Microspheres based on biodegradable functionalized poly(alpha-hydroxy) acids for the controlled release of bioactive proteins and peptides

Amir Hossein Ghassemi

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Cover: Fluorescent microscope image of rhodamine methacrylate-loaded PLHMGA microspheres

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Microsferen gebaseerd op biodegradeerbare, gefunctionaliseerde poly(alpha-hydroxy) zuren voor de gecontroleerde afgifte van bioactive eiwitten en peptiden
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 12 september 2011 des middags te 2.30 uur

door

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geboren op 13 juli 1979, te Tehran, Iran

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Dr. C.F. van Nostrum

To my parents

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Chapter 1

General introduction

1.1. Pharmaceutical peptide/protein

With the discovery of insulin by Banting and Best in 1921 as a potent molecule for treatment of diabetic patients, pharmaceutical peptide/proteins from natural sources (e.g. porcine, bovine) were introduced for the treatment of patients. Many other diseases are based on the lack of endogenous proteins, but the introduction of therapeutic proteins from natural sources was more the exception (e.g., Factor VIII, growth hormone) than the rule. With advances in biotechnology and chemistry, therapeutic proteins such as cytokines, hormones, antibodies, as well as bioactive peptides including luteinizing hormone releasing hormone (LHRH) and octreotide have become commercially available in sufficient quantities to treat patients.

A major challenge with protein drugs is that these molecules are often highly instable and prone to degradation which may lead to their inactivation or induction of unwanted side effects and immunogenic reactions (1-6). Therefore, preparation of protein formulations and delivery of these protein drugs are not without problems. The main function of a successful drug delivery system is to deliver the drug at the right time to the active site at a therapeutically effective concentration while preserving the efficacy of the drug with the lowest possible side effects and highest patient compliance. Many peptide/protein drugs on the market fail to meet one or more of these criteria because of the many obstacles involved in protein delivery, such as biological barriers to transport, (enzymatic) degradation and stability issues during formulation (5).

At present, the administration of peptide/protein drugs for systemic delivery is almost exclusively either by injection or by infusion. Other routes of administration have been extensively explored but have been met, so far, with little success. Therefore, in this thesis the focus will be on optimizing parenteral delivery strategies for proteins and peptides. Upon injection, the short half life of many of these molecules requires repetitive injection to achieve the desired therapeutic effects. Short injection intervals and high peak/trough ratios in the circulation can be associated with (severe) side effects and are inconvenient to patients. Therefore, there is a need for continuous release of these biologically active molecules from a delivery system which keeps the peptide/protein intact before drug release. The most commonly used systems for sustained peptide/protein delivery are particulate drug delivery systems which will be briefly introduced in section 2 of this chapter.

1.2. Particulate systems for peptide/protein delivery

Particulate peptide/protein delivery systems for parenteral use are made from biodegradable and biocompatible materials. They can appear in the form of emulsions, liposomes, hydrogels, nanospheres and microspheres (7-10). Bjerregaard et al. (11) found a prolonged release of aprotinin incorporated in a water-in-oil emulsion, a 6.5 kDa protein, in mice upon intraperitoneal injection and showed that the emulsion was tolerated with low acute toxicity. However, formulation and protein stability issues as well as low encapsulation efficiency, incomplete release and poor control over release kinetics limit the use of emulsions as protein delivery system. Liposomal formulations demonstrated the capability of releasing encapsulated peptide and proteins by adjusting the physical and chemical characteristics of liposomes e.g. the lipid composition (12-14). One of the major drawbacks of the use of liposomes is the fast elimination from the blood by the reticulo-endothelial cells and difficulties to achieve tailorable controlled release (14). Proteins and peptides can be physically incorporated in the hydrogel matrix and unlike other delivery systems (e.g. emulsions, liposomes, microspheres) where preparation conditions are sometimes detrimental to proteins (e.g use of organic solvents), mild conditions are used in hydrogel preparation procedure. Nevertheless, an initial burst release of proteins and peptides is one of the major challenges by using hydrogels (15,16). The initial burst release can be partially decreased by tailoring hydrogels cross-link density via changes in polymer architecture, molecular weight or concentration. Preformed, macroscopic hydrogels have to be administered by surgical intervention which is another drawback of hydrogels. To circumvent this hurdle, injectable hydrogels (which are characterized as polymer solutions prior to injection and turn into visco-elastic system at the site of administration) have been investigated for drug delivery. To increase the stability of injectable hydrogels after forming gel, cross-linking methods (photopolymerization, Michael addition, stereocomplexation, etc.) have been exploited (17-19). Other particulate systems are nanospheres and microspheres-based drug delivery systems which are advantageous because of their injectability without considerable changes in geometry and stability of particles after injection. These formulations are usually made from different biodegradable polymers such as poly(D,L-lactide-co-glycolide) (PLGA), poly (lactic acid) (PLA), poly(ε-caprolactone) (PCL) and chitosan. Among the abovementioned polymers, PLGA is one of the most frequently investigated biodegradable systems by both academic and industrial research groups for peptide/protein delivery. PLGA degrades via hydrolysis of the ester bonds and the degradation products are lactic and glycolic acids which are endogenous compounds. Years of experience have

shown that materials in the form of microspheres and implants have a good biocompatibility and degrades in vivo by bulk erosion in a timeframe of 2-4 months, depending on the molecular weight of the polymer, the size of the device and the site of implantation (20,21). Microspheres based on PLGA, showed sustained release of encapsulated peptide and proteins such as human growth hormone (22), leuprolide acetate (23) and octreotide (24). Microspheres of this polymer have also been used for the entrapment and release of antigens for development of so-called single shot vaccines (25, 26). These PLGA particles have been mostly prepared by solvent diffusion (27), solvent evaporation (28), multiple emulsion (29) and solvent displacement methods (30).

In general, one of the major drawbacks of PLGA microsphere formulations is incomplete and difficult to tailor release of the encapsulated biopharmaceuticals which may influence their potential as drug delivery systems (31,32). Peptides/proteins are essentially immobile in homogeneous/non-porous PLGA matrices. Saying that, release/transport of encapsulated peptide/protein molecules can only occur when the matrix is porous (it is very difficult to control porosity and it is irreproducible) and the pores are interconnected or when the matrix degrades (2-4 months). Moreover, the drop of the pH inside the microspheres of PLGA due to the accumulation of degradation products (lactic and glycolic acid as well as their oligomers) (33) can cause the unfolding/aggregation of the proteins which can trigger unwanted side effects such as immunogenicity and toxicity (2,34). Furthermore, chemical reactions such as deamidation, oxidation and acylation of peptide and proteins can occur in the acidic microenvironment generated in degrading PLGA matrices (35). Several attempts have been made to stabilize peptides and proteins inside PLGA microspheres among which pegylation and addition of excipients to prevent the drop of the pH are well known (36-40). Although these approaches (partly) overcome some drawbacks of PLGA systems, there is still a need of polymers that degrade more rapidly than PLGA and that similarly as PLGA yield non-toxic degradation products. Importantly, the new polymers should be more hydrophilic to increase the water absorbing capacity of the polymer matrices to facilitate the release of degradation products (lactic and glycolic acid and their oligomers), thereby preventing the pH drop which is detrimental for peptide and protein activity. All these parameters (faster degradation, no pH drop, increased hydrophilicity) may lead to a complete release of peptide/proteins with preserved structural integrity. Recently, with the introduction of functional polyesters, a new chapter for biomacromolecular drug delivery has been opened (chapter 2).

1.3. Functional polyesters

These polymers are mainly based on aliphatic polyesters (e.g. PLGA, PDLA, PCL) with enhanced hydrophilicity resulting in higher degradation rates. Importantly, it is expected that due to the more hydrophilic nature of these polymers, matrix acidification during degradation is prevented by enhanced hydration of the matrix and thus faster diffusion of acidic degradation products out of the matrix. Many functionalized polyesters have been synthesized in recent years (41-43). However, despite their obvious advantages over their non-functionalized counterparts, applications of these polymers have been rarely studied in the biomedical and pharmaceutical areas. In a previous project carried out in our department, Leemhuis et al. synthesized and characterized functionalized polyesters, poly(lactide-co-hydroxymethyl glycolide) (PLHMGA) (Fig. 1) (44,45). The degradation of films made from copolymers varying in monomer molar ratios was investigated and it was shown that the degradation of the films made from PLHMGA copolymer could be tailored from a few days to two months (46). In the present thesis, the suitability of PLHMGA for the controlled release of proteins and peptides is explored.

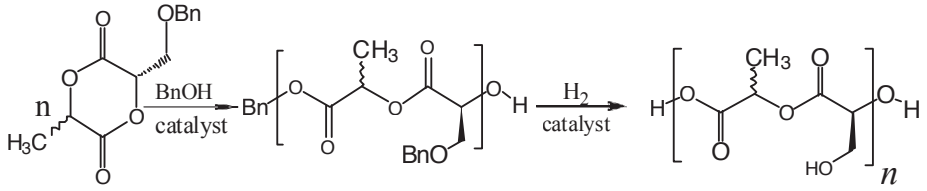

Fig. 1. Synthesis of hydrophilic aliphatic polyesters based on lactic acid and glycolic acid with pendant hydroxyl groups, poly(lactic-co-hydroxymethyl glycolic acid) (44).

1.4. Aim of the thesis

This thesis focuses on the preparation of microspheres based on the functional polyester PLHMGA and their application for sustained delivery of peptides and proteins. Microspheres were prepared with the well-known double emulsion evaporation extraction technique and model proteins (lysozyme and BSA) as well as therapeutic peptides (octreotide and F991) were used to study release characteristics. The structural integrity of the released proteins and peptides was investigated with different methods. Also the pH inside of the PLHMGA microspheres was measured and compared with that of PLGA based microspheres.

1.5. Outline of the thesis

Chapter 2 gives an overview of recent approaches in the synthesis of functional polyesters and their application in the biomedical and pharmaceutical field. The polymers are synthesized following one of three major routes of synthesis: polycondensation, ring opening polymerization and enzymatic polymerization. Their application in protein delivery, tissue engineering and gene delivery is reviewed as well.

Chapter 3 describes formulation of dextran-blue and lysozyme-loaded PLHMGA microspheres and investigates the *in vitro* release of these macromolecules compared to that of PLGA microspheres. The effect of processing parameters on porosity of the microspheres and strategies to decrease porosity and consequent burst release was investigated. Structural integrity of the released lysozyme and the reasons for incomplete release were studied. It was hypothesized that the formation of slowly degrading and highly crystalline oligomers of L-lactic acid was one of the reasons for the observed incomplete release.

In **Chapter 4** D,L-lactide instead of L-lactide was used for copolymerization with benzyloxymethyl methyl glycolide (BMMG) to yield a copolymer that upon degradation resulted in the formation of amorphous oligomers of L and D-lactic acid that further fully degraded into L and D lactic acid. BSA was used as a model protein to investigate the *in vitro* release. Microspheres were made from copolymers with different molar ratios of D,L-lactide and BMMG (50/50, 65/35 and 75/25) and it was shown that the release was governed by the degradation of microspheres. The effect of polymer concentration and molecular weight on release was also studied. Stability of the released protein was investigated by SEC and fluorescence spectroscopy.

The possibility of using PLHMGA microspheres for peptide release was investigated in **Chapter 5** using octreotide as a therapeutic peptide. The *in vitro* release of octreotide from PLHMGA microspheres with different copolymer composition was studied and compared with that of Sandostatin LAR®, the commercially available octreotide-loaded microspheres based on PLGA-glucose star polymer. Octreotide related substances formed during the release from PLHMGA formulations and Sandostatin LAR® were analyzed by HPLC and mass spectroscopy.

One of the major reasons to use PLHMGA was the introduction of the extra hydroxyl group in the PLHMGA backbone which makes it more hydrophilic than its counterpart PLGA. Therefore, we were expecting less acidification during degradation of the microspheres and the results of the experiments of testing this hypothesis are reported in **Chapter 6**.

Chapter 7 summarizes the findings and conclusions of this thesis. In addition, perspectives and suggestions for future research in this area are given.

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Chapter 2

Functional aliphatic polyesters for biomedical and pharmaceutical applications

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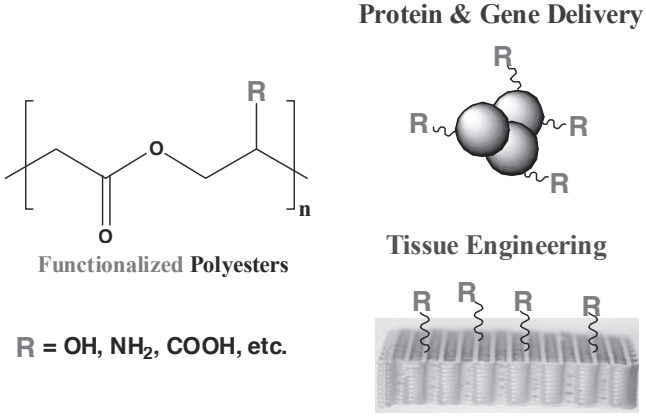
Abstract

Functional aliphatic polyesters are biodegradable polymers with many possibilities to tune physico-chemical characteristics such as hydrophilicity and degradation rate as compared to traditional polyesters (e.g. PLLA, PLGA and PCL), making the materials suitable for drug delivery or as scaffolds for tissue engineering. Lately, a large number of polyesters have been synthesized by homopolymerization of functionalized monomers or co-polymerization with other monomers mainly via ring-opening polymerization (ROP) of cyclic esters. This review presents the recent trends in the synthesis of these materials and their application for protein delivery and tissue engineering.



2.1. Introduction

Aliphatic polyesters belong to the category of biodegradable polymers and have been broadly used in medical products such as sutures, bone screws, tissue engineering scaffolds and drug delivery systems (1-3). With the advancement of biotechnology that has resulted in increased number of available therapeutic peptide/proteins, traditional aliphatic polyesters such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) have been exploited for the development of controlled release systems for peptide/proteins (4,5). Polyesters used for biomedical applications are mainly derived from glycolide (GA), lactide (LA), β-butyrolactone (β-BL), ε-caprolactone (ε-CL), 1,5-dioxepan-2-one (DXO) and trimethyl carbonate (TMC) (2,6). There are three major routes for the synthesis of aliphatic polyesters. The first route is by polycondensation of a hydroxy acid or of a diol and a diacid (1). However, this method has some drawbacks including the low degree of polymerization resulting in low molecular weight polymers (1,7) and block copolymers can not be synthesized. The second route is via ring opening polymerization (ROP) of lactones and other cyclic diesters such as lactide and glycolide which under proper conditions can result in polyesters with high molecular weights and ROP is associated with limited side reactions such as racemization (8, 9). Moreover, it has been shown that block copolymers can be obtained by living ROP (10,11). Enzymatic polymerization is the third route to obtain polyesters and is carried out under mild conditions avoiding the use of toxic reagents and with the possibility to recycle the catalyst (12). Additionally, regional and stereo selectivity of enzymes provides attractive possibilities for the direct synthesis of functional polyesters avoiding the use of protected monomers and block copolymers can be synthesized by using enzymatic polymerization (13). However, the major drawback of the enzymatic synthesis of polyesters is the relatively low molecular weight of the obtained polymers (2,12).

The degradation of aliphatic polyesters generally proceeds via hydrolysis of main chain ester bonds and the rate and extent of degradation depends on the character of polymer (e.g. hydrophilicity and crystallinity). They degrade either by bulk erosion or surface erosion (1,14,15). Although aliphatic polyesters have been used for many years for biomedical and pharmaceutical applications, there is still need for improvements. To illustrate, PLGA has been extensively investigated for development of microspheres with the aim to get a controlled release of the entrapped therapeutic agent (e.g. a low molecular weight drug, a pharmaceutical protein or pDNA) for a prolonged time. Particularly, emphasis has been given in the last two decades on the development of protein loaded PLGA formulations. However, instability, aggregation and chemical

modification of the loaded proteins due to a pH drop in the microspheres upon their degradation has been reported (16-20). Importantly, the resulting aggregates can cause unwanted immunogenic reactions (21-23). Another example of an aliphatic biodegradable polyester is PCL which has been extensively used for the development of biomedical devices and tissue engineering scaffolds. Its slow degradation (2 to 4 years) and intrinsic hydrophobicity are major drawbacks for use in tissue engineering (24). The introduction of functional groups into commonly used polyesters such as PLA and PCL provides polymers with tunable degradation behavior by suppression of crystallinity and enhanced hydrophilicity which favors cell adhesion to the surfaces important for tissue engineering purposes (25). Further, an increasing hydrophilicity results in a greater water absorbing capacity of the polymers, thereby increasing the degradation rate and probably preventing a pH drop inside the degrading matrices and hence prevent aggregation and incomplete release of encapsulated proteins (26). Moreover, coupling peptide ligands with the RGD (Arg-Gly-Asp) sequence to functionalized polymers improves cell adhesion which in turn will trigger cell growth and proliferation (27,28).

Many functionalized polyesters have been synthesized in recent years (29-32). However, despite their obvious advantages over their non-functionalized counterparts, applications of these polymers have been rarely studied in the biomedical and pharmaceutical areas. This article reviews recent developments of functionalized aliphatic polyesters and discusses their applications for tissue engineering and protein/pDNA delivery.

2.2. Synthesis of functional polyesters

2.2.1 General aspects

Functional polyesters can be synthesized by post-polymerization functionalization. This can be established by abstraction of protons from the polyester by treatment with a base, such as lithium diisopropyl amide (LDA), followed by subsequent addition of an electrophilic reagent, such as a halogen- or a carbonyl-containing compound (33,34). The main drawback of this method is the possibility of side reactions, such as chain scission and racemization. Besides, the preparation of block copolymers using this strategy is not straightforward. Therefore post-polymerization functionalization is not the preferred route to obtain functional polyesters. A more frequently used route to functional polyesters is to homopolymerize functional (protected) monomers or copolymerize such monomers with commercially available non-functionalized monomers which can be performed via three major polymerization routes as

pointed out in the introduction sections. The functional groups in the monomers are normally protected to avoid side reactions and consequently a deprotection step after polymerization has to be carried out to obtain polymers bearing functional groups (32,35).

The most frequently studied functional monomers are based on lactones (8, 36-39). Highly pure monomers are the key factor to tailor the molecular weight of aliphatic polyesters from these monomers and to obtain high molecular weight polymers (38). The main advantage of lactones for polyester synthesis is the possibility to purify them by recrystallization or distillation prior to polymerization. Recent reviews on the different synthesis routes to functionalized lactones can be found elsewhere (8,40).

2.2.2. Polycondensation polymerization

Step-growth polymerization refers to the condensation of hydroxyl-acids or mixtures of diacids and diols. The major drawbacks of this method are the high temperatures and long reaction times which may lead to side reactions, such as racemization (38). The molecular weight of polycondensates is indeed usually limited to a few tens of thousands (M_n < 30 kDa), because completion of the reaction is a (kinetic) problem and deviation of the stoichiometry of diacids and diols detrimentally affects the chain length (7,38).

There are only a few publications on the synthesis of functionalized polyesters via polycondensation. For instance, Brown et al. (41) reported on the synthesis of amorphous aliphatic polyesters containing unsaturated double bonds which were in a subsequent reaction converted to ether or amine functional groups. They used Diels-Alder reactions to synthesize new difunctional monomers via the reaction of fumaric acid or maleic anhydride and a variety of dienes (Fig.1).

