven staan hadden tot doel om de mechanismen van eiwitstabilisatie met behulp van suikerglastechnologie te onderzoeken, om met suikerglas gestabiliseerde eiwitten in innovatieve, biologisch afbreekbare polymeermatrices te incorporeren door middel van HME, en om de eiwitafgifte uit deze matrices in kaart te brengen. Het onderzoek richtte zich op twee belangrijke processtappen:

1. Evaluatie van de sproeidroogtechnologie voor stabilisatie van eiwitten met behulp van suikerglazen, gevolgd door het inbouwen van de gestabiliseerde eiwitten in biologisch afbreekbare polymeren.

2. Evaluatie van nieuwe multiblok-copolymeren als matrix voor de gecontroleerde afgifte van eiwitten. HME werd gekozen als methode voor het vervaardigen van depotformuleringen met geïncorporeerde eiwitten.

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van de HME techniek en de gebruikte polymeren. De toepassing van HME voor de bereiding van

verschillende formuleringen voor orale, sublinguale, transdermale, intra-oculaire en parenterale toediening wordt geëvalueerd. Biologisch afbreekbare en niet-biologisch afbreekbare polymeren die algemeen worden toegepast voor deze techniek staan beschreven en de belangrijkste technieken voor het karakteriseren van op polymeren gebaseerde HME-producten zijn samengevat.

In **hoofdstuk 3** is de rol onderzocht die verglazing en het zogenaamde watervervangingsmechanisme spelen bij eiwitstabilisatie met behulp van suikerglazen. Hierbij werd alkalische fosfatase als een modeleiwit gebruikt. Het eiwit werd door middel van sproeidrogen ingebouwd in een matrix van inuline en trehalose in de glastoestand. Ammediol werd gebruikt als buffer, maar diende tevens als weekmaker door het glasvormende vermogen van deze stof. Door het variëren van de suiker/buffer verhouding werden gesproeidroogde producten verkregen met verschillende glasovergangstemperaturen. De opslagstabiliteit van het eiwit bij deze verschillende glasovergangstemperaturen werd onderzocht. Er werd aangetoond dat wanneer de glasovergangstemperatuur onder de opslagtemperatuur is, eiwitstabilisatie plaatsvindt via het verglazingmechanisme. Wanneer de glasovergangstemperatuur boven opslagtemperatuur is, is juist het watervervangingsmechanisme dominant.

In **hoofdstuk 4** wordt de synthese en karakterisering beschreven van een nieuwe hydrofiel multiblokcopolymeer die bestaat uit twee fase-gescheiden blokken. Het ene blok bestond uit semi-kristallijn poly(ε-caprolacton) (PCL) (30% w/w van het copolymeer), het andere uit amorf poly(ethyleenglycol)-poly(ε-caprolacton) (PCL-PEG) (70% w/w van het copolymeer). Een groot voordeel van dit copolymeer is dat extrusie mogelijk is bij een veel lagere temperatuur (55 °C) dan die nodig is voor het veelgebruikte poly(lactide-co-glycolide) (> 85 °C). Daarnaast werd onderzocht of lysozym (als modeleiwit) kan worden geïncorporeerd in het polymeer met behulp van HME, zonder dat de biologische activiteit hierdoor verminderde.

Waterige oplossingen van lysozym werden gesproeidroogd met en zonder inuline, zodat poeders werden verkregen met een lysozym/inuline gewichtsverhouding van 1/0, 1/1, 1/2, 1/3. De gesproeidroogde poeders hadden een vergelijkbare deeltjesgrootteverdeling (gemiddelde diameter was 1,09-1,50 μm). Vervolgens werden de poeders in het polymeer geïncorporeerd, met totale beladingen (lysozym en inuline) van respectievelijk 10, 20, 30 en 40% w/w, waarbij de lysozymbelading constant werd gehouden. Tevens werd lysozym als uitgangsproduct (gemiddelde diameter was 20,82 μm) in het polymeer geïncorporeerd om het effect van de deeltjesgrootte te bestuderen. In alle gevallen bleef de enzymatische activiteit van lysozym intact tijdens HME. De *in-vitro* lysozymafgiftekinetiek alsmede met de enzymatische activiteit van het eiwit gedurende 260 dagen incubatie in fosfaatbuffer (pH 7,4) van 37 °C werden onderzocht. Co-incorporatie van verschillende hoeveelheden inuline resulteerde in een verhoogde lysozymafgiftesnelheid, wat