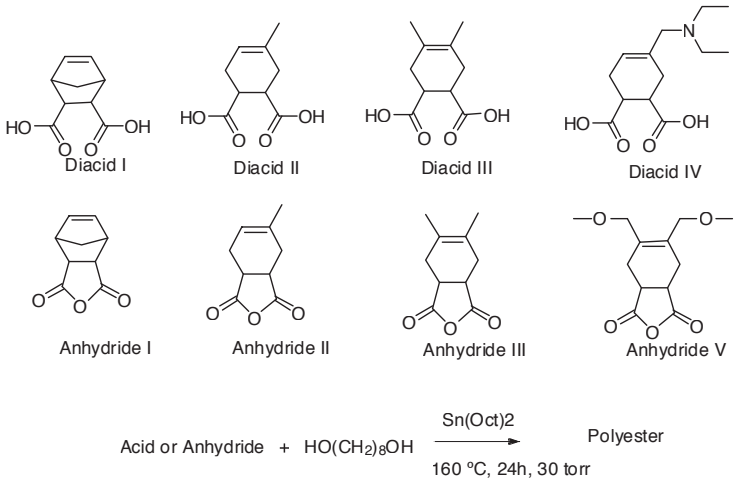


Fig. 1. Synthesis of unsaturated aliphatic polyesters via polycondensation (41).

The resulting unsaturated dicarboxylate monomers were incorporated into aliphatic polyesters (M_n ranging from 11 to18 kDa) by step growth polycondensations with di-alcohols (e.g. 1,8-octanediol). In a subsequent reaction, thermal crosslinking of these polymers was performed by mixing the polyesters with AIBN and heating at 130 °C for 24h, to yield degradable elastomers. Since these materials are intended for biomedical applications, the cured elastomers were tested for their cytotoxic response, using L-929 mouse fibroblast cells and it was shown that these materials were non-toxic.

Wang et al. (42) synthesized an elastomer through polycondensation of glycerol and sebacic acid (Fig. 2) in which the molar ratio of glycerol/sebacic acid was 1:1. This reaction resulted in a colorless elastomer, featuring small number of crosslinks (crosslink density of 38.3 ± 3.4 mol/m^3) in which the hydroxyl groups were directly attached to the network backbone.

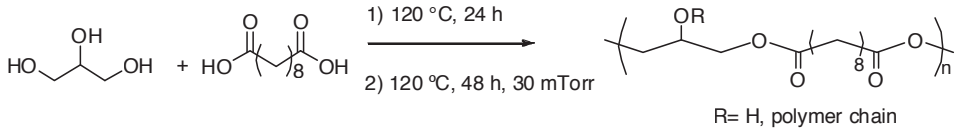


Fig. 2. Synthesis of poly(glycerol-sebacate) (42).

Yang et al. reported on the synthesis of a biodegradable elastomer, i.e. poly(1,8-octanediol-co-citric acid), with a controllable number of crosslinks, tailorable elasticity and biodegradability (43). They first prepared a low molecular weight (M_w ~1100) pre-polymer by a controlled condensation reaction

between 1,8-octanediol and citric acid (citric acid:1,8-octanediol molar ratio of 1:1), and then crosslinked the formed polymer under various conditions. They showed that the polymer that was crosslinked under mild conditions (low temperatures, e.g. 60 or 80 °C, no vacuum) had a significantly faster degradation rate than the polymer crosslinked under relatively tougher conditions (high temperature, e.g. 120 °C, 2 Pa vacuum). The crosslinked polymers synthesized at 60 °C completely degraded within 6 months of incubation in phosphate buffered saline (PBS) at 37 °C, while the crosslinked polymers synthesized at higher reaction temperature were more stable and showed only 50 % weight loss during this time.

2.2.3. Ring opening polymerization

High-molecular weight aliphatic polyesters can be prepared by ring opening polymerization (either in melt or solution) of lactones of different ring-size, with or without (protected) functional groups (39,40). Many of the reported ROP reactions to synthesize functional polymers are based on ε-CL derivatives. This monomer can be derivatized at different CH_2 positions and with a variety of functional groups (Fig. 3).

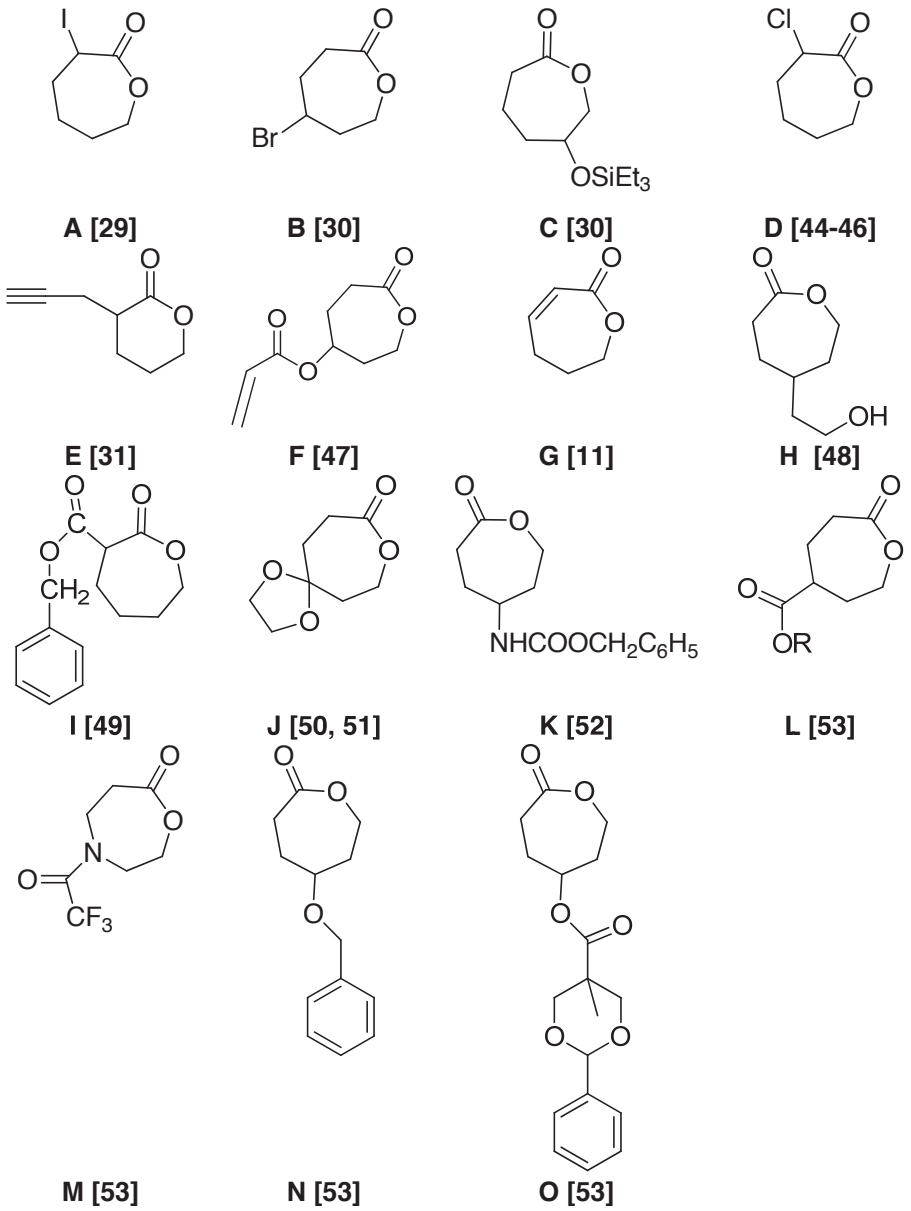


Fig. 3. Functionalized monomers based on ε-CL derivatives (except E) suitable for ROP.

Halogen-bearing CL monomers are the most frequently used functional monomers in ROP reactions. As an example, an iodine-functionalized caprolactone monomer (Fig. 3A) was synthesized by Habnouni et al. through activation of caprolactone with a non-nucleophilic strong base (lithium

diisopropyl amide), followed by an electrophilic substitution with iodine monochloride (29). This monomer was copolymerized with ε-caprolactone at 100 °C in toluene, initiated by methanol and catalyzed by $SnOct_2$, to yield an iodine bearing copolymer. The authors suggested that these iodinated polyesters open the way to new functional polyesters by substitution of the iodine group by other functional groups and to yield polymers that can potentially be applied in temporary reconstruction materials or drug delivery systems.

Gautier et al. (30) synthesized random copolyesters by controlled ROP polymerization of ε-caprolactone with γ-bromo-ε-caprolactone (Fig. 3B) or γ-triethylsiloxy-ε-caprolactone (Fig. 3C) using aluminum triisopropoxide as initiator. The obtained copolyesters were quaternized by reaction of the γBrCL units with pyridine and deprotected by acidic hydrolysis of γEt_3SiOCL units to yield p(CL-co-γPyCL) and p(CL-co-γOHCL), respectively. Further, they prepared stable nanoparticles by co-precipitation of poly(D,L-lactide) with small amounts of PCL partly substituted with pyridinium, with the aim to obtain degradable colloidal carriers with ability to bind molecules of interest.

Lenoir et al. synthesized α-chloro-ε-caprolactone (Fig.3D), and copolymerized this monomer with ε-caprolactone (44). They used the pendant chlorides of this copolymer to graft poly(methyl methacrylate) or poly(butenyl benzoate) blocks by ATRP.

Riva et al. (45,46) copolymerized the same monomer, α-chloro-ε-caprolactone Fig. 3D), with ε-CL and substituted the pendant chlorides by azide. These azide functionalized polymers were subsequently reacted under mild conditions by Huisgen's cycloaddition reaction (47) with alkyne functionalized reagents having ester, amine, and ammonium groups, as well as well with alkyne-derivatized PEO chains.

Parrish et al. (31) synthesized aliphatic polyesters with pendant acetylene groups by controlled ROP of α-propargyl-δ-valerolactone (Fig. 3E) with ε-caprolactone. They subsequently grafted azide-terminated PEG-1100 monomethyl ether and an azide functionalized GRGDS peptide to the polyester by click chemistry. They also showed that these polymers were biocompatible using in-vitro cytotoxicity assays.

Mecerreyes et al. (48) reported the synthesis of a functional lactone containing a pendant acrylate group (Fig. 3F). The homopolymers of 3F with different molecular weights (M_n of 7 kDa to 20 kDa) were synthesized via ROP at 25 °C in dry toluene and the polymers had a T_g of -60 °C. Random 6-arm star copolymers of 4-(acryloyloxy)-ε-caprolactone and ε-caprolactone were synthesized in bulk at 110 °C using the hydroxyl hexafunctional dendrimer of bis-MPA (2,2'-bis(hydroxymethyl)propionic acid) and $Sn(Oct)_2$, as initiator and catalyst, respectively. Poly(L,L-$lactide_{0.85}$-co-4-(acryloyloxy)-ε-$caprolactone_{0.15}$)

was dissolved in dichloromethane in the presence of benzoin diethyl ether as radical photoinitiator followed by UV irradiation to yield degradable networks. Crosslinking was also established by dissolving the polymer in toluene and using AIBN as a thermal radical initiator. However, the characteristics (e.g. cell compatibility and degradation kinetics) of the obtained networks were not reported.

Another unsaturated cyclic ester, 6,7-dihydro-2(5H)-oxepinone (DHO) was synthesized by Lou et al. (11) (Fig. 3G). They copolymerized this monomer with ε-CL in toluene at room temperature, using $Al(O^iPr)_3$ as initiator to obtain unsaturated aliphatic polyesters. Thermal analysis of the copolymers showed that an increasing amount of DHO has a strong effect on the melting temperature of the polymer; T_m decreases from 57 to 29 °C at F_{DHO}=0.66, followed by an increase up to T_m of the homopolymer of DHO (35 °C). The unsaturated polyester backbone offers sites for crosslinking, which is useful for the synthesis of biodegradable networks.

Liu et al. reported a new approach for synthesis of hyperbranched polymers in which ring-opening polymerization is combined with some of the features of self-condensing vinyl polymerization (SCVP) (49). They synthesized an AB type monomer, containing ε-caprolactone ring as well as an alcohol initiating group (a so-called inimer (=initiator/monomer), i.e. 4-(2-hydroxyethyl)-ε-caprolactone, Fig. 3H). This monomer was polymerized at 110 °C in bulk using stannous octoate ($SnOct_2$) as catalyst, yielding a hyperbranched polymer with a M_w of 65 - 85 kDa containing a large number of hydroxyl group chain ends. These hydroxyl groups can be used to attach biologically active molecules.

In a recent paper, Wolf et al. (50) described the synthesis of branched and hyperbranched poly(L-lactide) copolymers with hydroxyl end groups by ROP of L-lactide and a hydroxyl-functional lactone inimer, i.e. 5-hydroxymethyl-1,4-dioxane-2-on (5HDON). The polymerization was performed both in bulk and solution, and was catalyzed by either $Sn(Oct)_2$ or an organic base, 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD). They showed that both the degree of branching and the molecular weight could be tailored by the monomer/inimir ratio. The authors suggested that these potentially biodegradable and biocompatible branched/dendritic structures are useful for polymer modification reactions and surface functionalization.

Different biodegradable PEO-b-poly(ester) block copolymers having functional pendant α-benzyl carboxylate or carboxyl groups were prepared by ROP of α-benzyl carboxylate-ε-caprolactone (Fig. 3I) with ε-caprolactone using methoxy poly(ethylene oxide) as initiator, to yield PEO-b-PBCL which after catalytic debenzylation yielded PEO-b-PCCL (51). It was shown that these amphiphilic polyesters were able to form spherical micelles with nanoscopic

dimension (average diameter of 62 and 20 nm for PEO-b-PBCL and PEO-b-PCCL, respectively) in water. These micelles are potentially suitable as delivery systems for controlled and /or targeted delivery of therapeutic agents.

5-Ethylene ketal ε-caprolactone (Fig. 3J) was synthesized by Tian et al. (52,53) and they copolymerized this monomer with ε-caprolactone at 25 °C in toluene, using $Al(O^iPr)_3$ as initiator, to yield copolymers bearing acetal pendant groups. Deprotection of the copolymers was carried out with triphenylcarbenium tetrafluoroborate and the formed ketone groups were reduced to yield hydroxyl groups by sodium borohydride. They showed that these polymers form stable nanoparticles in water with a mean size below 100 nm potentially suitable for drug delivery.

An amine-functionalized PCL was synthesized by copolymerization of the functional monomer γ-(carbamic acid benzyl ester)-ε caprolactone (Fig. 3K) with ε-CL at 130 °C using $SnOct_2$ as catalyst (54). Subsequently, the synthesized polymer was deprotection by hydrogenation using Pd/C as catalyst, to yield PCL with pendant amine groups (Fig. 4). Biotin (a model compound) was attached to these amine groups to show the accessibility of these functional groups for further conjugation with bioactive molecules.

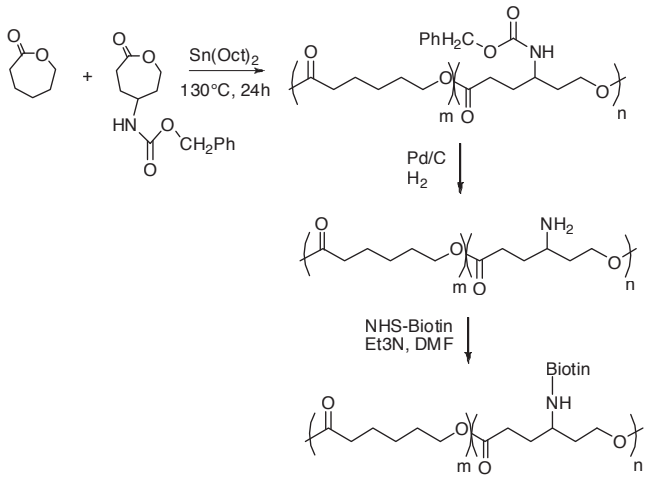


Fig. 4. Synthesis and biotinylation of poly(CL-co-ACL) (54).

Trollsås et al. synthesized several functionalized caprolactone monomers (Fig. 3L, M, N, and O) which were synthesized by the Bayer-Villiger oxidation of the corresponding cyclohexanone derivatives (55). Homopolymerization of these protected functionalized ε-caprolactone derivatives (hydroxyl-, bis-(hydroxyl)-, amino- and carboxyl-substituted) were performed either in bulk or in toluene as solvent (Fig. 5) at 110 °C to minimize possible transesterification

reactions. They showed that the obtained polymers had a M_n close to their targeted molecular weights (5000-15000 g/mol) with narrow polydispersities (1.20-1.35).

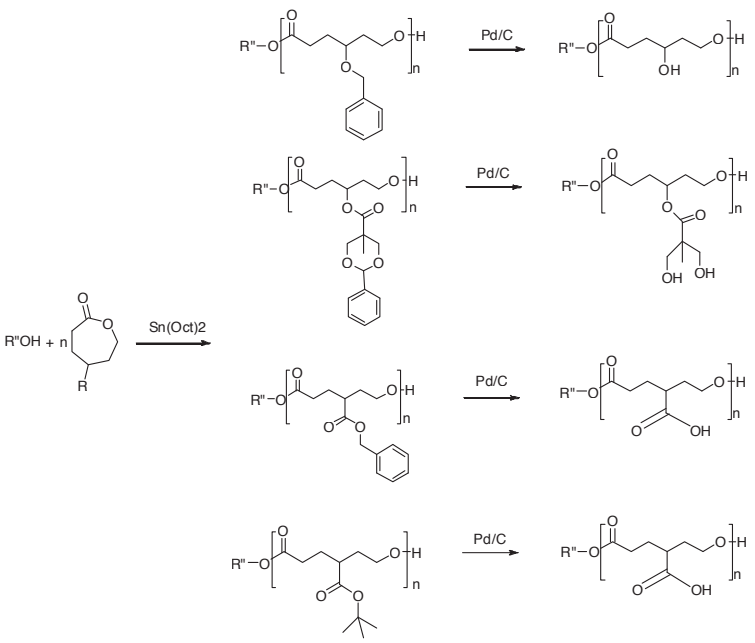


Fig. 5. ROP of different functional lactones followed by deprotection (55).

John et al. (56,57) synthesized poly(ε-caprolactone-co-glycolic acid-co-L-serine) from the corresponding monomer, e.g. 3-(O-benzyl)-L-serinylmorpholine-2,5-dione (Fig. 6). First, copolymerization of ε-caprolactone and the aforementioned monomer was performed using $Sn(Oct)_2$ as initiator. The obtained copolymer was subsequently deprotected by catalytic hydrogenation and acrylic derivatives of the copolymer were synthesized by reacting the PCL(Glc-Ser) copolymer with acryloyl chloride. Crosslinked scaffolds and microspheres of this polymer were prepared by means of photopolymerization.

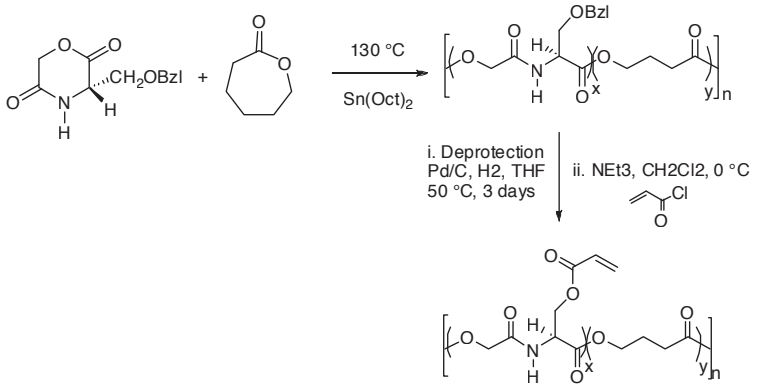


Fig. 6. Synthesis of acrylated PCL(Glc-Ser) (56).