betekent dat inuline fungeerde als porievormer. De monsters met 10 en 20% w/w eiwit/suikerbelading gaven, na een hoge initiële afgifte, het eiwit geleidelijk af gedurende 260 dagen met een kinetiek die leek op een eerste-ordekinetiekprofiel. Dit suggereert dat, na de hoge initiële afgifte, de eiwitafgifte bij lage beladingen geregeld werd door diffusie door de polymeermatrix. Bij hogere belading werd lysozym zeer snel afgegeven. Dit wijst erop dat de lysozym/inulinedeeltjes verbonden waren via een percolerend netwerk, waardoor het eiwit snel naar buiten diffundeerde. Tevens werd waargenomen dat de initiële afgifte van lysozym sterk afhangt van de deeltjesgrootte van het eiwit. Een formulering bestaande uit 10% w/w gesproeidroogd lysozym had een kleinere initiële afgifte dan een formulering die dezelfde hoeveelheid lysozym met grotere deeltjesgrootte bevatte (het uitgangsproduct). Na 70 dagen afgifte was de eiwitafgiftesnelheid van beide formuleringen vergelijkbaar, wat aangeeft dat in de latere fase de afgifte niet afhankelijk was van de deeltjesgrootte. Na 180 dagen resteerde een enzymactiviteit van 80%. Daarbij bleek uit scanning elektronenmicroscopie dat de polymeermorfologie na 180 dagen afgifte intact was. Dit is een indicatie dat het polymeer zeer langzaam degradeerde. Samenvattend, in dit onderzoek werd aangetoond dat niet alleen de eiwit/suikerbelading, maar dat ook de deeltjesgrootte van het eiwit in beschouwing moet worden genomen bij het ontwerpen van een polymeerproduct met gereguleerde afgifte.

In **hoofdstuk 5** werd de afbraakkinetiek geëvalueerd van het multiblokcopolymeer dat wordt beschreven in hoofdstuk 4. Voorts werd onderzocht of de afbraaksnelheid van dit copolymeer kan worden verhoogd door het vergroten van amorfe fractie (PCL-PEG) in het polymeer. Hiertoe werden polymeren met verschillende *x*[PCL-PEG_{1500}]/*y*[PCL]-blokverhoudingen (*x*/*y* zijnde 30/70, 50/50, 70/30 (w/w)) gesynthetiseerd en gekarakteriseerd en werd het *in-vitro* afbraakgedrag geëvalueerd van implantaten bestaande uit enkel polymeer gedurende 140 dagen incubatie in fosfaatbuffer (pH 7,4) van 37 °C. Er werd waargenomen dat de afbraaksnelheid van deze copolymeren werd verhoogd bij een toenemende [PCL-PEG] / [PCL] verhouding. De effecten van het molecuulgewicht van het eiwit en de verhouding tussen de polymeerblokken in relatie tot de afgiftekinetiek werden eveneens onderzocht. Aangezien de studies beschreven in hoofdstuk 4 aantoonden dat zowel eiwitdeeltjesgrootte als eiwitbelading de afgiftekinetiek beïnvloeden, werden in deze studie eiwitten met verschillende molecuulgewichten (gosereline (1.2 kDa), insuline (5.8 kDa), lysozym (14 kDa), koolzuuranhydrase (29 kDa), runderserumalbumine (66 kDa)) gesproeidroogd met inuline in gelijke gewichtsverhouding (1/10) en gelijke procescondities om zodoende gelijke deeltjesgrootteverdelingen te krijgen. De poeders werden in gelijke belading (eiwit + inuline) (11% w/w) geïncorporeerd in de polymeren. Alle formuleringen werden geëxtrudeerd bij lage temperaturen (50 - 55 °C).