Protected functional glycolide-type monomers, i.e. 3S-(benzyloxymethyl)-6S-methyl-1,4-dioxane-2,5-dione and 3S-benzyloxymethyl-1,4-dioxane-2,5-dione, were synthesized by Leemhuis et al. (32,58) (Fig. 7). These functional monomers were copolymerized with L-lactide to form, after deprotection, the corresponding hydroxyl functionalized poly(lactide-co-hydroxymethyl methyl glycolide) (poly(HMMG-L)). In a follow-up paper (59), Leemhuis et al. studied the hydrolytic degradation of these functionalized polyesters of different copolymer compositions in phosphate buffer (174 mM, pH 7.4) at 37 °C. It was observed that the degradation times ranged from less than 1 day (for the homopolymers of hydroxymethyl methyl glycolide) to 2 months (copolymers with lactide containing 25% of functionalized monomer). The degradation rates increased with increasing hydroxyl density of the polymers, which was associated with a switch from bulk to surface erosion.

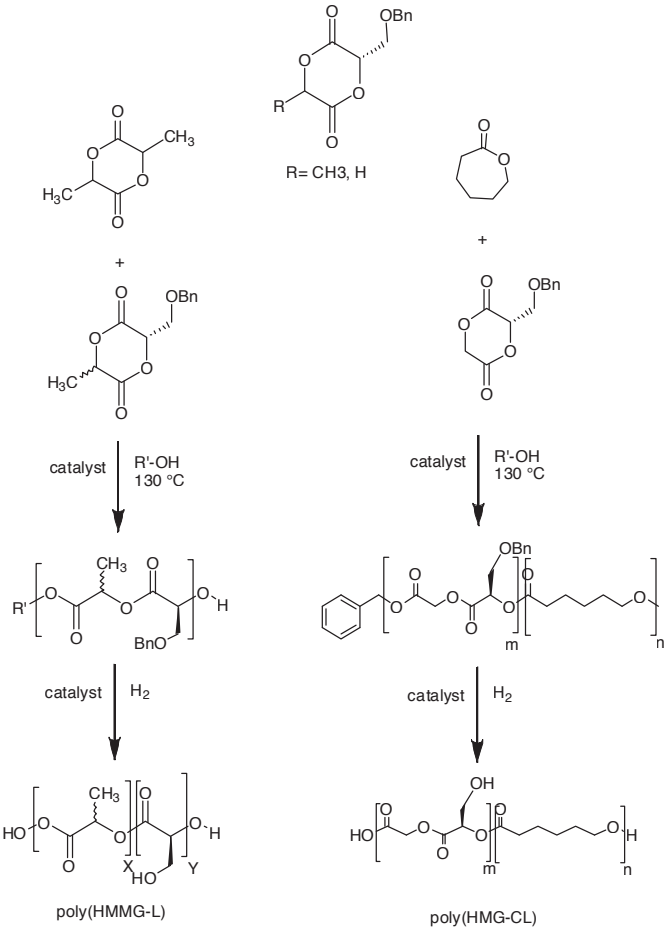


Fig. 7. Synthesis of poly(HMMG-L) (26) and poly(HMG-CL) (60).

Loontjens et al. (61) showed that functional random copolymers of these monomers with ε-caprolactone (poly(BHMG-co-CL)) synthesized at 110 °C, 130 °C and 150 °C (containing more than 40 mol % of the protected BHMG monomer) had T_g's of -16, -29 and -28 °C, respectively. It was shown that at all these temperatures, ε-CL started to polymerize when BHMG was almost fully converted. Deprotection of these copolymers resulted in a subtle increase in the T_g which indicates a decrease in the mobility of the polymer chains due to stronger interpolymer interactions of the hydroxyl groups.

Leemhuis et al. (62) synthesized an ally-functionalized glycolide monomer (i.e. 3-allyl-1,4-dioxane-2,5-dione) and copolymerized this monomer with L-lactide by ROP in the melt using benzyl alcohol and $Sn(Oct)_2$ as initiator and catalyst, respectively. They showed that the obtained polymers were amorphous materials and their T_g increased with increasing lactide content, ranging from 19

to 42 °C, when the lactide content increased from 25% to 75%. Further, the allyl groups of poly(allylglycolide) copolymers were oxidized using m-chloroperoxy benzoic acid (mCPBA) to yield the corresponding epoxidated polymers in high yield. These polymers were amorphous as well and their T_g varied from 21 to 24 °C, depending on their copolymer composition.

PLA-based polymers with ε-amine side groups (poly(lactic acid-co-lysine), PLAL) were synthesized by Elisseeff et al. (63) (Fig. 8). Poly(aspartic acid) side chains were grafted to the amine functionalities of the polymers to obtain PLAL-ASP. Different percentages of aspartic acid units of this polymer were partially converted to the methacrylate mixed anhydride by varying the duration of the substitution reaction at 37 and 60 °C. Hydrogels of these polymers were prepared by UV polymerization in bulk. The swelling degree of networks prepared from polymers containing 5-22% methacrylate groups, was ~5.5 in PBS. The compression moduli of these networks ranged from 1.4 to 3.1 kPa having mechanical characteristics suitable for biomaterials/tissue engineering applications.

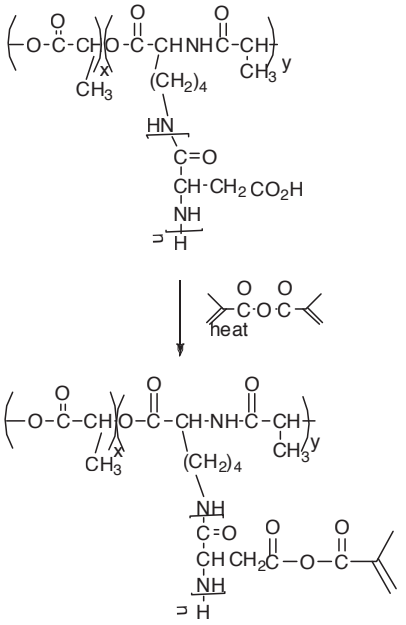


Fig. 8. Synthesis of poly(lactic acid-co-lysine) grafted with poly(aspartic acid) and modified with methacrylate groups (63).

Hu et al. (64) synthesized a functionalized polymer (PEG-b-P(LA-co-PTO)) via copolymerization of a functionalized/protected monomer, [9-phenyl-2,4,8,10-tetraoxapiro[5,5]undecan-3-one] (PTO) with LA, in the presence of

monohydroxyl poly(ethyleneglycol) as macro initiator and $Sn(Oct)_2$ as catalyst (Fig. 9). The benzylidene protecting groups of this polymer were removed by catalytic hydrogenation to obtain PEG-b-P(LA-co-DHP). Micelles of the protected polymer were formed in aqueous solution and it was shown that CMC decreased with increasing PTO content (from 12.3 to 1.0 mg/L when the PTO content increases from 5% to 20%). A cytocompatibility study was carried out by investigating the adhesion and spreading of Vero cells on films of both protected and deprotected polymers. After 24 hours, almost all cells adhered to the polymer films and they proliferated and covered the whole surfaces after 48 hours. They also studied the enzymatic degradation of these polymers in presence of proteinase K at 37 °C and compared that to PLA. After 40h, the weight loss was 89% for the deprotected polymer while it was 77% and 70% for the protected polymer and PLA, respectively. They derivatized the free hydroxyl groups with biotin to demonstrate the potential of this polymer for drug conjugation.

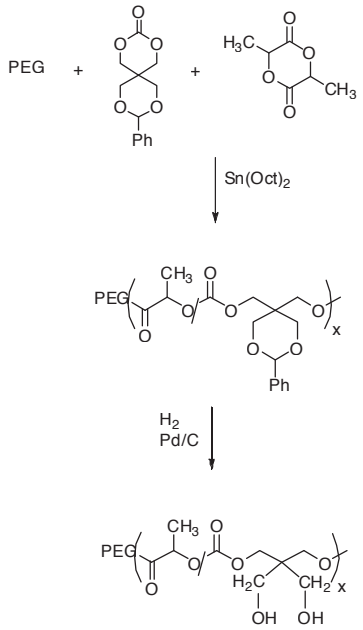


Fig. 9. Synthesis of PEG-b-P(LA-co-PTO) block copolymers followed by deprotection (64).

2.2.4. Enzymatic polymerization

Enzymatic polymerization, particularly using lipase, is an efficient method for the synthesis of aliphatic polyesters (65). The advantage of this polymerization

method is its regioselectively and there is no need for protection/deprotection (66). The mechanism of the lipase-catalyzed polymerization of a lactone is schematically shown in Fig. 10.

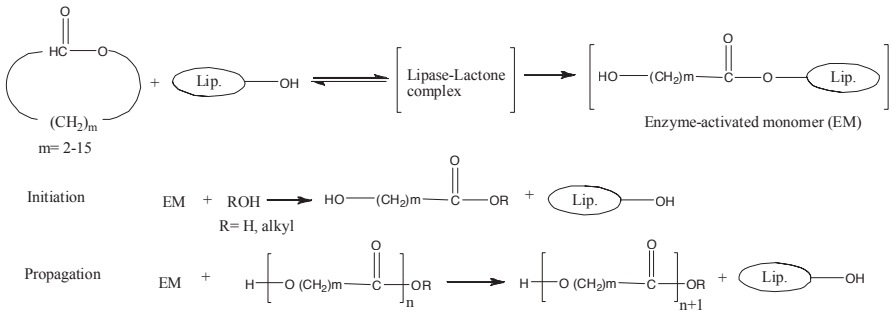


Fig. 10. Schematic presentation of enzymatic polymerization by lipase (65).

Reaction of the lactone with lipase causes ring-opening to give an acyl-enzyme intermediate (enzyme-activated monomer, EM). Initiation is by nucleophilic attack of enzyme-absorbed water molecules onto the acyl carbon of the intermediate to yield an α,ω-hydroxycarboxylic acid. In the propagation stage, the EM is nucleophilically attacked by the terminal hydroxyl group of a propagating polymer chain to yield a one-unit-more elongated chain. A kinetic investigation of the polymerization showed that the overall rate-determining step is the formation of the EM and, therefore, the polymerization proceeds via a "monomer-activated mechanism" (65). As an example of functionalized polyesters synthesized in this way, Al-Azemi et al.(67) reported on the lipase-catalyzed synthesis of a water-soluble polycarbonate having pendant carboxyl groups on the main chain using 5-methyl-5-benzyloxycarbonyl-1, 3-dioxin-2-one as monomer (Fig. 11). They hypothesized that these polymers have enhanced biodegradability, but they have not reported the degradation data, yet.

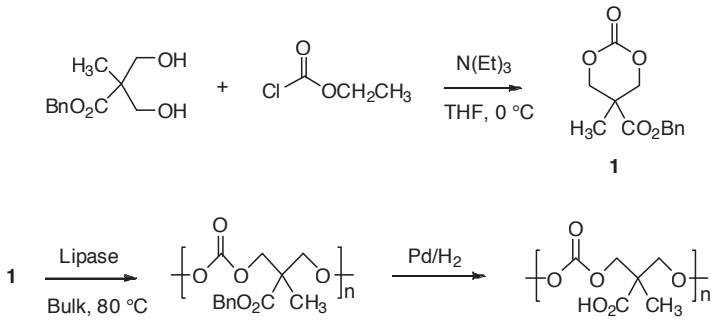


Fig. 11. Synthesis of 5-methyl-5-benzyloxycarbonyl-1,3-dioxane-2-one and its enzymatic polymerization using lipase (67).

Veld et al. (68) synthesized two novel monomers, ambrettolide epoxide and isopropyl aleuriteate, containing functional groups. The homopolymers of ambrettolide epoxide were synthesized using Novozym 435 as catalyst to obtain a polymer of M_n = 9.7 kg/mol and PDI = 1.9, while the epoxide groups remained unaffected during the polymerization. Selective polymerization of isopropyl aleuriteate using Novozym 435 was performed as well and a polymer with moderate molecular weight (M_n = 5.6 kg/mol, PDI = 3.2) was obtained. Copolymerization of isopropyl aleuriteate with e-CL at different ratios resulted in soluble, hydroxy functional polymers with M_n's ranging from 10 to 27 kg/mol in a good yield (~75 %). The secondary hydroxy groups of the polymer reacted easily with hexyl isocyanate, to demonstrate that these groups are assessable for post-polymerization modifications

2.3. Applications

2.3.1. Protein delivery

For the past two decades, non-functionalized aliphatic polyesters such as PLLA, PLDLA and PLGA have been extensively studied for the controlled delivery of therapeutic peptides/proteins (16,69,70). However, due to the drawbacks associated with these systems (e.g. unfavourable protein-polymer interactions (71), acidification of the matrix during degradation (17), acylation of the encapsulated proteins (72) and difficult to tailor the release (73)), it is questionable whether the systems are generally applicable for the development of well-performing formulations. In recent studies, microspheres based on functionalized polyesters, i.e. the previously mentioned poly(L-lactide-co-hydroxymethyl glycolide) (poly(HMMG-L) (Fig. 7), were investigated for encapsulation and release of a model protein(lysozyme) (26). Poly(HMMG-L) microspheres were prepared by multiple emulsion (w/o/w) solvent evaporation technique and, depending on the polymer concentration used for the preparation, 50 to 70% of lysozyme was released in a sustained manner from the microspheres. In comparison, PLGA microspheres showed hardly any sustained release of lysozyme after an initial small burst release (Fig. 12). Importantly, the released lysozyme was fully enzymatically active and preserved its structural integrity (CD analysis). However, insoluble residues remained which as demonstrated by DSC and FTIR analysis contained lactic acid oligomers and aggregated lysozyme which likely explains the observed incomplete release of the protein.

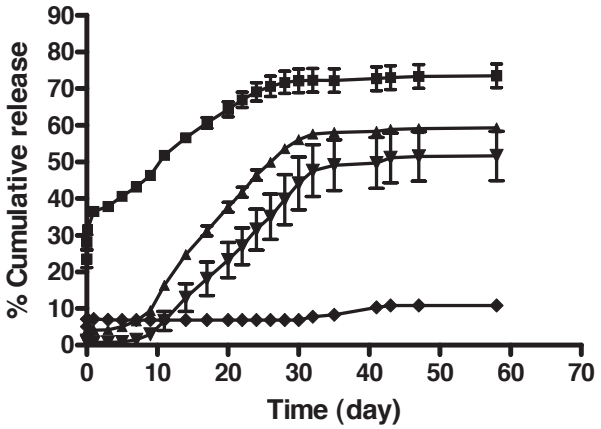


Fig. 12. Lysozyme release from poly(HMMG-L) microspheres prepared with different polymer concentration (10 (■), 15 (▲) and 20% (▼) in the organic phase). Also the release from PLGA microspheres is shown (♦) (26).

Replacing L-lactide by D,L-lactide in the copolymer prevented the formation of insoluble degradation products of crystalline oligomers of lactic acid. In this case, bovine serum albumin (BSA) was encapsulated with high efficiency (>85%) and was quantitatively released from microspheres of poly(D,L-lactide-co-hydroxymethyl glycolide). It was shown that the release from 2 weeks to 2 months was governed by degradation of the microspheres and depended on the copolymer composition (Fig. 13) and, importantly, no insoluble residues remained (74).

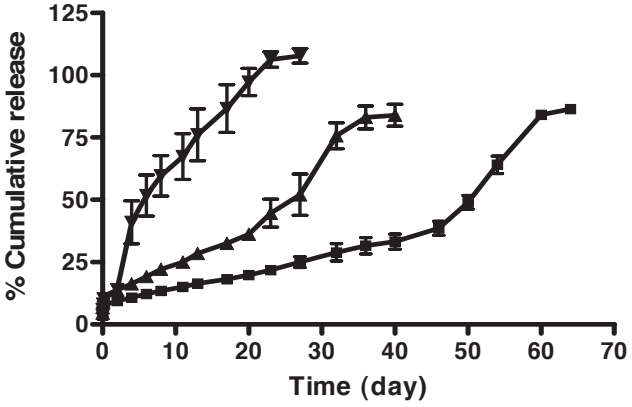


Fig. 13. BSA release from poly(HMMG-L) microspheres prepared from copolymers with different copolymer composition; (▼) 50/50, (▲) 35/65 and (■) 25/75 (74).

In a recent study, Cristian et al. studied the encapsulation of BSA in microspheres of PCL bearing pendant acryloyloxy or methacryloyloxy groups (75). Microspheres were prepared by a double emulsion evaporation method and the final emulsion was UV-irradiated in the presence of a photinitiator to crosslink the particles. They showed successful encapsulation of BSA in crosslinked polymer particles with raman spectroscopy. However, no data on either the loading efficiency nor the release and stability of the encapsulated BSA were reported, yet.

2.3.2. Tissue engineering

Degradable polymers that are frequently used for tissue engineering applications are 'normal' aliphatic polyesters such as PCL, PLA and PLGA. Drawbacks of these polyesters are their hydrophobicity and lack of functional groups, which limits cell adhesion that is an important factor when constructing polymeric scaffolds (35,76). Another drawback is their slow hydrolytic degradation (77). Although several functionalized polyesters have been synthesized in recent year (described in section 2) only a limited number of functionalized polyesters has been evaluated for tissue engineering applications so far.

Wang et al. (42) evaluated both the in vitro and in vivo biocompatibility of a functionalized elastomer, (poly(glycerol-sebacate), PGS) (Fig.2), to get insight into the potential of this material for tissue engineering. They showed that NIH 3T3 fibroblast cells cultured on PGS-coated glass petri dishes were viable, showed normal morphology and had a higher growth rate in comparison with cells cultured on PLGA films as control. After subcutaneous implantation in rats it was shown thatPGS implants completely absorbed within 60 days without granulation or formation of scar tissues.

Yang et al. (43) investigated the suitability of poly(1,8-octanediol-co-citric acid) (POC) for cardiovascular tissue engineering applications. They showed that the water contact angles on POC films decreased from 76° to 38° after 30 minutes exposure to water, indicating that the surface becomes more hydrophilic in time. The adhesion and proliferation of human aortic smooth muscle cells and endothelial cells seeded on POC films was investigated as well and it was observed that growth and viability of both cell types was at least as good as or better than that observed on PLLA films.

Seyednejad et al. synthesized poly(hydroxymethylglycolide-co-caprolactone) (Fig. 7) and investigated the suitability of this polymer for tissue engineering applications (60). It was shown that the wettability of these polymers was tunable by the percentage of the functional monomer in the structure and they showed

that incorporation of the functional monomer in the copolymer structure resulted in a decrease in receding contact angle from 64° to 40° after 20 min exposure to water, likely caused by exposure of the hydroxyl groups of the polymer at the water/polymer interface. Importantly, the increased hydrophilicity of copolymer films resulted in the improved adherence of human mesenchymal stem cells (hMSCs) along with the ability of the seeded cells to differentiate towards osteogenic lineage, when compared to hydrophobic PCL. In a follow up study (78), it was shown that three-dimensional scaffolds can be made from these functional polyesters by means of 3D-printing and the scaffolds showed superior properties in comparison with PCL scaffolds regarding mechanical properties as well as hMSCs adherence and proliferation.

2.3.3. Gene delivery

In recent years, gene therapy has become an important modality for the treatment of patients with acquired or inherited genetic diseases (79-81). Viral gene delivery systems including adenoviruses and retroviruses demonstrated high transfection efficiency (82-84), but their application is limited due to their side effects such as immunogenicity and mutagenesis caused by the cell-infected viruses (85). Functional water-soluble cationic polyesters have been used as non-viral vectors for the binding and subsequent intracellular release of plasmid DNA (86). For instance, poly(α-(4-aminobutyl)-L-glycolic acid) (PAGA) synthesized by Lim et al. (87,88) is able to form PAGA/DNA polyplexes and it was shown that these polyplexes had a 3-fold higher transfection activity in vitro compared to the polyamide analogue of PAGA, i.e. poly-L-lysine (pLL). Importantly, PAGA showed no cytotoxicity at 100 μg/ml while pLL is highly cytotoxic at this concentration. In another study, Vromen et al.(89) investigated copolymers of ε-caprolactone and γ-bromo-ε-caprolactone quarternized by pyridine for gene delivery. They observed that the cytotoxicity and transfection efficiency was comparable to polyethylenimine 50 kDa (90-92) which is a non-biodegradable but commonly used polymer for gene delivery. A series of modified hyperbranched polymers based on 2,2-bis-(methylol)propionic acid (bis-MPA) with tertiary amines were synthesized by Reul et al.(93). They showed that the toxicity of these hyperbranched polyesters was very low as compared to polyethyleneimine (PEI) and the transfection activity of the polymers increased with amine density. In a recent study, Liu et al. (94) synthesized degradable poly(β-aminoester)s with pendant aminoethyl groups and demonstrated that polyplexes of these polymers had higher transfection efficiencies than branched PEI 25 kDa in 293T cells.

2.4. Conclusions

In this contribution, the recent approaches to synthesize biodegradable and biocompatible functional aliphatic polyesters and their pharmaceutical and biomedical applications are reviewed. It is shown that functional polyesters with well-defined characteristics can be synthesized by homo- and copolymerization of (protected) functional monomers. Importantly, the characteristics of copolymers such as T_g, hydrophilicity, solubility and degradability can be tuned by the copolymer composition. Although a limited number of studies regarding the applications of functional aliphatic polyesters have been published, the results obtained so far encourage future investigations. The increasing number of accessible functional aliphatic polyesters provides the opportunity to study relationships between structure and functionality of these polymers such as degradability and cell adhesion in vitro and in vivo as well as to develop applications of these potential materials for drug delivery in the form of micro- and nanoparticles, implants, or scaffolds in tissue engineering.

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Chapter 3

Preparation and characterization of protein loaded microspheres based on a hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid)

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Abstract

The purpose of this study was to investigate the suitability of a novel hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid) (PLHMGA), as controlled release system for pharmaceutical proteins. Dextran Blue (as a macromolecular model compound) and lysozyme-loaded PLHMGA and PLGA (control formulation) microspheres were prepared by a solvent evaporation technique. The Dextran Blue and lysozyme loaded PLHMGA microspheres prepared with 10 % polymer solution showed, because of a high porosity, a high burst release (35-75 %) and the remaining content was released in a sustained manner for 15-20 days. The microspheres prepared with 15 and 20 % polymer solution had a lower porosity and showed a pulsed release after day 8 and in 27 days they released more than 90 % of Blue Dextran. The release of lysozyme was incomplete, likely due to aggregation of part of the encapsulated protein. Spectroscopic analysis of the released lysozyme indicated fully preserved secondary/tertiary structure and an enzyme activity assay showed that the specific activity of the released protein was maintained. An in vitro degradation study showed that the release of Blue Dextran and lysozyme is essentially controlled by the degradation of the microspheres. This study shows that microspheres made of the hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid), are promising systems for the controlled release of pharmaceutical proteins.

3.1. Introduction

Biotech products such as proteins, peptides, pDNA and siRNA are potent molecules for the treatment of patients suffering from different chronic and life-threatening diseases. Since oral administration of these labile molecules for obvious reasons does not result in therapeutic effects, these molecules are almost exclusively administered to patients via injection. In the last 25 years tremendous efforts have been made to the development of systems which give sustained release of therapeutic proteins over a predetermined time. In particular, poly(lactic-co-glycolic acid) (PLGA) e.g. in the form of implants, microspheres and nanospheres has been investigated frequently as a biodegradable protein delivery system (1-7). However, there are several drawbacks associated with the use of PLGA as protein release system. These include incomplete and difficult-to-tailor release of entrapped protein (8,9). Importantly, during degradation of the polymer acidic low molecular weight degradation product accumulate in the matrix, which causes a drop in the pH in the matrix (10,11). This low pH in turn might induce unfolding/aggregation of the entrapped protein which is one of the reasons for the frequently observed incomplete release of proteins from PLGA microspheres (12,13). Furthermore, amide bond hydrolysis, deamidation and oxidation of peptides and protein drugs can also occur in this acidic microenvironment (14). Finally, protein aggregates may cause unpredictable and highly unwanted side effects, such as immunogenicity and toxicity (15-17) . Several approaches have been investigated in order to tailor release profiles of proteins from PLGA microspheres and to prevent protein denaturation/aggregation such as entrapment of pegylated proteins and addition of urea to the formulation (18-20). Also, additives like magnesium hydroxide have been co-entrapped to prevent acidification of the degrading matrix (10,21). Although some progress has been made in recent years, it is obvious that these approaches are not the general solution for the protein/PLGA incompatibility and other options need to be explored.

Recently, we reported on new hydrophilic aliphatic polyesters based on lactic acid and glycolic acid with pendant hydroxyl groups (Fig. 1), poly(lactic-co-hydroxymethyl glycolic acid) (22). We showed that the degradation times of these polyesters were much shorter than those of PLGA and that the degradation time could be tailored by the copolymer composition (ranging from a few hours to two months in comparison to 2-4 months for PLGA) (23). The aim of the present study was to study whether these hydrophilic polyesters can be used for the preparation of microspheres for controlled release of pharmaceutical proteins/macromolecules, by using a conventional double emulsion extraction-evaporation method. Dextran Blue, as a stable and inert macromolecular

hydrophilic compound and lysozyme, as a model protein, were encapsulated in microspheres based on this hydrophilic polyester and their in vitro release kinetics was determined. PLGA microspheres with these compounds were used for comparison. Moreover, the degradation of the microspheres and the integrity of the released protein were studied.

3.2. Materials and Methods

3.2.1 Materials

N,N´-Dimethylformamide and methyl-tert-butyl ether (MTBE), peptide grade dichloromethane (DCM), chloroform and tetrahydrofurane (THF) were purchased from Biosolve (Valkenswaard, The Netherlands). Benzyl alcohol was obtained from Merck (Darmstadt, Germany). Toluene (Acros, Geel, Belgium) was distilled from P_2O_5 and stored over 3 Å molecular sieves under argon. O-Benzyl-L-serine was purchased from Senn Chemicals AG (Dielsdorf, Switzerland). N,N´-Dimethylaminopyridine (DMAP) and sodium azide (NaN_3, 99%) were obtained from Fluka (Zwijndrecht, The Netherlands). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}\ H_2O$) were purchased from Merck (Darmstadt, Germany). PLGA (50:50, intrinsic viscosity 0.4 dl/g) and L-lactide were obtained from Purac, The Netherlands. Polyvinylalcohol (PVA; Mw 30,000-70,000; 88 % hydrolyzed) and tin (II) 2-ethylhexanoate ($SnOct_2$) were from Sigma-Aldrich, Inc., USA. Hen egg-white lysozyme was purchased from Fluka, Belgium. BCA reagent was from Interchim, USA. Pd/C (Palladium, 10 wt % on activated carbon, Degussa type E101 NE/W) was purchased from Aldrich, Zwijndrecht, The Netherlands. Dextran Blue (Mr ~ 2×10^6) was from Fluka, Sweden. Micrococcus lysodeikticus was obtained from Sigma, St.Louis, MO, USA. Unless otherwise stated, all chemicals were used as received.

3.2.2. Synthesis of copolymers of 3S-(benzyloxymethyl)-6S-methyl-1, 4-dioxane-2, 5-dione with L-lactide

3S-(benzyloxymethyl)-6S-methyl-1, 4-dioxane-2, 5-dione (BMMG, Fig. 1) was synthesized according to Leemhuis et al. (22).

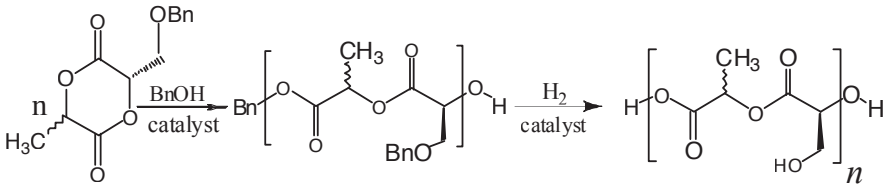

Fig. 1. Synthesis of hydrophilic aliphatic polyesters based on lactic acid and glycolic acid with pendant hydroxyl groups, poly (lactic-co-hydroxymethyl glycolic acid) (22).

In a typical procedure, a copolymer of BMMG and L-lactide (monomer ratio 35/65 % mol/mol) was synthesized by melt copolymerization as follows: BMMG (960 mg, 3.8 mmol) and L-lactide (1027 mg, 7.1 mmol) were transferred into a dried Schlenk tube under a dry nitrogen atmosphere. Benzyl alcohol (11.86 mg; 50 µL from a 237 mg/ml toluene stock) and $SnOct_2$ (22.2 mg; 100 µL from a 222 mg/ml toluene stock solution) were added. Toluene was removed by putting the tube under vacuum for 2 h. The tube was closed and heated to 110 °C using an oil bath for 16 h while stirring. After cooling to room temperature, the formed copolymer was dissolved in around 5 ml chloroform, precipitated into 250 ml of cold methanol and vacuum dried after filtration to give poly(lactic acid-ran-benzyloxymethyl glycolic acid) (PLBMGA) as a white solid (1.95 g). The copolymer was then dissolved in distilled THF (300 ml) and 10 % w/w of Pd/C was added to remove the protecting benzyl group. After stirring at room temperature for 16 h under hydrogen atmosphere, the catalyst was removed by using a Hyflo filter. Evaporation in vacuo yielded 1.55 g of poly(lactic-co-hydroxymethyl glycolic acid) (PLHMGA).

3.2.3. Physico chemical characterization of PLBMGA and PLHMGA

^{1}H NMR measurements of PLBMGA and PLHMGA dissolved in $CDCl_3$ were performed at 298 K on a Varian Gemini-300 NMR machine, operating at 300 MHz.

Differential scanning calorimetry (DSC) analysis was done on a TA Instruments DSC Q2000 machine. PLBMGA and PLHMGA (approximately 5 mg) were loaded into aluminum pans and heated from room temperature to 200 °C at a heating rate of 5 °C/min. Next, the samples were cooled down to -50 °C and heated to 200 °C with a rate of 5 °C/min. The melting temperature (T_m) was determined from the endothermic peak of the DSC thermogram in the first heating scan and the glass transition temperature (T_g) was determined from the thermogram recorded in the second heating scan.

Gel permeation chromatography (GPC) was carried out on a Waters Aliance system, with a Waters 2695 separating module and a Waters 2414 Refractive Index detector. Two PL-gel 5 µm mixed-D columns fitted with a guard column (Polymer Labs, M_w range 0.2-400 kDa) were used. Calibration was done with polystyrene standards using THF as the mobile phase (1 ml/min) for PLBMGA. For the GPC analysis of PLHMGA, a 10 mM solution of LiCl in DMF with the flow rate of 0.7 ml/min was used as eluent and the columns were calibrated with PEG standards (22).

3.2.4. Preparation of the microspheres

Dextran Blue loaded microspheres of PLHMGA or PLGA were prepared essentially as described by Wang et al. (24). In short, 200 µl of Dextran Blue solution in water (50 mg/ml) were mixed either with 800 µl of a solution of 10, 15 and 20 % (w/w) PLHMGA solution in DCM or 15 % (w/w) PLGA in the same solvent. The water/DCM two phase system was emulsified by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 s at the highest speed. Subsequently, 800 µl of 1 % PVA solution were added and the mixture was vortexed for 30 s at the maximum speed. The resulting w/o/w emulsion was then transferred into 8 ml of an aqueous PVA (0.5 % w/w) solution and stirred for 1 hour at room temperature to evaporate DCM. After that the microspheres were separated by centrifugation (Laboratory centrifuges, 4K 15, Germany) at 5000 g for 5 min, washed 3 times with 100 ml reversed osmosis water and freeze dried overnight. Lysozyme loaded microspheres were prepared using the same method as the Dextran Blue-loaded microspheres except that the resulting w/o/w emulsion was transferred into either 80 ml or 8 ml of an aqueous PVA (0.5 % w/w) solution (V_{DP}/V_{CP} were 1/100 and 1/10, respectively; V_{DP} = volume of dispersed phase, V_{CP} = volume of continuous phase) and stirred for 1 hour at room temperature to evaporate DCM. In addition, blank microspheres of PLHMGA were prepared with a V_{DP}/V_{CP} of 1/10.

3.2.5. Characterization of the microspheres

The average size and size distribution of the microspheres were measured using an Accusizerth 780 (Optical particle sizer, SantaBarbara, California, USA). The morphology of the microspheres was studied by scanning electron microscopy (Phenom^{TM}, FEI Company, The Netherlands). Microspheres were glued on 12 mm diameter aluminum sample holder using conductive carbon

paint (Agar scientific Ltd., England) and coated with palladium under vacuum using an ion coater. DSC analysis of the microspheres was done by loading approximately 5 mg of the freeze-dried microspheres into an aluminum pan. Thermograms of the microspheres were recorded by using the same heating/cooling/heating method as described in section 2.3 for PLHMGA.

The loading efficiency (LE) of Dextran Blue loaded microspheres was investigated by dissolving 20 mg of microspheres in DMSO and measuring the absorbance at 620 nm. Calibration was done with Dextran Blue dissolved in DMSO (concentration ranging from 10-200 µg/ml).

The lysozyme loading efficiency was determined by a micro-BCA method (25). Briefly, 20 mg of freeze-dried microspheres were dissolved in 2 ml DMSO and 10 ml of a 0.05 M NaOH solution containing 0.5 % (w/v) SDS (sodium dodecyl sulfate) were added and incubated at room temperature for 1 hour. Calibration was done with lysozyme dissolved in DMSO/0.05 M NaOH (volume ratio 1/5, lysozyme concentration ranging from 10-200 µg/ml) also containing 0.5 % (w/v) SDS. Twenty five µl of standards and samples were pipetted into a 96 well plate and 200 µl of a BCA working reagent (a freshly prepared solution of 50 parts of a BCA stock solution with 1 part of 4% $CuSO_4.5H_2O$) were added. The plates were incubated for 2 hours at 37 °C. The absorbance of the solutions in the wells was measured by a Novapath Microplate Reader at a wavelength of 550 nm.

To investigate whether lysozyme was present in the insoluble residues present in the release samples after 60 days, the residues were washed twice with RO (reverse osmosis) water and after freeze-drying, they were dissolved in DMSO (200 µl). After 1 hour, 1 ml of 0.05 M NaOH containing 0.5 % (w/v) SDS was added and the amount of lysozyme was determined by the micro-BCA assay both in the water fraction and the DMSO/NaOH/SDS solution. Additionally, the presence of protein was studied by the FTIR analysis of the freeze dried residues using a BIO-RAD FTS6000 FT-IR (BIO-RAD, Cambridge, MA, USA) and KBr (26). The FTIR spectra were measured at room temperature and a total of 32 scans at a resolution of 2 cm^{-1} were averaged.

3.2.6. In vitro degradation of PLHMGA microspheres

A series of blank microspheres (20 mg) prepared from PLHMGA with 65 % lactide content were transferred into eppendorf tubes (10 tubes per microsphere batch) and 1.5 ml of PBS (pH 7.4, 0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05 % (w/w) NaN_3) was added. Samples were incubated while gently shaken at 37 °C. At predetermined time points, 0.5 ml of buffer was replaced by fresh buffer. Samples of microspheres were taken out at

predetermined time points and washed twice with 1 ml RO water and after freeze drying, the weight of samples was measured. NMR, DSC and GPC were used to analyze the remaining polymer.

3.2.7. In vitro release studies

About 20 mg of Dextran Blue-loaded microspheres were suspended in 1.5 ml of PBS and incubated at 37 °C under mild agitation. At different time points, the samples were centrifuged (5000 g for 1 min) and 0.5 ml of supernatant was removed and replaced by 0.5 of fresh buffer. The absorbance at 620 nm was measured and calibration was done with Dextran Blue in PBS (10-300 µg/ml).

For lysozyme loaded microspheres, 20 mg of microspheres were suspended in 1.5 ml of PBS and the sampling was done as for the Dextran Blue-loaded microspheres. The protein content in the supernatant was determined by ultra performance liquid chromatography (Acquity UPLC®) using a BEH300 C18 1.7 µm column. Elution was performed at a flow rate of 0.25 ml/min, using a gradient starting with 75 % eluent A (95 % H_2O, 5 % ACN and 0.1% TFA) and 25 % eluent B (100 % ACN and 0.1% TFA). The eluent linearly changed to 60 % A / 40 % B during 4 min and the volume ratio was set back at 75 % A / 25 % B after 5 minutes. The injection volume was 5 µl and detection was done at 280 nm. A series of lysozyme standards (10-500 µg/ml) was used for calibration.

3.2.8. Lysozyme structural analysis

The released lysozyme was analyzed by far-UV CD spectroscopy (260-200 nm) at ambient temperature in a quartz cell (path length 0.05 cm) with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instruments System, Bogart, GA, USA). Five scans of each sample were averaged.

Tryptophan fluorescence spectra of lysozyme (50 µg/ml) were measured on a Fluorolog®-3 spectrofluorometer (HORIBA Jobin Yvon Inc., New Jersey, USA) with an excitation wavelength of 295 nm. Emission wavelength range was 300 to 450 nm and the average of 3 scans was taken.

The enzymatic activity of lysozyme released from the microspheres was determined spectrophotometrically by using Micrococcus lysodeikticus as substrate (27). In short, a 0.2 mg/ml substrate cell suspension was prepared in 66 mM phosphate buffer pH 6.2 (7.59 g $NaH_2PO_4 \cdot H_2O$, 1.96 g $Na_2HPO_4 \cdot 2H_2O$). Next, 10 µl of a sample of released lysozyme (protein concentration adjusted to 50 µg/ml) were mixed with 1.3 ml of substrate solution and the turbidity decrease was measured for 3 min at 450 nm. Enzymatic activity of the released

lysozyme is expressed as a percentage of activity of a reference lysozyme solution.

3.3. Results and Discussion

3.3.1. Synthesis and characterization of PLHMGA

We previously showed that PLHMGA degraded in 20 or 60 days under physiological conditions for the copolymer with 50 mol % or 75 mol % of L-lactide, respectively (23). Therefore, in order to obtain a system capable to degrade in approximately 30 days, BMMG was copolymerized in the melt with 65 % L-lactide (mol/mol) using BnOH and $SnOct_2$ as initiator and catalyst, respectively. PLBMGA was obtained in almost quantitative yield and the copolymer composition as determined by NMR matched the feed ratio (Table 1).

Table 1. Characteristics of PLBMGA and PLHMGA

	Feed ratio M/L[a]	Copolymer composition (NMR analysis)	Yield %	M_n theoretical (kg/mol)	M_n measured[b] (kg/mol)	M_w/M_n	Tg (°C)
PLBMGA	**35/65**	**38/62**	**95**	**19**	**14**	**1.4**	**35**
PLHMGA	**35/65**	**32/68**	**79**	**15**	**11**	**1.3**	**38**

[a] M = monomer BMMG, L = lactide; [b] Determined with GPC using the following calibration standards: polystyrene for PLBMGA (solvent: THF) and PEG for PLHMGA (solvent: DMF/LiCl)

Further, in agreement with our previous data (22), DSC analysis showed that PLBMGA was completely amorphous with a T_g of 35 °C. The protecting benzyl group of BHMMG was removed by catalytic hydrogenation using Pd/C as catalyst. ^1H NMR analysis confirmed complete removal of the protecting group by the disappearance of the peak at ~ 7.3 ppm. The deprotected copolymer (abbreviated as PLHMGA) was obtained in a high yield (~ 80%) with a T_g of 38 °C and the copolymer compositions (determined by NMR analysis) were close to the feed ratio.

3.3.2. Preparation and characterization of PLHMGA microspheres

Dextran Blue and lysozyme-loaded PLHMGA as well as blank microspheres were prepared using a double emulsion extraction-evaporation method. Table 2 summarizes the characteristics of obtained microspheres.