De structurele integriteit van de eiwitten tijdens extrusie bleef intact. Verder werd aangetoond dat de eiwitafgiftesnelheid toenam met afnemend molecuulgewicht van het eiwit en met toenemende [PCL-PEG]/[PCL] verhouding in het polymeer. Geconcludeerd werd dat eiwitten met een laag molecuulgewicht voornamelijk vrijkomen door diffusie; echter wanneer het eiwit groter is dan de grootte van de poriën tussen de polymeerketens dan bepalen zowel eiwitdiffusie als polymeerafbraak de eiwitafgifte. Een belangrijke conclusie is dat het molecuulgewicht van het eiwit en de [PCL-PEG]/[PCL] verhouding in het polymeer beide essentieel zijn voor het ontwerpen van een optimaal depotpreparaat met gereguleerde eiwitafgifte.

Hoofdstuk 6 beschrijft de synthese van nieuwe multiblokcopolymeren, opgebouwd uit semi-kristallijne blokken van poly(ε-caprolactone) (PCL) en amorfe blokken van poly(D,L-lactide) (PDLA) en poly(ethyleenglycol) (PEG) [PDLA-PEG]. De blokverhouding in deze [PDLA-PEG]-b-[PCL] multiblokcopolymeren werd gevarieerd en de afbraak van implantaten van deze polymeren (bereid met HME) werd vergeleken met die van implantaten bestaande uit 30[PCL-PEG_{1500}] -70[PCL], een copolymeer dat in de studies in de vorige hoofdstukken werd gebruikt.. Daarnaast werd de afgifte onderzocht van de modeleiwitten lysozym en runderserumalbumine na incorporatie in de polymeerimplantaten.

De copolymeren bestaande uit [PDLA-PEG]-b-[PCL] vertoonden een verhoogde afbraaksnelheid in de beginfase, die gestuurd kon worden door de [PDLA-PEG] / [PCL] blokverhouding aan te passen. Volgens verwachting werd gevonden dat de eiwitafgiftesnelheid toeneemt bij verhoogde polymeerafbraaksnelheid. De eiwitafgifte uit [PDLA-PEG]-b-[PCL] copolymeren was echter onvolledig, in tegenstelling tot [PCL-PEG]-b-[PCL] copolymeren die langzame en continue afgifte vertoonden. De onvolledige eiwitafgifte werd toegeschreven aan ofwel irreversibele interactie tussen de eiwitten en de polymere afbraakproducten ofwel inclusie van de eiwitdeeltjes in de semi-kristallijne PCL-matrix. De volledige, continue eiwitafgifte uit het langzaam degraderende [PCL-PEG]-b-[PCL] polymeer gedurende de gehele studie suggereert dat eiwit-polymeerinteracties of irreversibele eiwitinclusie niet optreden bij dit type copolymeer.

Conclusies en perspectieven

Er zijn diverse micro-incoporatiemethoden gebaseerd op emulsies beschikbaar om moleculen in polymeermatrices in te bouwen. In tegenstelling tot deze methoden is HME een oplosmiddelvrije methode, die hierom interessant is voor de ontwikkeling van toedieningsvormen. Ofschoon HME vele voordelen kent (besproken in **hoofdstuk 2**), zijn er echter vaak hoge temperaturen vereist en treden er schuifspanningen op die

schadelijk kunnen zijn voor instabiele stoffen zoals eiwitten. In dit proefschrift werd daarom een combinatie van twee strategieën onderzocht met als doel eiwitafbraak tijdens incorporatie in polymeermatrices door HME te voorkomen: 1) pre-stabilisatie van eiwitten met behulp van suikerglastechnologie en 2) toepassing van nieuwe polymeren die bij relatief lage temperaturen geëxtrudeerd kunnen worden.