Table 2. Characteristics of Dextran Blue and lysozyme loaded PLHMGA and PLGA microspheres (n=3). Unless stated otherwise, microspheres prepared with V_{DP}/V_{CP} = 1/10

ingredient	Polymer	concentration in DCM %(w/w)	volume weight mean(µm)	LE (%)*	LC (%)†	Yield %
Dextran Blue	PLHMGA	10	5.0±0.5	50±4	5.5±0.5	75.8±0.8
		15	7.0±0.5	84±4	6.0±0.1	73.3±1.6
		20	8.0±1.0	85±5	4.3±0.2	73.3±2.4
	PLGA	15	13.0±2.0	72±3	4.7±0.1	78.9±1.2
lysozyme¥	PLHMGA	15	10.5±0.2	78±3	5.0±1.0	76.0±0.6
lysozyme	PLHMGA	10	6.6±1.1	66±1	7.5±0.2	73.3±2.3
		15	7.7±0.3	81±5	5.1±0.4	78.7±1.7
		20	12.4±0.1	85±1	3.7±0.1	83.0±1.2
lysozyme	PLGA	15	19.6±1.6	67±7	4.3±0.2	78.3±3.8

¥ V_{DP}/V_{CP} = 1/100

* LE (Loading Efficiency) expressed as encapsulated Dextran Blue or protein divided by the total amount of Dextran Blue or protein used for encapsulation.

† LC (loading Capacity) expressed as encapsulated amount of Dextran Blue or protein divided by the total dry weight of the microspheres

The volume-weight mean diameter of the microspheres slightly increased from 5 to 8 µm for Dextran Blue-loaded microspheres and from 6 to12 µm for

lysozyme-loaded microspheres with increasing PLHMGA concentration in DCM. Likely, a higher viscosity of the oil phase (due to higher polymer concentration) results in larger emulsified droplets, which in turn yields larger microspheres, as explained before (28,29). The loading efficiency (LE) of Dextran Blue and lysozyme increased from 50-60 % to 85 % with increasing polymer concentration. The V_{DP}/V_{CP} ratio had no significant effect on the LE but the particle size was slightly increased when this ratio was decreased from 1/10 to 1/100. The found LEs are in agreement with those reported for bovine serum albumin and a nine-amino acid peptide loaded in PLGA microspheres (30,31). The observed increase in LE with increasing polymer concentration can be explained as follows. First, at higher polymer concentration a faster precipitation of the polymer occurs during the solvent evaporation process, which in turn will retard diffusion of the protein into the continuous phase (30). Secondly, as pointed out by Bodmeier et al. a higher viscosity of the polymer solution in DCM will reduce the protein mobility in the w/o emulsion droplets (32).

The surface morphology of lysozyme-loaded PLHMGA microspheres prepared at V_{DP}/V_{CP} 1/10 and 1/100 and with 15 % (w/w) PLHMGA in DCM was studied by SEM analysis. Microspheres made at a V_{DP}/V_{CP} of 1/100 were rather porous (Fig. 2a, b), whereas microspheres with a V_{DP}/V_{CP} of 1/10 were essentially non-porous (Fig. 2c, d). The large pores in the microspheres prepared at a low V_{DP}/V_{CP} ratio were also reported by Jeyanthi et al. and can likely be explained by a rapid extraction of dichloromethane from the dispersed phase into the continuous phase, resulting in a faster polymer precipitation (33). PLGA microspheres prepared at a V_{DP}/V_{CP} of 1/10 showed a surface morphology comparable to those of PLHMGA microspheres (Fig. 2e, f).

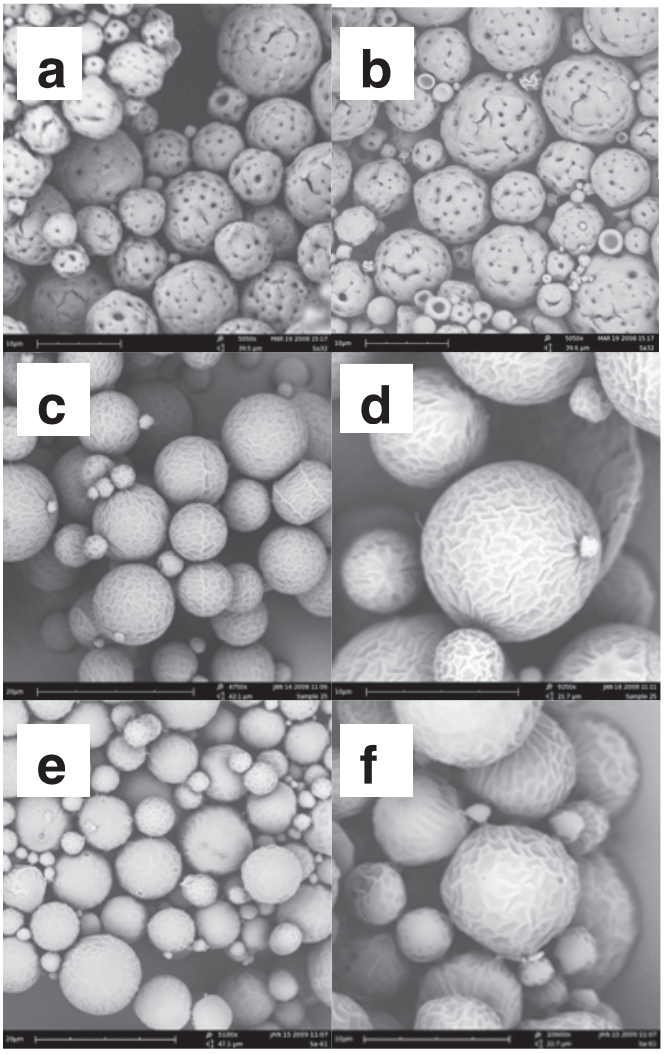

Fig. 2. Scanning electron microscopy photographs of PLHMGA and PLGA microspheres prepared from a 15 % polymer solution in DCM and different V_{DP}/V_{CP} ratios: (a, b) PLHMGA microspheres with V_{DP}/V_{CP} of 1/100; (c, d) PLHMGA microspheres with V_{DP}/V_{CP} of 1/10; (e, f) PLGA microspheres with V_{DP}/V_{CP} of 1/10.

3.3.3. In vitro degradation of PLHMGA microspheres

Microspheres of PLHMGA incubated at 37 °C and pH 7.4 showed a continuous weight loss in time; after 50 days less than 20 % of their initial weight remained (Fig. 3 a). This Fig. also shows that the polymer mass loss was independent of the polymer concentration in DCM used to prepare the PLHMGA

microspheres, which suggests that the microspheres degrade via bulk erosion, in agreement with previous data (23). The M_n of the non-dissolved polymer as determined by GPC, showed a gradual decrease in time (Fig. 3b).

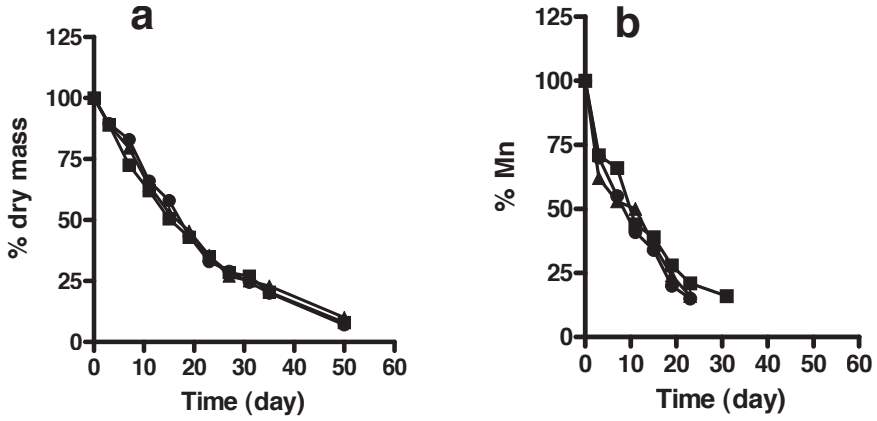

Fig. 3. (a) Relative mass decrease of the PLHMGA microspheres and (b) number average molecular weight (M_n) decrease of PLHMGA as a function of time; microspheres were prepared with an initial polymer concentration in DCM of (■) 10 %, (▲) 15 % and (●) 20 % (w/w). Degradation was done in PBS at 37 °C.

The degradation of the microspheres, however, was not complete in the time frame studied (60 days) and some insoluble residues remained. ^{1}H NMR analysis of the residues showed that the duplet peak around 4 ppm attributed to the protons of the methylene group of hydroxymethyl glycolic acid had disappeared, and oligomers of lactic acid remained (Fig. 4). This means that during degradation preferential removal of the HMGA units occurred during degradation. Likely, due to the hydration of the hydroxyl groups of these units, hydrolysis preferentially occurs at the hydroxyl enriched sites in the polymer (23,34).

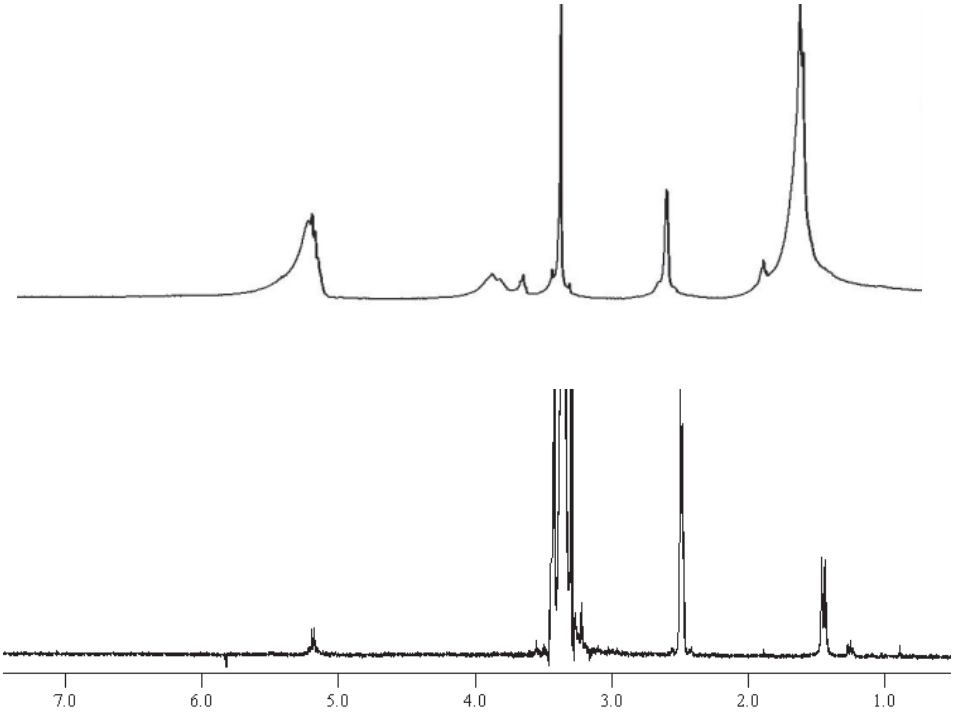

Fig. 4. ^{1}HNMR spectrum of the PLHMGA (top) and the insoluble residues isolated after 50 days of degradation (bottom); samples were dissolved in DMSO.

DSC analysis of PLHMGA microspheres showed a lower T_g (25 °C) in comparison to PLHMGA polymer (38 °C) which is likely caused by PVA that can act as plasticizer and decrease the T_g of the microspheres (35). Moreover, PLHMGA microspheres were completely amorphous before degradation (Fig. 5), while crystalline fragments of lactic acid were formed during degradation (Fig. 5, T_m= 102 ° C, ΔH = 48 J/g). Based on a previous report, the degree of polymerization of these lactic acid oligomers can be estimated to be around 10 (36).

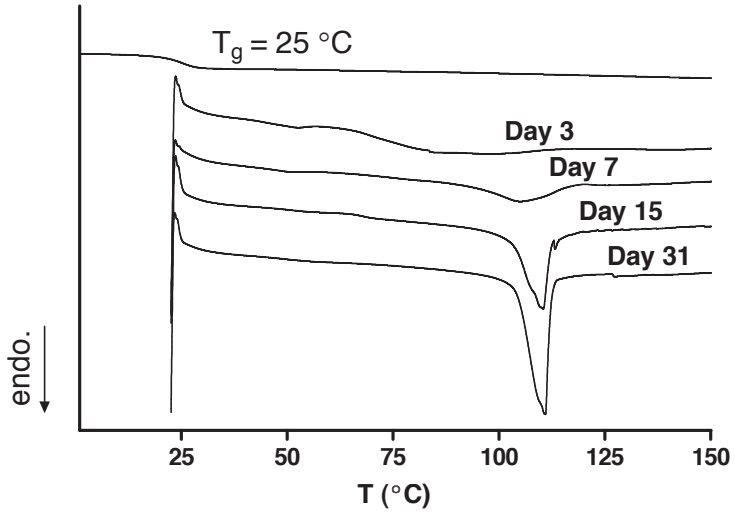

Fig. 5. DSC thermograms of the PLHMGA microspheres (made from a 15 % (w/w) polymer solution) before degradation, recorded in the second heating run (top) and after 3, 7, 15 and 31 days of degradation recorded in the first heating run. ΔH values of the melting peak are 19, 39 and 50 J/g for day 7, 15 and 31, respectively.

3.3.4. In vitro release of Dextran Blue and lysozyme from PLHMGA microspheres

Fig. 6 (a) shows the release of Dextran Blue from different PLHMGA microspheres in PBS. Microspheres made with 10 % polymer solution in DCM, showed a high burst release (> 70 %) followed by a gradual release, reaching 90 % of the loaded amount, over the next 14 days. Increasing the initial polymer concentration to 15 and 20 % resulted in microspheres showing a decreased initial burst of 20 and 10 %, respectively. This lower burst release can be attributed to the lower porosity of these microspheres (37,38). After this small burst, hardly any Dextran Blue was released during the first few days. Around day 6, Dextran Blue started to be released, reaching completeness (>90 % of the loaded amount) in 27 days. In contrast, after a low burst release, PLGA microspheres showed hardly any release of Dextran Blue during 27 days. After 40 days, as polymer degradation progressed, they started to release Dextran Blue up to 65 % in 65 days, which is in agreement with the release behavior of FITC-dextran from PLGA microspheres (39). The difference between Dextran Blue release from PLHMGA and PLGA microspheres can likely be explained by the

different degradation rate of PLHMGA and PLGA, which is more rapid for PLHMGA.

Fig. 6 (b) shows that the release of lysozyme from PLHMGA microspheres also depended on the polymer concentration in the DCM phase used for the preparation of microspheres. The microspheres prepared from a 10 % polymer solution showed a burst release of around 35 % followed by a sustained release for 20 days. A small burst release, 3 and 1 %, was observed for the microspheres prepared from a 15 and 20 % polymer solution, respectively, and these microspheres started to release the protein after a certain delay time (around 8-10 days). Thereafter, as microsphere degradation progressed, the protein was released with almost zero-order kinetics for the next 25 days. In agreement with a study of Jiang et al. (8), PLGA microspheres showed an initial burst release (7 %) followed by a very slow release reaching 11 % of the loaded amount after 60 days (Fig. 6 b). Several reasons for the incomplete release of encapsulated proteins from PLGA microspheres have been given. Particularly, aggregation of the entrapped protein, chemical degradation or interactions between protein and polymer are most frequently suggested (13,14,40).

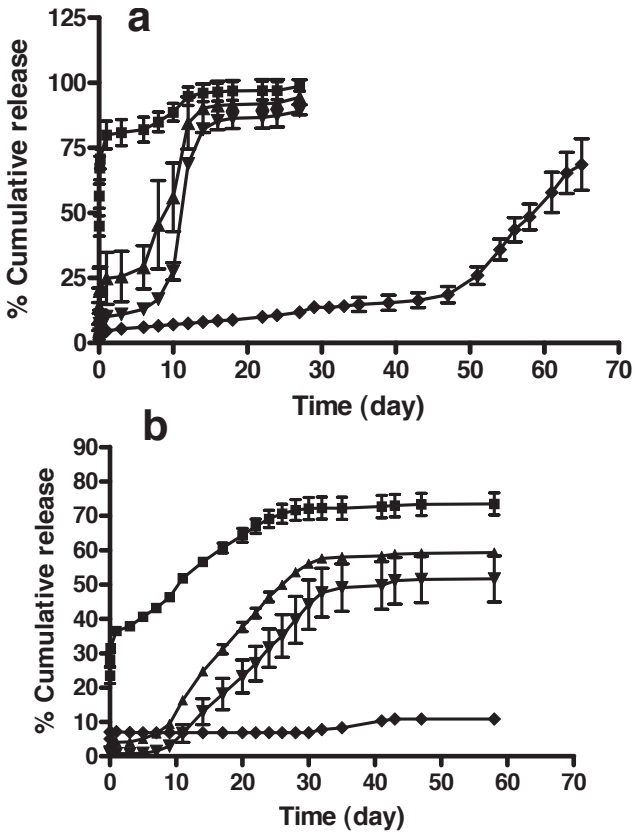

Fig. 6. Dextran Blue release (a) and lysozyme release (b) from PLHMGA microspheres in PBS (V_{DP}/V_{CP} of 1/10, n=3) prepared from a 10 (■), 15 (▲) and 20 % (▼) polymer solution; PLGA microspheres (♦).

The effect of the rate of solvent removal on lysozyme release from the resulting PLHMGA microspheres (prepared from a 15 % polymer solution) was investigated by comparing the release from microspheres prepared with a V_{DP}/V_{CP} of 1/10 and 1/100 (Fig. 7).

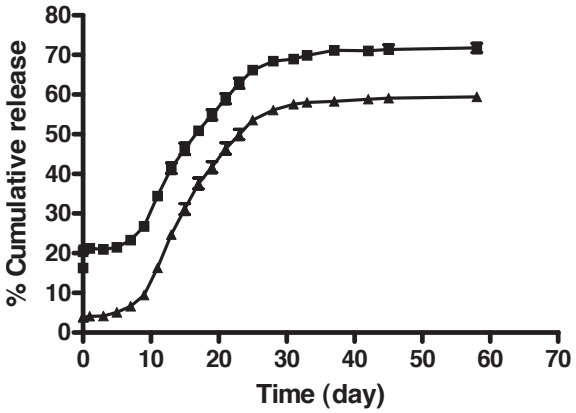

Fig. 7. Release of lysozyme (n=3) from PLHMGA microspheres prepared from a 15 % polymer solution in DCM at V_{DP}/V_{CP}=1/100 (■) and V_{DP}/V_{CP}=1/10 (▲).

Microspheres prepared with a low V_{DP}/V_{CP} (1/100), showed a burst release of around 20 % followed by a sustained release of lysozyme up to 70 % of the loaded dose for 30 days. Microspheres with a higher V_{DP}/V_{CP} (1/10), in agreement with the Dextran Blue loaded microspheres, showed a low burst release (< 5 %) and 60 % of the loaded dose was released in the next 30 days. This difference in release kinetics can be attributed to the porosity of the microspheres. Microspheres prepared at a V_{DP}/V_{CP} of 1/100 were rather porous (Fig. 2 a, b) in contrast to microspheres prepared with a V_{DP}/V_{CP} of 1/10 (Fig. 2c, d) (33).

Figs. 6 (b) and 7 show that the release of lysozyme from PLHMGA microspheres prepared from PLHMGA solutions of 10, 15 and 20 % in DCM and with different V_{DP}/V_{CP} ratios was incomplete (reaching 50-70 % of the loaded amount). To investigate this incomplete release, the insoluble residues present in the release samples after 60 days of study were washed with water and thereafter freeze dried and dissolved in DMSO. The protein amount in both the wash fractions and the DMSO solutions was measured (Table 3).

Table 3. Lysozyme specific activity and recovery from PLHMGA and PLGA microspheres. Data are expressed as mean ± SD (n=3).

polymer	polymer concentration in DCM %(w/w)	lysozyme released from microspheres after 60 days (%)	lysozyme extracted from insoluble residues (%)	recovery (%)	specific activity of the released lysozyme (%)
PLHMGA	10	73 ± 4	10 ± 4	83 ± 8	111 ± 13
	15	59 ± 1	20 ± 4	80 ± 4	103 ± 13
	15*	72 ± 1	18 ± 4	91 ± 1	105 ± 6
	20	52 ± 9	26 ± 1	78 ± 9	118 ± 3
PLGA	15	11 ± 1	68 ± 2	79 ± 2	102 ± 5

* V_{DP}/V_{CP} = 1/100 (others with V_{DP}/V_{CP} = 1/10)

Lysozyme could not be detected in the wash fractions, however 10-26 % of the loaded protein was recovered after dissolution of the freeze dried insoluble residues in DMSO/NaOH/SDS, whereas for PLGA microspheres even 68 % of the entrapped protein was found in this fraction. This incomplete recovery is most probably caused by protein denaturation/aggregation, resulting in insoluble structures which can not be detected by the BCA method.

The presence of protein in the remaining insoluble residues was investigated by FTIR. Fig. 8 shows the inverted 2nd derivative spectra of the amide I region of lysozyme that remained in the residues from PLHMGA and PLGA microspheres, together with the spectrum of native lysozyme. Whereas native lysozyme did not show an absorption band in the region around 1625 cm^{-1}, lysozyme in the PLHMGA and PLGA residues did show an absorption peak in this region. This band is typical for the presence of aggregated protein (41).

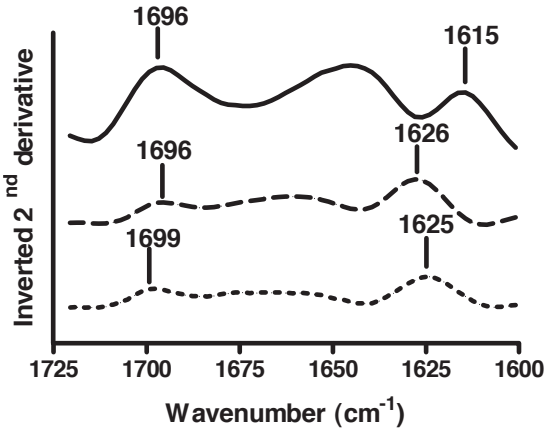

Fig. 8. Inverted 2nd derivative spectra in the Amide I region of native lysozyme (solid line), lysozyme in the insoluble residues isolated after 60 days from the microspheres of PLHMGA (dashed line) and PLGA (dotted line).

It should be investigated in future studies whether aggregation of lysozyme occured during preparation of the protein loaded microspheres or during degradation of the PLHMGA of the microspheres yielding the crystalline PLLA residues in which the protein might be entrapped.

3.3.5. Structural integrity of the released lysozyme

Table 3 shows that the biological activity of the released lysozyme from PLHMGA microspheres was fully preserved. Also, the released lysozyme from the PLGA microspheres was also fully bioactive. This demonstrates, as reported previously, that lysozyme is a very stable enzyme (42,43). Further, the far-UV CD spectrum as well as the fluorescence emission spectrum of the released lysozyme overlapped almost completely with that of native lysozyme (results not shown), indicating that the secondary and tertiary structure of released lysozyme was preserved. These findings demonstrate that released lysozyme retained its structural and functional integrity.

3.4. Conclusions

Microspheres can be successfully made from the hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid) by the double emulsion extraction-evaporation method. The in vitro release study showed that the release is governed by degradation. Importantly, the structural and functional integrity of

the released lysozyme was preserved. This study shows that PLHMGA microspheres are promising systems for the controlled release of pharmaceutical proteins. Our present studies focus on the evaluation of other polymer compositions and other proteins and strategies to circumvent protein aggregation.

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Chapter 4

Hydrophilic polyester microspheres: effect of molecular weight and copolymer composition on release of BSA

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Abstract

To study the release of a model protein, bovine serum albumin (BSA), from microspheres of an hydroxylated aliphatic polyester, poly(lactic-co-hydroxymethyl glycolic acid) (PLHMGA). BSA-loaded microspheres were prepared by a double emulsion solvent evaporation method. The effect of copolymer composition and the molecular weight of the copolymer on in vitro release and degradation were studied. The integrity of the released BSA was studied by fluorescence spectroscopy and size exclusion chromatography (SEC). Microspheres prepared from PLHMGA with 50% hydroxymethyl glycolic acid (HMG) showed a burst release followed by a sustained release in 5-10 days. PLHMGA microspheres prepared from a copolymer with 35% and 25% HMG showed a sustained release of BSA up to 80% for 30 and 60 days, respectively. The release of BSA was hardly affected by the molecular weight of the polymer. Fluorescence spectroscopy and SEC showed that the released BSA preserved its structural integrity. Microspheres were fully degradable and the degradation time increased from ~20 days to 60 days when the HMG content decreased from 50% to 25%. Taken the degradation and release data together, it can be concluded that the release of BSA from PLHMGA microspheres is governed by degradation of the microspheres.

4.1. Introduction

Poly(lactic-co-glycolic acid) (PLGA) has been extensively investigated for the delivery of hormones, antigens, antibodies and enzymes (1,2). Lactic acid/glycolic acid copolymers degrade via hydrolysis of the ester bounds connecting the monomer units, and the degradation products are lactic and glycolic acid which can be metabolized and/or excreted by the kidneys (3). However, due to the instability of proteins during preparation, storage and release, PLGA systems have drawbacks for protein delivery. One major concern of PLGA microspheres for the controlled release of proteins is the acidic pH that is generated in the degrading polymer matrix (4-7) which leads to denaturation and aggregation of encapsulated proteins and results in incomplete release (8). Furthermore, PLGA is rather hydrophobic and proteins might adsorb onto surfaces of this polymer which can be associated with protein unfolding and aggregation (9-11). Finally, it has been reported that acylation and deamidation of proteins might occur in degrading PLGA matrices (12-16). In our previous study (17) we investigated the suitability of microspheres of a hydrophilic polyester, poly(L-lactic-co-hydroxymethyl glycolic acid) (PLHMGA), based on L-lactide and glycolide with pendant hydroxyl groups for the release of macromolecular compounds (18). We showed that the release of a water-soluble polysaccharide (dextran blue) and a model protein (lysozyme) was governed by degradation of the microspheres. However, the release of lysozyme, depending on the copolymer concentration used for microspheres formulation, varied from 50% to 70% of the loaded amount, and some insoluble residues composed of aggregated protein and insoluble crystalline L-lactic acid oligomers remained after 30 days. In order to circumvent the formation of these crystalline oligomers during degradation and to obtain complete degradation of the microspheres, in the present study, we used D,L-lactide instead of L-lactide for synthesis of copolymers with HMG. Microspheres were prepared using a double emulsion solvent evaporation method. The degradation of the microspheres made from copolymers of D,L-lactide and HMG with different copolymer composition and molecular weight was studied. The release of a model protein, bovine serum albumin (BSA), from PLHMGA microspheres was investigated. Furthermore, the structural integrity of the released protein was studied.

4.2. Materials and Methods

4.2.1. Materials

O-Benzyl-L-serine was purchased from Senn Chemicals AG (Dielsdorf, Switzerland). Bovine serum albumin (BSA) was obtained from Sigma Chemical Company (St Louis, USA). D,L-lactide was obtained from Purac, The Netherlands. N,N´-Dimethylformamide and methyl-tert-butyl ether (MTBE), peptide grade dichloromethane (DCM), methanol, chloroform and tetrahydrofurane (THF) were purchased from Biosolve (Valkenswaard, The Netherlands). Benzyl alcohol was obtained from Merck (Darmstadt, Germany). Toluene (Acros, Geel, Belgium) was distilled from P_2O_5 and stored over 3 Å molecular sieves under argon. N,N´-Dimethylaminopyridine (DMAP) and sodium azide (NaN_3, 99%) were obtained from Fluka (Zwijndrecht, The Netherlands). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) were purchased from Merck (Darmstadt, Germany). Polyvinyl alcohol (PVA; Mw 30,000-70,000; 88 % hydrolyzed) and tin (II) 2-ethylhexanoate ($SnOct_2$) were from Sigma-Aldrich, Inc., USA. BCA reagent was from Interchim, USA. Pd/C (Palladium, 10 wt % on activated carbon, Degussa type E101 NE/W) was purchased from Aldrich, Zwijndrecht, The Netherlands. Unless otherwise stated, all chemicals were used as received.

4.2.2. Synthesis of copolymers of 3S-(benzyloxymethyl)-6S-methyl-1,4-dioxane-2, 5-dione with D, L-lactide

3S-(benzyloxymethyl)-6S-methyl-1,4-dioxane-2,5-dione (BMMG) was synthesized according to Leemhuis et al.(18). The synthesized monomer BMMG was copolymerized with D,L-lactide at different monomer molar ratios (25/75, 35/65 and 50/50 % mol/mol) by melt copolymerization (Fig. 1) (18).

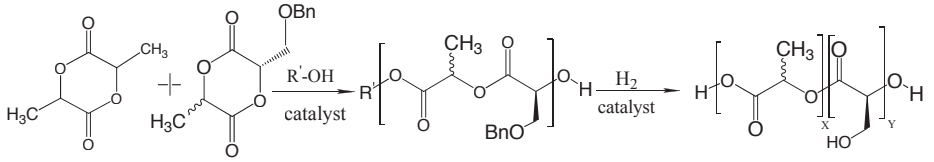

Fig. 1. Synthesis of hydrophilic aliphatic polyesters based on lactic acid and glycolic acid with pendant hydroxyl groups, poly(lactic-co-hydroxymethyl glycolic acid).

Briefly, for the synthesis of the 25/75 copolymer, BMMG (726 mg, 2.87 mmol) and D,L-lactide (1253 mg, 8.66 mmol) were transferred into a dried Schlenk tube under a dry nitrogen atmosphere. Benzyl alcohol (12.54 mg; 37 µl from a 352 mg/ml toluene stock solution) and $SnOct_2$ (23.5 mg; 71 µl from 330 mg/ml toluene stock solution) were added. After evaporation of toluene under vacuum, the tube was sealed and immersed into a 130 °C oil bath for 16 h while stirring. After cooling to room temperature, the obtained copolymer, poly(D,L-lactic acid-ran-benzyloxymethyl glycolic acid) (PLBMGA), was dissolved in chloroform and subsequently precipitated in cold methanol. After filtration, the polymer was vacuum dried to yield a 1.86 g white solid material which was dissolved in 300 ml distilled THF, followed by the addition of 10 % w/w Pd/C catalyst to remove the benzyl protecting groups. The solution was stirred under hydrogen atmosphere for 16 h and then the catalyst was removed by Hyflo filter. Evaporation of THF yielded 1.5 g of poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA). Copolymers of 35/65 and 50/50 BMMG and D,L-lactide were synthesized using the same method by adjusting the amounts of BMMG and D,L-lactide. Two copolymers of BMMG and D, L-lactide (35/65) with different molecular weights were synthesized at monomers to initiator (M/I) of 100 and 10.

4.2.3. Polymer characterization

^{1}H NMR analysis of PLBMGA and PLHMGA was performed using a Gemini-300 MHz spectrometer at 298 K. Samples were dissolved in $CDCl_3$.

The molecular weights of the obtain polymers were determined using GPC (Waters Alliance system), with a Waters 2695 separating module and a Waters 2414 refractive index detector. Two PL-gel 5 µm Mixed-D columns fitted with a guard column (Polymer Labs, M_w range 0.2-400 kDa) were used and calibration was done using polystyrene standards with narrow molecular weight distributions. THF was used as the mobile phase (1 ml/min) (17).

The thermal properties of the protected (PLBMGA) and deprotected copolymers (PLHMGA) were measured using differential scanning calorimetry (TA instrument, Q2000). Approximately 5 mg of either PLBMGA or PLHMGA was loaded into aluminum pans and after heating from room temperature to 120 °C, with a heating rate of 5 °C/min, samples were cooled down to -50 °C. Thereafter they were heated to 120 °C with temperature modulation at ±1 °C and a ramping rate of 1 °C/min. The second cycle was used to determine the glass transition temperature (T_g) of synthesized polymers.

4.2.4. Preparation of microspheres

BSA-loaded microspheres were prepared using a solvent evaporation technique (17,19,20). Briefly, 125 µl of a BSA solution in water (50 mg/ml) was emulsified with 500 µl of a solution of 20% (w/w) PLHMGA solution in DCM by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30s at the highest speed to get an w/o primary emulsion. An emulsion stabilizer (500 µl 1% PVA solution) was then added and the mixture was vortexed for 30s at maximum speed. The formed emulsion was subsequently transferred into a 5 ml 0.5% PVA aqueous solution and stirred for 1 h at room temperature to evaporate DCM. Microspheres were collected by centrifugation (Laboratory centrifuge, 4 K 15 Germany) at 3000 g for 3 min and washed 3 times with 100 ml reversed osmosis (RO) water and freeze dried overnight. The dried microspheres were stored at -25 °C.

4.2.5. Microspheres characterization

The microspheres size distributions were analyzed by an Accusizerth 780 (Optical particle sizer, Santa Barbara, California, USA). Results are reported as volumetric mean diameter.

The morphology of microspheres was analyzed using a Phenom^{TM} SEM (FEI Company, The Netherlands). The samples were mounted onto 12 mm diameter aluminum specimen stubs (Agar Scientific Ltd., England) using double-sided adhesive tape. The samples were sputter coated with a platinum coating prior to analysis.

4.2.6. Protein loading of the microspheres

Protein loading of the microspheres was determined by dissolving about 10 mg of freeze dried microspheres in 1 ml DMSO. After 1 h incubation at room temperature, 5 ml of a 0.05 M NaOH solution containing 0.5 % (w/v) SDS (sodium dodecyl sulfate) was added, essentially as described by Hongkee et al.(21). The resulting solution was then analyzed for the total protein content by a BCA-microplate assay. BSA loading efficiency (LE) is reported as the amount of protein entrapped in the microspheres divided by the amount of protein added during the preparation of microspheres times 100%. Loading capacity (LC) is expressed as encapsulated amount of BSA divided by the total dry weight of the microspheres.

4.2.7. In vitro release studies

BSA-loaded microspheres (20 mg) were suspended in a 1.5 ml PBS (pH 7.4, 0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05 % (w/w) NaN_3) and incubated at 37 °C under mild agitation. Samples were collected at different time points after centrifugation of the microspheres dispersion, followed by removing 1 ml of the supernatant and replacing it with 1 ml of fresh buffer. The samples were analyzed for their protein concentration by UPLC (Acquity UPLC®) equipped with a BEH 300 C18 1.7 µm column. A gradient elution method was used with mobile phase A (95% H_2O, 5% ACN and 0.1% TFA) and mobile phase B (100% ACN and 0.1 % TFA). The eluent linearly changed from 80:20 (A: B) to 40:60 (A: B) over 2 min and set back to 80:20 (A: B) in 3.5 min, with a flow rate of 0.250 ml/min. The injection volume was 5 µl and UV absorbance was measured at 210 nm. BSA standards (10-200 µg/ml) were used for calibration.

4.2.8. In vitro degradation of PLHMGA microspheres

The in vitro degradation was studied by transferring 20 mg of the microspheres into eppendorf tubes (10 tubes per microspheres batch) to which 1.5 ml of PBS buffer (pH 7.4, 0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05 % (w/w) NaN_3) was added. The samples were incubated at 37 °C while gently shaken. In order to keep the pH constant, at regular time points 1 ml of the buffer was replaced by fresh buffer. Samples of the microspheres were removed at different time points and after washing them twice with 1 ml RO water, they were freeze dried and the weight of samples was measured. The remaining insoluble residues were characterized using NMR, DSC and GPC.

4.2.9. Protein stability

Fluorescence spectroscopy was used following the procedure described earlier (17) to investigate possible changes in the tertiary structure of the released protein. Excitation was at 295 nm and fluorescence emission spectra of the released samples (300-450 nm) were measured in 1-cm quartz cuvettes on a Fluorolog®-spectrofluorometer (Horriba Jobin Yvon, Edison, NJ, USA) and average of 3 scans was taken.

A GPC system comprised a Waters 2475 Multi λ Fluorescence Detector (excitation at 295/emission at 348) and a Superdex 200 10/300 GL column (GE Healtcare, Piscataway, USA) was used to investigate the presence of possible soluble protein aggregates in the released samples. The mobile phase was a

phosphate buffer (pH 6.8, 0.1 M), the flow rate was 0.5 ml/min and the injection volume was 50 µl. The run time was 70 min and the area under the curve (AUC) was measured to calculate the percentage of monomer, dimer and aggregates in standard (50 µg/ml) and released samples (ca. 50 µg/ml).

4.3. Results and Discussion

4.3.1. Synthesis and characterization of PLHMGA differing in copolymer composition and molecular weight

Random copolymers of benzyl protected hydroxyl methyl glycolide (BHMG) and D, L-lactide with different monomer molar ratios (25/75, 35/65 and 50/50) were synthesized in the melt at 130 °C by ring opening polymerization using BnOH and $SnOct_2$ as initiator and catalyst, respectively. The obtained copolymers (PLBMGA) were obtained in high yields (> 90%) and the copolymer composition, as determined by NMR analysis, closely matched that of the feed (Table I). The DSC thermograms showed that PLBMGA's were completely amorphous (supporting information S_1) with a T_g of 38, 37 and 34 °C for the 25/75, 35/65 and 50/50 copolymer, respectively, in agreement with our previous findings (17, 18). Complete removal of the protecting benzyl group was confirmed by NMR analysis. The deprotected PLHMGA copolymers were obtained in a high yield (> 80%) with a T_g of 36, 40 and 37°C for 25/75, 35/65 and 50/50 copolymer, respectively.

Table I. Characteristics of the PLBMGA and PLHMGA.

	Polymer	Feed ratio M/D,L[b]	Copolymer Composition (NMR)	M_n (kg/mol)	M_w (kg/mol)
PLBMGA	P_1	25/75	21/79	14	22
	P_2*	25/75	22/78	16	35
	P_3	35/65	30/70	11	16
	P_4*	35/65	35/65	21	41
	[a] [c] P_5*	35/65	32/68	10	16
	[a] P_6*	35/65	31/69	18	42
	P_7	50/50	44/56	10	15
	P_8*	50/50	46/54	20	51
PLHMGA	P_9	25/75	20/80	11	19
	P_{10}*	25/75	20/80	13	30
	P_{11}	35/65	31/69	8	13
	P_{12}*	35/65	33/67	20	37
	[a] [c] P_{13}*	35/65	33/67	10	15
	[a] P_{14}*	35/65	30/70	19	40
	P_{15}	50/50	47/53	4	7
	P_{16}*	50/50	46/54	19	45

[a] Copolymers used to study the effect of M_n on BSA release
[b] M=monomer BMMG, D, L=D,L-lactide
[c] M/I=10
* synthesized by using recrystallized monomer

Table I shows that the molecular weights of the synthesized polymers with different copolymer composition (P_9, P_{11} and P_{15}) were rather low, which might be ascribed to the presence of e.g. hydroxyl containing impurities in the monomer that might act as initiator of the ring opening polymerization. In order to obtain copolymers with higher molecular weight, BMMG was therefore recrystallized from MTBE to reduce the impurities (supporting information S_2). It was shown that this recrystallized monomer had a higher melting point (T_m=94 °C, ΔH=99 J/g) in comparison to the crystallized monomer (T_m=83 °C, ΔH=78 J/g) demonstrating the recrystallization resulted in a monomer batch with less impurities (supporting information S_3). The results showed an increase in M_n and M_w for the copolymer 35/65 (M_n from 8 to 20 kg/mol, M_w from 13 to 37 kg/mol,

compare P_{11} and P_{12}) and 50/50 (M_n from 4 to19 kg/mol, M_w from 7 to 45 kg/mol, compare P_{15} and P_{16}) using the recrystallized monomer, demonstrating that a reduction of the impurities resulted in polymers with higher molecular weights.