In de literatuur wordt de disaccharide trehalose vaak omschreven als de gouden standaard voor eiwitstabilisatie tijdens vries- of sproeidroogprocessen en daaropvolgende opslag. De oligosaccharide inuline is eveneens bekend als een uitstekende eiwitstabilisator. Om deze redenen hebben we de stabiliserende capaciteiten van trehalose en inuline onderzocht en vergeleken (**hoofdstuk 3**). Hoewel eiwitstabilisatie vaak wordt toegeschreven aan verglazing of watervervanging, hebben wij aangetoond dat het verschil tussen de glasovergangstemperatuur (Tg) en de opslagtemperatuur bepaalt volgens welk mechanisme eiwitstabilisatie plaatsvindt. Wanneer de Tg van de gesproeidroogde producten gelijk werd gemaakt door toevoeging van ammediol als weekmaker, bleek trehalose beter te stabiliseren dan inuline. Omdat zuiver inuline een hogere Tg heeft dan zuiver trehalose, kan inuline wel een aantrekkelijk alternatief zijn voor trehalose, vooral wanneer monsters worden bewaard bij hoge relatieve luchtvochtigheid en hoge temperatuur. Daarnaast biedt de hogere Tg van inuline betere eiwitstabilisatie tijdens processen die worden uitgevoerd bij hoge temperatuur, zoals HME.

Het nieuwe, hydrofiele multiblokcopolymeer gebaseerd op PCL en PEG (**hoofdstuk 4**) heeft gunstige eigenschappen voor het gebruik als matrix voor de incorporatie van eiwitten met HME. Deze polymeer kan bij 55 °C geëxtrudeerd worden, wat 30 °C lager is dan de extrusietemperatuur die nodig is voor PLGA. De lage temperatuur is een groot voordeel voor het incorporeren van thermolabiele stoffen zoals eiwitten en peptiden. Met het modeleiwit lysozym werd aangetoond dat er sprake was van continue afgiftekinetiek in plaats van de bifasische afgifte die meestal wordt waargenomen met PLGA preparaten. De lysozymafgifte was met name diffusiebepaald en het polymeer bleek slechts zeer beperkt te degraderen gedurende de afgifteperiode (260 dagen) (**hoofdstuk 5)**. De langzame degradatie van deze polymeren kan gunstig zijn wanneer stoffen met een vereiste afgifte van langer dan 1 jaar moeten worden geïncorporeerd. Voor moleculen kleiner dan een bepaalde hydrodynamische diameter is de afgifte sneller dan de polymeerafbraaksnelheid. Voor deze moleculen zou de ontwikkeling van een polymeer met versnelde afbraakkinetiek gunstig kunnen zijn, waardoor de levensduur van een implantaat beter overeenkomt met de duur van eiwitafgifte.

Snellere degradatie kan worden verkregen door het vervangen van het amorfe blok van [PCL-PEG] in [PCL-PEG]-[PCL] door [PDLA-PEG]. Wij hebben echter aangetoond dat eiwitten irreversibel binden met afbraakproducten van de

[PDLA-PEG]-[PCL] polymeer (**hoofdstuk 6**). Eiwit-polymeerinteracties zijn eerder beschreven als een belangrijke oorzaak van onvolledige *in-vitro* eiwitafgifte uit op PLGA gebaseerde depotsystemen. Voorbeelden van zulke interacties zijn eiwitaggregatie, eiwitadsorptie aan het hydrofobe PLGA, elektrostatische interacties tussen eiwitten en polymeren en chemische modificaties van eiwitten, bijvoorbeeld acylering. We hebben gespeculeerd dat mogelijk de vorming van polymere afbraakproducten verantwoordelijk is voor de eiwit-polymeerinteracties of dat het eiwit gevangen bleef in het semi-kristallijne blok tijdens de dissolutie / degradatie van het amorfe blok. Het exacte mechanisme van deze onvolledige afgifte zal echter nog verder onderzocht moeten worden. Een van de mogelijkheden om de aard van de covalente eiwit-polymeerbinding verder in kaart te brengen is de analyse van monsters met behulp van een combinatie van vloeistofchromatografie met massaspectroscopie (LC-MS)nadat ze zijn geïncubeerd in een oplossing van NaOH in water. Met behulp van deze methode kan worden onderzocht of er nieuwe verbindingen zijn ontstaan en wat het molecuulgewicht van deze verbindingen is. Daarnaast kunnen eiwitlabeling en daaropvolgende visualisatie na extrusie en tijdens *in-vitro* afgifte meer informatie geven over de verdeling van het eiwit in de implantaten.

Omdat onvolledige eiwitafgifte een gevolg kan zijn van polymeerafbraak, is het discutabel of polymeerafbraak geleidelijk dient te gebeuren tijdens eiwitafgifte, of liever massaal nadat het eiwit volledig is afgegeven om het risico op irreversibele eiwit-polymeerinteracties te minimaliseren. In de studie beschreven in hoofdstuk 5 werd waargenomen dat een zekere mate van polymeerdegradatie (of -erosie) nodig is voor de vorming van onderling verbonden kanalen, waardoor ook grote eiwitten kunnen worden afgegeven. Echter, om het risico op interactie tussen eiwitten en de polymere afbraakproducten te voorkomen, dienen eiwitten idealiter vrij te komen uit het polymeerdepot door diffusie. Diffusie van het eiwit uit de polymeermassa kan worden bevorderd door co-integratie van porievormers in de polymeermatrix. Dit kan bijvoorbeeld worden bereikt door toevoeging van PEG aan de polymeermatrix, waardoor de zwelbaarheid van de matrix toeneemt (**hoofdstuk 5 en 6**) of door co-incorporatie van inuline als porievormer (**hoofdstuk 4**).

Wanneer er toch polymeren met extrusietemperaturen ver boven 55 °C nodig zijn voor de bereiding van met eiwit beladen polymeerdepots dan zou inuline mogelijk voldoende bescherming kunnen bieden tijdens het HME proces. Toekomstige studies die zich hierop richten worden daarom aanbevolen. Omdat eiwitstabiliteit gedurende het incorporeren en de gereguleerde afgifte van geneesmiddelafgiftesystemen van groot belang is, is het gebruik van *in-line* technieken voor eiwitkarakterisering (FTIR, CD, Raman) tijdens het extrusieproces of tijdens de eiwitafgifte relevant om de structurele integriteit van het eiwit te volgen.

Ondanks dat wij in dit proefschrift hebben laten zien dat inuline de eiwitintegriteit waarborgt tijdens extrusie blijft het de vraag in welke mate de eiwitten *in-vivo* wordt gestabiliseerd door inuline na het inbrengen van de implantaten, waardoor ze in contact komen met een waterige omgeving. Hierdoor zullen de inulinedeeltjes worden bevochtigd en zullen ze (deels) oplossen voordat de volledige eiwitinhoud is afgegeven wat zou kunnen leiden tot een verminderde stabilisatie. *In-vivo* studies met geschikte diermodellen zijn nodig om een antwoord op deze vraag te vinden. Bovendien is *in-vivo* farmacokinetisch onderzoek nodig om de voorspellende waarde van de *in-vitro* afgifteprofielen bevestigen.

Voor de verdere ontwikkeling van met eiwit beladen polymeermatrices en de optimalisatie van de eiwitstabiliteit in deze systemen moet eerst worden bepaald in welk stadium de eiwitafbraak plaatsvindt en moeten de stressfactoren worden geïdentificeerd die de eiwitstabiliteit verminderen. Vervolgens kan een rationele stabilisatiestrategie worden gevolgd om de veiligheid en werkzaamheid van de met eiwit beladen polymeerdepots te waarborgen. Een en ander moet leiden tot het klinisch beschikbaar komen van implanteerbare geneesmiddelafgiftesystemen met therapeutische eiwitten.