PLHMGA with 35 mol% BHMG and 65 mol% D,L-lactide of different molecular weights (M_n=10 and 19 kg/mol) were synthesized using recrystallized BMMG with M/I ratio of 10 and 100, respectively (Table I, P_{13} and P_{14}).

4.3.2. Preparation and characterization of microspheres

BSA-loaded PLHMGA microspheres were prepared using a double emulsion evaporation method. The characteristics of the microspheres prepared using copolymers with different copolymer composition (25/75, 35/65 and 50/50) and molecular weights are shown in Table II.

Table II. Characteristics of BSA-loaded PLHMGA microspheres (n=3) prepared using PLHMGA copolymers with different copolymer composition and molecular weights

Polymer (Table I)	Formulation	Polymer concentration in DCM % (w/w)	Volume weight mean (μm)	[a]LE (%)	[b]LC (%)
P_9	F_1	20	10.3±0.9	84.1±1.2	3.6±0.1
P_{10}	F_2	25	15.0±1.7	82.9±3.2	2.6±0.1
P_{11}	F_3	20	9.6±1.1	85.0±1.4	3.6±0.1
P_{12}	F_4	25	10.8±3.1	81.6±3.5	2.7±0.1
P_{13}	[c] F_5	20	5.4±0.3	89.1±4.8	3.8±0.1
P_{14}	[c] F_6	20	7.0±2.4	93.3±4.8	4.0±0.2
P_{15}	F_7	20	10.6±0.5	49.0±2.8	2.2±0.2
P_{16}	F_8	25	13.8±0.5	86.5±2.2	3.0±0.1

[a] LE = Loading efficiency, [b] LC = Loading capacity
[c] Microspheres used to study the effect of M_n on BSA release

Table II shows that the size of the particles ranged from 5 to 15 μm. The loading efficiency was high (> 80%) and independent of the polymer concentration and polymer molecular weight, except for microspheres prepared using the copolymer 50/50 at a low polymer concentration and low M_n (F_7, LE

~49%). The high LE is probably caused by a fast precipitation of the polymer during evaporation of the solvent which in turn hampers the drug diffusion into the continuous phase (22). Table II also shows that the particle size and LE were not dependent on the molecular weight of the copolymer used for preparation of the microspheres (F_5 and F_6).

4.3.3. In vitro release of BSA from PLHMGA microspheres; effect of copolymer composition and molecular weight

Fig. 2 shows the BSA release from microspheres of PLHMGA with different copolymer composition.

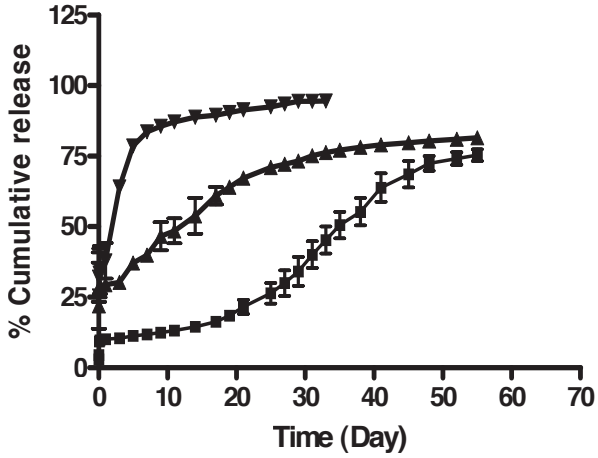

Fig. 2. BSA release from PLHMGA microspheres prepared from copolymers with different copolymer composition (Table II; F_1, F_3 and F_7); (▼) 50/50, (▲) 35/65 and (■) 25/75.

Microspheres made from PLHMGA 50/50 showed a burst release of BSA (~40% of the loaded amount) and thereafter more than 90% of the protein was released within 2 weeks. The burst release from PLHMGA microspheres decreased to 30% when the molar ratio of the hydrophilic monomer decreased to 35%. After the burst, for these microspheres a sustained release for 30 days was observed. PLHMGA 25/75 microspheres showed a low burst (<10%) and after a non-phase release (~20 days), as polymer degradation progressed, BSA was released for the next 35 days. However, the release from microspheres of copolymer 35/65 and 25/75 reached ~80% of the loaded amount. Likely, the possible deleterious conditions for the protein during degradation of the

microspheres might have led to denaturation and/or aggregation of BSA. Excipients can be used to counteract these adverse effects (23, 24).

BSA-loaded microspheres were also prepared from copolymers with higher molecular weight (Table I; P_{10}, P_{12} and P_{16}) and the copolymer concentration of the DCM solution used to prepare the microspheres was increased from 20% (w/w) to 25% (w/w). The BSA release profiles from the prepared microspheres are shown in Fig. 3.

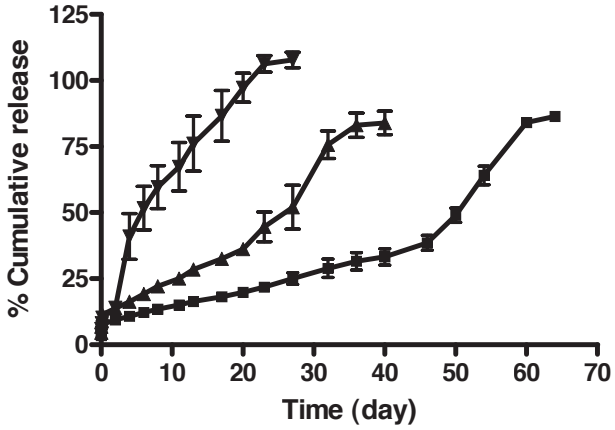

Fig. 3. BSA release from microspheres prepared from copolymers synthesized from recrystallized BMMG (Table II; F_2, F_4 and F_8) with the copolymer concentration of 25% (w/w); (▼) 50/50, (▲) 35/65 and (■) 25/75.

This figure shows that the microspheres prepared with higher copolymer concentration and molecular weight showed a substantially lower burst release (~10%) than those of Fig. 2 (10-40%). A possible explanation for this decrease in burst release is that an increasing polymer concentration and molecular weight resulted in a decrease of the porosity of the microspheres (25, 26). Indeed, microspheres formulation F_7 were highly porous whereas those of F_8 were non-porous (SEM analysis, supporting information S_4). A quantitative release of BSA from microspheres of 50/50 copolymer in 20 days was observed. BSA was released in a sustained manner for 35 days from microspheres of copolymer 35/65 (acceleration of release around day 25, due to degradation of the particles; see Fig. 6 and its discussion in the text) while microspheres based on copolymer 25/75 showed a slow release for 40 days. Thereafter a faster release was observed between day 50 and 70, which is caused by degradation of the microspheres (Fig. 6).

The degradation controlled release of BSA from PLHMGA microspheres opens the possibility to have release for a prolonged time (not feasible with only

one type of microspheres) using cocktails of microspheres. As an example, the calculated release of BSA from a 1/1/1 mixture of 50/50, 35/65 and 25/75 is shown in Fig. 4. This figure shows that after a small burst, the protein is released with almost zero order kinetics for 65 days.

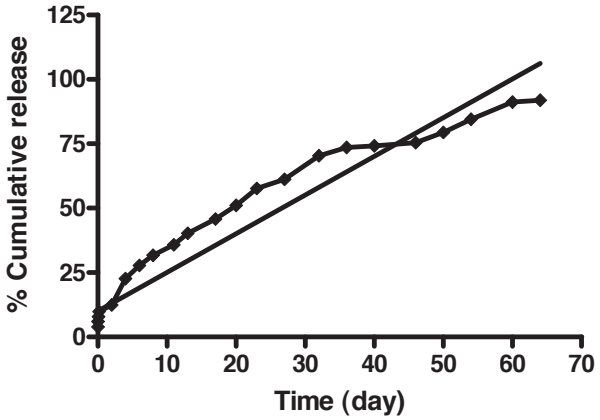

Fig. 4. Calculated release profile of BSA from a cocktail of microspheres (F_2, F_4, F_8, ratio 1/1/1). The straight line gives the zero-order release profile after a burst of 10%.

Fig. 5 shows the release of BSA from PLHMGA microspheres prepared with copolymers with different molecular weight.

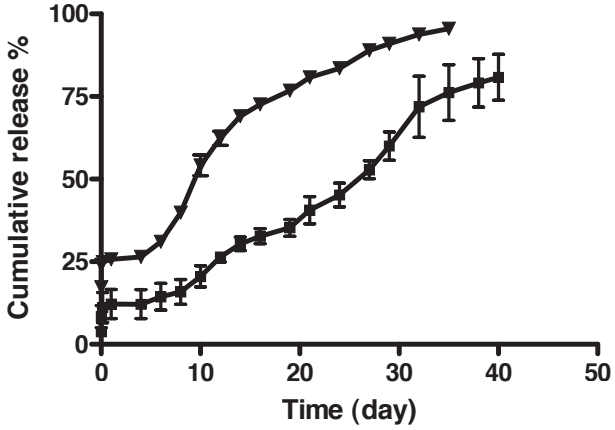

Fig. 5. BSA release from PLHMGA microspheres prepared from 35/65 copolymers (Table II; F_5 and F_6) with different M_n; (▼) 10 kg/mol (P_{13}, Table I) and (■) 19 kg/mol (P_{14}, Table I).

The microspheres prepared from copolymers with a rather low M_n (10 kg/mol) showed a burst around 30% followed by a sustained release for 30 days. Moreover, a quantitative release of BSA was observed in 35 days. Increasing the M_n to 19 kg/mol decreased the burst to 10% and thereafter a sustained release of BSA up to 80% was observed for 40 days. The decrease in burst release with increasing molecular weight of the copolymer used for the preparation of the microspheres has also been reported by other researchers (25,27) and is most likely caused by the decrease in porosity of the microspheres. However, differences in porosity using SEM could not be seen (supporting information S_5); likely the pores are smaller than detectable by SEM. The results also showed that there is hardly any effect of the polymer molecular weight on the duration of the release.

4.3.4. In vitro degradation of PLHMGA microspheres

It is generally accepted that the degradation of PLGA and related polymers proceeds via chemical hydrolysis of the ester bond (3,28,29); enzyme catalysis hardly contributes to the degradation of implants (30) and microspheres (31). It can therefore be expected that the degradation of PLHMGA microspheres is mainly controlled by hydrolytic degradation, given the resemblance in structure between this polymer and PLGA.

Microspheres of PLHMGA with different copolymer composition incubated in a phosphate buffer (155 mM, pH 7.4) at 37 °C showed a continuous weight loss in time (Fig. 6a). Microspheres of 50/50 copolymer completely degraded in less than 20 days, in agreement with Leemhuis et al. (32). Decreasing the BMMG ratio to 35% and 25% prolonged the complete degradation process to 30 and 55 days, respectively, again in line with previous findings (32). Fig. 6b shows that the M_n of the non-dissolved polymer decreased in time, which indicates that microspheres degrade via bulk erosion (17,32).

In contrast to our previous study (17) where insoluble residues composed of crystalline oligomers of lactic acid remained, the PLHMGA microspheres degraded completely. Because in the present study D,L- lactide instead of L-lactide was used to synthesize the copolymers, upon degradation amorphous D,L-lactic acid fragments are formed (DSC analysis, supporting information S_6) which in contrast to their crystalline counterparts are susceptible to further hydrolytic degradation (28,33).

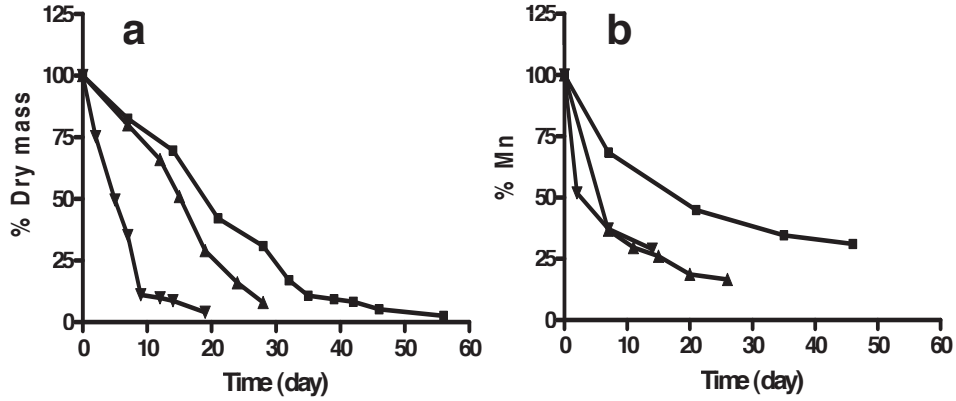

Fig. 6. (a) Relative mass decrease of the PLHMGA microspheres and (b) number average molecular weight (M_n) of PLHMGA as a function of time; The copolymer composition was 50/50 (▼), 35/65 (▲) and 25/75 (■).

Scanning electron micrographs of the PLHMGA microspheres revealed changes in microspheres morphology during degradation. Fig. 7 shows that the microspheres of the rapidly degrading 50/50 copolymer lost their spherical shape and became porous after 2 days of degradation; after 7 days mainly particle aggregates remained. SEM analysis at day 14 showed the presence of particle residues, whereas no residues were found at day 20. SEM pictures of microspheres composed of the 35/65 copolymer were partly fused at day 7, but they remained essentially non porous. As the degradation proceeded, they lost their spherical shape and were porous at the day 14. The particles were fully degraded at day 30. The microspheres based on the 25/75 copolymer slowly degraded. At day 14, the particles showed some porosity and they were completely degraded at day 60.

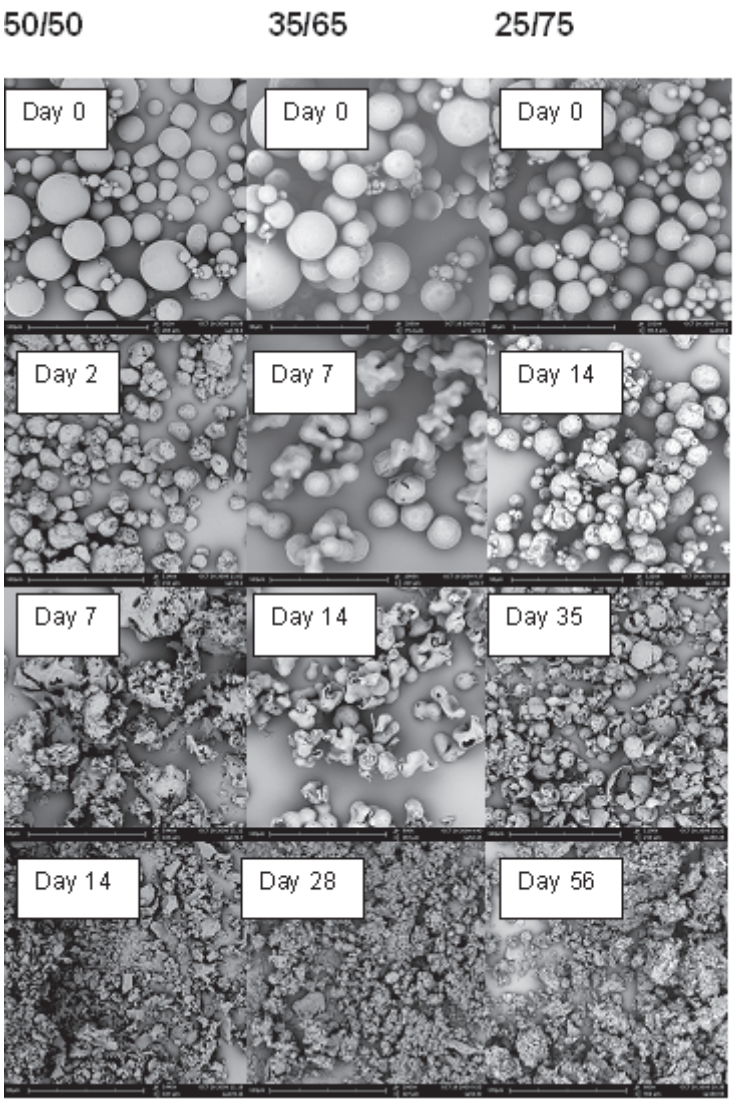

Fig. 7. Scanning electron micrographs of the PLHMGA microspheres during in vitro degradation.

The degradation of the PLHMGA microspheres prepared from copolymers with different M_n was studied. The microspheres showed a continuous weight loss in time (Fig. 8a) and they were completely degraded in 30 days. The mass loss was accompanied by a gradual decrease in molecular weight (Fig. 8b). The initial molecular weight of the copolymer had no effect on neither the kinetics of

mass decrease nor on molecular weight decrease. It can be expected that particles prepared of a higher molecular weight polymer degraded slower, because more ester bonds have to be hydrolyzed before soluble products are formed. Obviously, the molecular weight of our copolymers differs not sufficiently to result in clear different degradation kinetics.

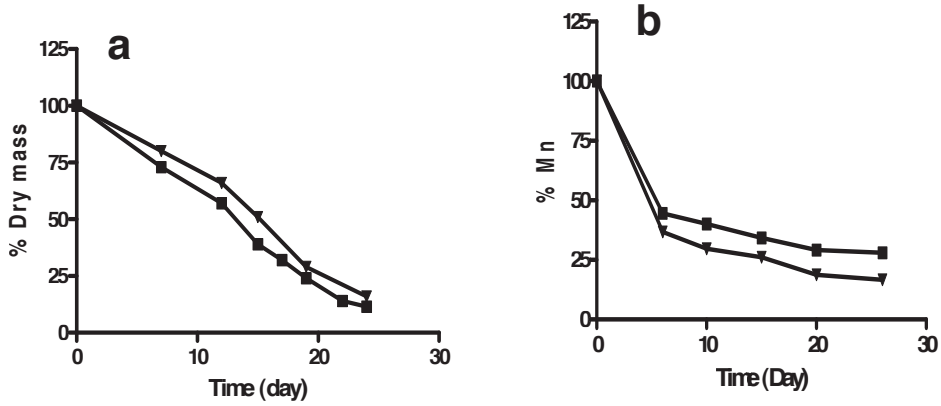

Fig. 8. (a) Relative weight decrease of PLHMGA microspheres prepared from copolymers with different molecular weights and (b) number average molecular weight decrease (M_n) of PLHMGA as a function of time; the M_n of the copolymers was 10 (■) and 19 (▼) kg/mol.

Combining the release (Fig. 2, 3 and 5) and the in vitro degradation data (Fig. 6 and 8), it can be concluded that the release of BSA is to a large extent governed by the degradation of the microparticles. Obviously once the protein molecules present in the pores of the particles are released (burst release), protein entrapped in the microsphere matrix has a very low mobility and is only released in the environment once the matrix has undergone substantial degradation.

4.3.5. Protein stability

The fluorescence emission spectrum of released BSA showed no difference in intensity and almost completely overlapped with that of native BSA (supporting information S_7). This means that no changes in the tertiary structure of the protein had occurred. Size exclusion chromatography analysis showed that the monomer/dimer ratio in released samples was the same as in freshly dissolved BSA. Further, extra peaks e.g. with retention times shorter than BSA dimer (corresponding to soluble aggregates) were not observed (supporting information S_8). This means that, although likely some insoluble BSA aggregates were

formed during release from the slowly degrading microspheres (Figure 3 and 5), the structural integrity of the released/soluble protein was fully preserved.