—Appendix II—

Acknowledgments

Acknowledgments

This PhD project was truly a journey on which many people have been important co-travelers. Along the journey there were moments of doubt, but from each of those moments I have learned important things about my own knowledge and capabilities. In my moments of disbelief, but also, fortunately, in many moments of happiness and success, I had companions who made my PhD research and my time in Groningen a valuable experience. I apologize in advance if I have not mentioned all of you here, but to each of you I am highly grateful.

First and foremost, I would like to express my sincere gratitude to my promoter, Prof.dr. Erik Frijlink, for his help and support. Erik, by accepting me for this PhD project you gave me a chance to discover and love research and at the same time to fight my own fears and doubts. You gave me the opportunity to learn from you and to develop myself within the scientific world. Your words of support were of great help to me with my job in Graz. I am sincerely thankful.

I would also like to express my great appreciation to my co-promoter, Dr. Wouter Hinrichs. Wouter, thank you for your incredible support and guidance during my PhD project. Thank you for the many scientific and non-scientific discussions we had, for your patience, for bearing with the insecurity I felt when I first came to Groningen and for helping me to develop confidence as a scientist. In spite of my stress, you always managed to encourage, motivate and teach me. I cannot imagine having a better mentor.

I would like to thank the assessment committee, Prof. dr. H.C.P. Kleinebudde, Prof dr. H.C. van der Mei, and Prof dr. A.A. Broekhuis, for taking time to evaluate my manuscript and for your valuable comments and suggestions.

Dr. Herman Woerdenbag, thank you for initiating the first contact that brought me to do my PhD in Groningen and thank you for reading and improving my Dutch summary.

My dear ladies, my paranymphs, hause and officemates and my friends, Dorenda and Jelena, thank you both for being so amazing. Jekić, we have known each other for more than 12 years and I am extremely grateful for our friendship. I am really happy you came to Groningen an year after me; it would certainly not be the same without you there ☺. Dorenda, "my roomy" thank you for always being so much fun, but also so understanding. It was always *gezellig* in the office and super fun. And thanks for keeping my "mafia" notes on your desk long after I have left Groningen ☺.

My dear colleagues and friends from the Department of Pharmaceutical Technology and Biopharmacy, you made my last four years there very pleasant.

BT, TT, Parinda, Tofan, Vinay, Senthil, Marcel, Naomi, Tobias, Maarten, Gaurav, thanks for being great colleagues and friends. Floris, thank you for your last minute support with the SEM photos. Niels, thank you for your patience and help during the initial stage of our PhD research. I am glad that our work together resulted in a nice publication. Hans, you were a great support during and after your post-doc position at our department. My dear Anne and Wouter, thanks for being great friends these years (did I say party loving also? ☺); Anne, thanks for fighting with my Dutch summary. Paul, Anko, Anne de Boer, Peter, Jan, Doetie, Lida, Marinella, Caroline, thanks for your assistance whenever it was needed and for the many interesting conversations we had. Anko, thank you for your help with HPLC and with other equipment I consider an enemy most of the time ☺, and thanks for the numerous SEM photos for my research. I knew for years that my "smiley-face happy polymer surface" will be on my cover. Christina, "my roomy", I am glad I have met you and shared the office and many, maaany interesting talks with you (some were even science-related ☺); but all the stories, laughs, tears and travels were priceless. Dear Sonja, you were an incredible help with all the administrative details, but above all I enjoyed our talks. I was really fortunate to be part of such a department.

I would like to thank my students, Gina, My Linh, Nathaly, Joop, Sander, Vera, and Jette, whose contribution is also included in pages of this thesis. Philip and Jasmine, thanks for your great support and friendship during the last 2 years of my PhD. Nothing could make me more proud than to attend your defense four years from now.

I would like to thank Rob, Christine and all the colleagues from InnoCore for providing polymers for my research and for sharing their knowledge about polymers. I am also thankful for your contributions to the publications. The last four years of working together resulted in better understanding of polymers, and I hope we all learned something from this collaboration. Johan, thanks for your constant help, and for your good humor and spirit; stay this way, it is just super nice! ☺ Maarten, Wilma, Laurens, Letitia, Lidia, Luisa, Catarina, Arjen, Evelien, Cees, Mark, Sonja, it was always so much fun - dinners, drinks, science and music together! Thanks for being always ready for a good time, even after I moved away from Groningen. Luisa, thanks for being a great friend and support! Cees, thank you for doing NMR and SEC measurements. Sonja thanks for providing polymers. Albert and Mark, thanks for introducing me to the beauties of extrusion ☺. Maarten, I am happy to have met someone with the same passion for playing music! The show must go on, next time recorded (maybe then I will forgive you testing the lab shower with me underneath it ☺). Evelien and Arjen, thanks for your help with HPLC when it was needed. You are all big contributors to this thesis.

Part of this thesis was completed during my work at the Research Center of Pharmaceutical Engineering (RCPE) in Graz. I would like to thank Prof. dr. Johannes Khinast and Dr. Thomas Klein for giving me the opportunity to work in RCPE while I was finalizing the thesis. Dear Massimo, thanks for your help and support. Dear Amrit and Simone, thank you for your understanding during the final stages of this thesis. Dear Anna, Shengqian, Diogo, thank you for your friendship and kindness. Dear Buchi, thanks for your help with the thesis cover.

I would also like to thank the Balkan community in Groningen for making the North feel more like a home, Vibiša, Kaća i Sara, Dejan, Sonja, Ivana, Miličica, Jelena, Ivan, Slobodan, Dule, Jelena i Relja, Višnja, Goća, Primzi, Danijela, Igor, Brana, Milena, Ena, Faris, Jovan..Živeli! Miličice i Višnjičice, zatvaramo mi naš klub izgleda, bar pod postojećim imenom☺. Vibiša, hvala ti što si pravi drugar, uvek bio i ostao☺! Duško i Jelena, thanks for giving amazing friendship and support from the very first moment I arrived in Groningen! I am happy to have met you all.

Maxitaji, you have been and still are my great support during these years. Thanks for being my sport buddy, my yoga teacher and my friend! Julia, Felix, Fede, Suzi, my dear HB Katia, Manuel, Helgi, Jaakko, Marcelo, Henri, you are amazing, caring, party-loving group of friends and I am really happy I got to know you in Groningen.

Beste Pape Seck, dank je wel voor het onderwijzen van de schoonheid van de Afrikaanse percussie en Djembe. De afgelopen 3 jaar heb ik uitgekeken naar elke les die je hebt gegeven. Jouw positieve energie en de muziek die je maakt waren mijn afleiding in alledaagse situaties en mijn PhD, het heeft me enorm geholpen.

I would like to thank my flatmates for being there for me and for sharing everyday life in Groningen. Chronologically, Petros and Rania, it was really nice to live together; Petros you helped me a lot during the first years of my PhD. Clement, I am so happy that we shared the house! It was always so much fun, and thanks to you and Jekić it always felt like home and a family.

My best friends in Serbia, Viki, Ana, Vanja, Milena, Neda and your families, for almost 20 years you have been an indispensible part of my life. Thanks for your faith in me, for listening to my stories and complaints, for your understanding and love and for being always proud of me! Stevo, Jeko, Bojana, Kirovcice, Stevco, thanks for your friendship. Mnogo, ali mnogo vas volim!

Liebe Gisela, Emmerich und Andi, Ich bin so froh, dass es Euch gibt und ihr zu meiner zweiten Familie in Österreich geworden seid. Danke für alle Eure Unterstützung. Ich bin sehr glücklich Euch hier zu haben!

My family, there are no words to express how thankful I am for your support and love. Ne bih nikada bila ovde, niti postigla sve ovo da nije vas. My mom and

my best friend Sonja, you had to deal with me and my never ending ambition and doubts- but your endless love, advice and positive attitude always helped me to take the next step. Najbolja si i volim te najviše na svetu celom! Rašo, hvala ti za svu podršku koju nam pružaš godinama. Laki, I love you more than anything on this earth, and I am the most grateful and proud person to have you as a brother! And the fact that you are beginning your studies in Graz and that you are finally going to be close to me again makes me enormously happy ☺. Baka Leno, hvala ti to veruješ u mene, uvek. Tata, baka Saveta i deda Brana, znam da se vi i dalje brinete o meni i da bi ste bili vrlo ponosni sad. Volela da mogu da podelim ovaj ali i svaki drugi trenutak sa vama. Na kraju dana, jedna Tokata i Fuga u D-molu sigurno će preneti bar deo emocija.

And finally, Stefan, I would need an infinite number of pages just to describe how grateful I am to have met you and how much I love you, but I think my writing must come to an end. Finally ☺. The last four years of our fight with the distance were certainly not always easy (not to mention cyclining thousands of kilometres during our precious holidays - instead of relaxing like, you know, normal people ☺), but our faith in US and your endless support overcame all the miles that separated us. Thank you for your patience during my PhD. You are the most incredible person I have ever met and I am endlessly thankful for your love and never-ending laughter!

—Appendix III—

Curriculum vitae

Curriculum vitae

Name: Milica Stanković

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2002 - 2008	Pharmacy studies Medical faculty, Department for Pharmacy, University of Niš, Serbia
2008 - 2009	Pharmacist, internship Pharmacy "Danifarm" Niš, Serbia
2009 - 2013	PhD candidate Department for Pharmaceutical technology and Biopharmacy, University of Groningen, The Netherlands; InnoCore Technologies, Groningen, The Netherlands
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List of publications and selected presentations

M. Stanković, H. de Waard, R. Steendam, C. Hiemstra, J. Zuidema, H.W. Frijlink and W.L.J. Hinrichs. *Low temperature extruded implants based on novel hydrophilic multiblock copolymer for long-term protein delivery.* Eur. J. Pharm. Sci, 2013.

M. Stanković, J.Tomar, C. Hiemstra, R. Steendam, J. Zuidema, H.W. Frijlink and W.L.J. Hinrichs. *Tailored protein release from biodegradable poly(ε-caprolactone-PEG)-b-poly(ε-caprolactone) multiblock-copolymer implants.* Eur. J. Pharm. Biopharm, 2014.

N. Grasmeijer, **M. Stanković**, H. de Waard, H.W. Frijlink, W.L.J. Hinrichs. *Unraveling protein stabilization mechanisms: vitrification and water replacement in a glass transition temperature controlled system.* Biochim. Biophys. Acta, 2013.

M. Stanković, C. Hiemstra , H.de Waard, J. Zuidema, R. Steendam, H.W. Frijlink, W.L.J. Hinrichs. *Incomplete protein release from hydrophilic poly(D,L-lactide-PEG)-b-poly(ε-caprolactone) implants.* Submitted for publication.

M. Stanković, H.W. Frijlink, W.L.J. Hinrichs. *Polymeric formulations for drug release prepared by Hot Melt Extrusion; Application and characterization.* Submitted for publication.

M. Stanković, 2012. *Low temperature extrusion - preparation of polymeric implants for controlled release of proteins.* Pharmacy day, Groningen, The Netherlands.

M. Stanković, 2012. *Low temperature extrusion of a novel biodegradable multiblock copolymer and a strategy to tailor release of proteins.* Biopharmacy Day, Utrecht, The Netherlands.

M. Stanković, 2014. *Tailored protein release from multiblock copolymer implants made by hot melt extrusion.* 6th International Congress on Pharmaceutical Engineering, Graz, Austria.

M. Stanković, H. de Waard, W.L.J. Hinrichs, R. Steendam, C. Hiemstra, J. Zuidema, H.W. Frijlink, 2011. *Novel multi-blockcopolymer for low temperature extrusion of protein depots.* 5th International Congress on Pharmaceutical Engineering, Graz, Austria.

M. Stanković, H. de Waard, C. Hiemstra, W.L.J. Hinrichs, R. Steendam, H.W. Frijlink, 2012. *Strategies to tailor protein release from novel hydrophilic multi block co-polymeric implants*. 39th Annual Meeting & Exposition of the Controlled Release Society, Québec City, Canada.