4.4. Conclusions

Microspheres prepared from PLHMGA showed, dependent on the copolymer composition a sustained release of BSA for 15 to 60 days. The microspheres are fully degradable and the degradation kinetics can be tailored by the copolymer composition. Increasing the BMMG content of the copolymer caused a faster degradation of the copolymer, which in turn resulted in a faster release of the encapsulated BSA. A quantitative release of BSA was observed from fast degrading microspheres (50/50 copolymer). However, 80% of BSA was recovered from microspheres composed of copolymer 35/65 and 25/75 which is possibly due to the formation of insoluble protein aggregates. Fluorescence spectroscopy and size exclusion chromatography showed that the released soluble BSA retained its structural integrity. These findings show that microspheres of PLHMGA are promising systems for the delivery of pharmaceutical proteins. In our future studies, we are focusing on suitable excipients for the stabilization of encapsulated proteins in order to achieve a complete release of proteins, particularly from the slowly degrading microspheres.

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Chapter 5

Controlled release of octreotide and assessment of peptide acylation from poly(D,L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres

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Abstract

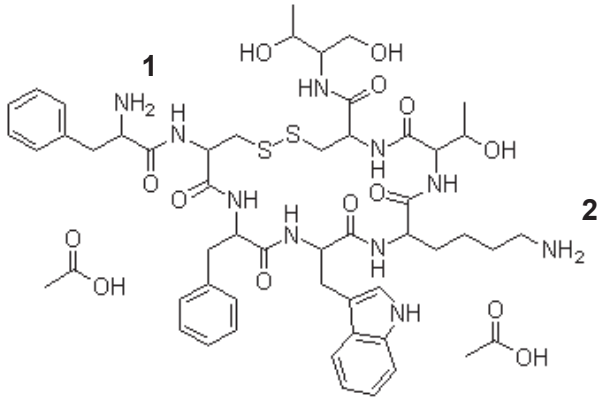
To investigate the in vitro release of octreotide acetate, a somatostatin agonist, from microspheres based on a hydrophilic polyester, poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA). Spherical and non-porous octreotide-loaded PLHMGA microspheres (12 to 16 μm) and loading efficiency of 60-70 % were prepared by a solvent evaporation technique. Octreotide release profiles were compared with that of the commercial PLGA formulation (Sandostatin LAR®) and possible peptide modification with lactic, glycolic and hydroxymethyl glycolic acid units was monitored. The PLHMGA microspheres showed a burst release (~20 %) followed by a sustained release for 20 to 60 days, depending on the hydrophilicity of the polymer. The percentage of loaded peptide that was released was high (70-90 %) and more than 60 % of released peptide was native octreotide. On the contrary, PLGA microspheres did not show peptide release for the first 10 days after which it was released in a sustained manner over the next 90 days and more than 75 % of the released peptides were acylated adducts. PLHMGA microspheres are promising controlled systems for peptides with excellent control over release kinetics. Moreover, substantially less peptide modification occurred in PLHMGA than in PLGA microspheres.

5.1. Introduction

Microspheres of biodegradable polymers with poly(D,L-lactide-co-glycolide) (PLGA) as main representative have been widely studied for the prolonged release of bioactive peptides (1-7). However, one of the issues in peptide delivery using PLGA formulations is the formation of peptide adducts as a result of acylation with lactic and glycolic units (8,9). Acylation of, particularly, lysine residues in peptides is catalyzed by the low pH that is generated inside degrading PLGA microspheres due to the accumulation of degradation products, i.e. lactic and glycolic acid and their oligomers (10-14). The investigated options to avoid chemical derivatization of peptides entrapped in PLGA matrices, such as PEGylation and co-encapsulation of water-soluble divalent cationic salts, resulted in less acylation (15-17). However, it is obvious that these approaches can not be generally applied and consequently other options need further exploration. Besides acylation, incomplete and difficult to tailor release of peptides from PLGA microparticles are other challenges hampering widespread clinical application of these peptide formulations (18,19). Within our Department, a new hydrophilic polyester, poly(lactide-co-hydroxymethyl glycolide) (PLHMGA), has been developed (20,21). Protein-loaded PLHMGA microspheres based on copolymers with different ratios of D,L-lactide and hydroxymethyl glycolide (HMG) (75/25, 65/35 and 50/50) were prepared using a double emulsion solvent evaporation method. Degradation of the microspheres and release of model proteins (lysozyme and BSA) was investigated. It was demonstrated that the release of model proteins was governed by degradation of the microspheres and that duration of the release could be tailored from 2 weeks to 2 months (22,23). Spectroscopic and chromatographic analysis as well as bioactivity measurements (lysozyme) showed that the released proteins retained their structural integrity.

In the present study we investigated the suitability of PLHMGA microspheres for the release of a therapeutic peptide, octreotide (structure shown in Fig. 1). This synthetic peptide mimics the peptide hormone somatostatin and has received FDA approval for the treatment of acromegaly (a condition in which excess amount of growth hormone is produced from the anterior pituitary) and gastroenteropancreatic neuroendocrine tumors (24,25). Because of its poor pharmacokinetics (plasma half-life of about 100 minutes following iv and sc injections (26,27)), sustained release octreotide formulations based on PLGA microspheres have been developed. One formulation named Sandostatin LAR® made with a PLGA-glucose star polymer is commercially available and used in clinics for the treatment of acromegaly and gastrointestinal tumors (28,29). However, previous studies with octreotide-PLGA microspheres showed an

incomplete and difficult to tailor release (30). Importantly, HPLC and mass spectrometric analysis demonstrated the presence of octreotide derivatives (lactoyl and glycoyl adducts) in the release samples (17,30). It was hypothesized that a nucleophilic attack of the primary amine groups present in the N-terminus and lysine residue of the peptide on the electrophilic carbonyl ester groups present in the PLGA backbone resulted in peptide acylation (9,31).



H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol

Fig. 1. Structure of octreotide acetate; acylation most likely occurs at position 1 and 2 (http://www.chemblink.com/products/83150-76-9.htm)

Due to steric factors, the nucleophilic attack of octreotide more readily occurred on glycolic acid rather than lactic acid units (9). Because PLHMGA lacks glycolic acid units, we hypothesized that octreotide encapsulated in PLHMGA microspheres is less susceptible to acylation. Moreover, the extra hydroxyl groups in PLHMGA increases the water absorbing capacity of the degrading polymer matrix and facilitates the release of acid degradation products, thereby preventing a pH drop (manuscript in preparation). It is further anticipated that, as previously shown for BSA (23), the release of the peptide can be tailored by the degradation kinetics of the polymer which in turn depends on the copolymer composition. Therefore, the aim of this study was to investigate PLHMGA microspheres of different composition for the controlled release of octreotide and compare the release kinetics with octreotide release from Sandostatin LAR®. Possible modification of the released peptide was investigated by HPLC and MALDI-TOF analysis.

5.2. Materials and Methods

5.2.1. Materials

O-Benzyl-L-serine was purchased from Senn Chemicals AG (Dielsdorf, Switzerland). D,L-lactide was obtained from Purac, the Netherlands. N,N´-Dimethylformamide and methyl-tert-butyl ether (MTBE), peptide grade dichloromethane (DCM), methanol, chloroform and tetrahydrofurane (THF) were purchased from Biosolve (Valkenswaard, the Netherlands). Benzyl alcohol was obtained from Merck (Darmstadt, Germany). Toluene (Acros, Geel, Belgium) was distilled from P_2O_5 and stored over 3 Å molecular sieves under argon. N,N´-Dimethylaminopyridine (DMAP) and sodium azide (NaN_3, 99 %) were purchased from Fluka (Zwijndrecht, the Netherlands). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) were obtained from Merck (Darmstadt, Germany). Polyvinyl alcohol (PVA; Mw 30,000-70,000; 88 % hydrolyzed) and tin (II) 2-ethylhexanoate ($SnOct_2$) were from Sigma-Aldrich, Inc., USA. Pd/C (Palladium, 10 wt % on activated carbon, Degussa type E101 NE/W) was purchased from Aldrich, Zwijndrecht, the Netherlands. Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; MW=1019 Da) was obtained from Feldan-bio (Quebec, Canada). Sandostatin LAR® (Batch No. S0090) was obtained from Novartis, Basel, Switzerland. According to the supplier, the loading capacity of this formulation was 20 mg peptide per 500 mg microspheres. Unless otherwise stated, all chemicals were used as received.

5.2.2. Synthesis of copolymers of 3S-(benzyloxymethyl)-6S-methyl-1,4-dioxane-2,5-dione with D,L-lactide

3S-(benzyloxymethyl)-6S-methyl-1,4-dioxane-2,5-dione (BMMG) was synthesized as described before by Leemhuis et al. (20). Copolymers of D,L-lactide and BMMG with different monomer ratios (75/25, 65/35 and 50/50 % D,L-lactide/BMMG mol/mol) were synthesized by melt copolymerization (Fig. 2) (20).

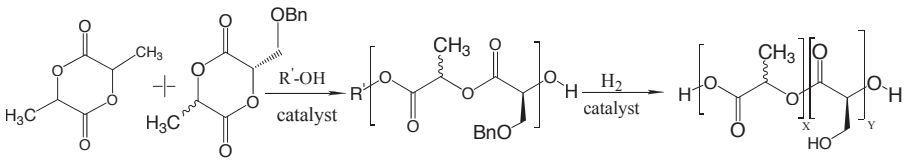


Fig. 2. Synthesis of hydrophilic aliphatic polyesters based on lactic acid and glycolic acid with pendant hydroxyl groups, poly (lactic-co-hydroxymethyl glycolic acid) (20).

As a representative example, for the 75/25 copolymer, D,L-lactide (1253 mg, 8.66 mmol) and BMMG (726 mg, 2.87 mmol) were transferred into a dried Schlenk tube under a dry nitrogen atmosphere. Benzyl alcohol (12.54 mg; 37 µl of a 352 mg/ml toluene stock solution) and $SnOct_2$ (23.5 mg; 71 µl of a 330 mg/ml toluene stock solution) were added as initiator and catalyst, respectively. Toluene was removed under vacuum and the tube was sealed and immersed into a 130 °C oil bath for 16 h while stirring. After cooling to room temperature, the obtained copolymer was dissolved in chloroform and subsequently precipitated in cold methanol. The precipitate was collected by filtration and vacuum dried to yield 1.86 g of poly(D,L-lactic acid-ran-benzyloxymethyl glycolic acid) (PLBMGA) as a white solid. The copolymer was dissolved in 300 ml distilled THF and 10 % w/w Pd/C was added. The solution was stirred under a hydrogen atmosphere for 16 h to remove the benzyl protecting group and the catalyst was subsequently removed by Hyflo filter. Evaporation of THF yielded 1.5 g of poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA). Copolymers of 65/35 and 50/50 (mol/mol D,L-lactide and BMMG) were synthesized using the same method by adjusting the amount of BMMG and D,L-lactide in the feed.

5.2.3. Polymer characterization

^1H NMR measurements of PLBMGA and PLHMGA dissolved in $CDCl_3$ were performed at 298 K on a Varian Gemini-300 MHz spectrometer. Molecular weights of the synthesized polymers were determined by GPC analysis (Waters Alliance equipped with a Waters 2695 separating module and a Waters 2414 refractive index detector). Two PL-gel 5 µm Mixed-D columns fitted with a guard column (Polymer Labs, M_w range 0.2-400 kDa) were used. Calibration was performed with polystyrene standards using THF as the mobile phase (1 ml/min). PLBMGA/PLHMGA was dissolved in THF (5 % w/v) and 50 µl was injected into the column (22). The thermal properties of the protected and deprotected copolymers were measured using differential scanning calorimetry (TA

instrument, Q2000) as described before (23). The second cycle was used to determine the glass transition temperature (T_g) of synthesized polymers.

5.2.4. Preparation of octreotide microspheres

Microspheres were prepared using a solvent evaporation technique (22,32). In an optimized protocol, 50 µl of an octreotide solution in reversed osmosis (RO) water (200 mg/ml) was emulsified with 500 µl of dichloromethane solution of PLHMGA (220 mg, 25 % w/w) by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 seconds at the highest speed (30000 rpm) to get the primary emulsion. Next, 500 µl of a PVA solution (1 % w/w in 30 mM phosphate buffer, pH 7.4) was added slowly and the mixture was vortexed for 30 seconds at 30000 rpm. The obtained w/o/w emulsion was then transferred into a 5 ml PVA solution 0.5 % (w/w) in 30 mM phosphate buffer pH 7.4 while stirring. Continuous stirring at room temperature for 2 hours resulted in extraction/evaporation of DCM. Finally, hardened microspheres were collected by centrifugation (Laboratory centrifuge, 4 K 15 Germany) at 3000 g for 3 min, subsequently washed 3 times with 100 ml RO water and freeze dried overnight. The dried microspheres were stored at -25 °C. Microspheres were also prepared with other formulation and processing parameters (see supporting information).

5.2.5. Microspheres characterization

The microspheres size distribution was analyzed by an Accusizerth 780 (Optical particle sizer, Santa Barbara, California, USA). Results are reported as volumetric mean diameter.

The morphology of the microspheres after freeze-drying was analyzed by scanning electron microscopy using a Phenom^{TM} SEM (FEI Company, the Netherlands). The samples were mounted onto a 12 mm diameter aluminum specimen stub (Agar Scientific Ltd., England) using double-sided adhesive tape and were sputter coated with platinum.

The octreotide loading of the microspheres (PLHMGA as well as the PLGA/Sandostatin LAR® formulation) was determined using an HPLC method according to Wang et al. (33). In detail, about 10 mg of PLHMGA microspheres were dissolved in 0.5 ml THF with gentle shaking. Thereafter, 2 ml of diluent (0.2 % w/v glacial acetic acid, 0.2 % w/v sodium acetate and 0.7 % w/v sodium chloride in water) was added to precipitate the polymer. Next, the mixture was

incubated at room temperature for 20 min and the precipitated polymer was spun down by centrifugation at 5000 g for 1 min. The protein content in the supernatant was measured by high performance liquid chromatography (HPLC) using a Prosphere HP C4-300 column. A gradient elution method was used with a mobile phase A (95 % H_2O, 5 % ACN and 0.1 % TFA) and a mobile phase B (100 % ACN and 0.1 % TFA). The eluent linearly changed from 100 % to 40 % A in 20 min with a flow rate of 1 ml/min. Octreotide standards (5-200 μg/ml, 20 μl injection volume) were used for calibration and detection was done at 210 nm. Loading efficiency (LE) of octreotide in microspheres is reported as the encapsulated peptide divided by the total amount of peptide used for encapsulation. Loading capacity (LC) is expressed as the encapsulated amount of octreotide divided by the total dry weight of the microspheres.

5.2.6. In vitro release studies

The release of octreotide from PLHMGA and Sandostatin LAR® microspheres was studied in PBS (0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05 % (w/w) NaN_3, pH 7.4). About 30 mg of microspheres was suspended into 1.5 ml of PBS buffer in eppendorf tubes and incubated at 37 °C under mild agitation using a circular mixer (ASSISTENT RM5). At the different time points, the dispersion was centrifuged (3000 g, 3 min) and 1 ml of the supernatant was replaced with 1 ml of fresh buffer. The microspheres were resuspended by gentle shaking and the dispersion was incubated at 37 °C. The peptide concentration in the release samples was measured by HPLC as described in section 2.5.3.

5.2.7. MALDI-TOF analysis

Different release samples were analyzed using MALDI-TOF (Applied Biosystems 4700, Foster City, CA, equipped with a 200 Hz Nd:YAG laser operating at 355 nm). Samples were diluted to 5 μg/ml in a solution of 3 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 1:1 acetonitrile/MilliQ (v/v) containing 0.1 % (v/v) trifluoro acetic acid. The sample (about 1 μl) was spotted onto a stainless steel matrix-assisted laser desorption/ionization (MALDI) plate. Analysis was performed in a reflectron positive ion mode and typically, 3000 shots/spectrum were acquired in the MS mode.

5.3. Results and Discussion

5.3.1. Synthesis and characterization of PLHMGA with different copolymer composition

Benzyl protected hydroxymethyl glycolide (BMMG) and D,L-lactide were copolymerized by ring opening polymerization at different feed ratios (75/25, 65/35 and 50/50 mol D,L-lactide/mol BMMG) in the melt at 130 °C using BnOH and $SnOct_2$ as initiator and catalyst, respectively. The protected copolymers (PLBMGA) were obtained in high yields (>90 %) and NMR analysis showed that the copolymer composition closely matched that of the feed (Table I). DSC analysis showed that the copolymers were fully amorphous with a T_g ranging from 33 to 41 °C (Table I), which is in agreement with previous findings (20,22,23). NMR analysis showed that catalytic hydrogenation of PLBMGA resulted in quantitative removal of the protecting benzyl groups to obtain PLHMGAs in a high yield. As for the protected polymers, PLHMGA of different composition were fully amorphous with a T_g of 47-49 °C (Table I). GPC analysis showed that the number average molecular weights of synthesized polymers before and after deprotection ranged from 13-24 kg/mol. The molecular weight distributions were similar (M_w/M_n ~2) to those of polyesters synthesized by ring opening polymerization of lactide/glycolide in the melt (34).

Table I. Characteristics of PLBMGA and PLHMGA used in this study.

polymer	feed ratio D,L[a]/M[b]	copolymer composition (NMR)[c]	M_n (kg/mol)	M_w (kg/mol)	T_g (°C)
PLBMGA	75/25	78/22 (11)	16	35	36
	65/35	70/30 (15)	24	51	41
	50/50	57/43 (21.5)	19	35	33
PLHMGA	75/25	80/20 (10)	13	30	49
	65/35	69/31 (15.5)	22	45	47
	50/50	51/49 (24.5)	17	31	48

[a]D,L = D,L-lactide
[b]M = Monomer BMMG (benzyloxymethyl methyl glycolide)
[c]The numbers in brackets are correspondent to either BMGA or HMGA

5.3.2. Preparation and characterization of octreotide-loaded microspheres

Octreotide-loaded microspheres were prepared using a double emulsion/solvent evaporation technique as described in section 2.4. The characteristics of the microspheres are summarized in table II.

Table II. Characteristics of octreotide-loaded microspheres (n=3) prepared using PLHMGAs of different composition and the commercial PLGA formulation (Sandostatin LAR®).

polymer	copolymer composition	volume weight mean diameter (μm)	LE[a] (%)	LC[b] (%)
Poly(D,L-lactide-co-hydroxymethyl glycolide)	75/25	11.6±2.2	57.8±3.5	3.2±0.2
	65/35	15.6±0.6	57.1±2.6	3.5±0.2
	50/50	12.6±1.5	67.1± 0.3	3.9±0.0
[c]PLGA (Sandostatin LAR®)	50/50	65.0	[d]N.A.	4.1±0.1

[a] LE = Loading efficiency, [b] LC = Loading capacity
[c] The results are from one single batch; LC was determined in triplicate
[d] N.A. = data not available

Table II shows that the PLHMGA microspheres had about the same size that ranged from 12-16 μm. The effect of several formulation parameters, which are known to have a possible influence on the loading efficiency, was investigated (polymer concentration (35), volume of the protein solution (36), addition of salt to the continuous phase (37), volume of continuous phase (38), use of methanol as solvent for octreotide (o/w method) (8) and buffering the pH of the PVA phase at pH 7.4). The results presented in the supplementary information show that the LE was low (<30 %) except for the formulation that was prepared with a neutralized external phase (LE was 50-60 %, Table II). Likely, the driving force for extraction of octreotide from the internal to the external aqueous phase during formation of the particles is low at pH 7.4 (which is close to the pI of octreotide (8.5)) because the aqueous solubility of the peptide is low, resulting in an enhanced loading efficiency and capacity. The LE was independent of copolymer composition and is in agreement with previous findings showing around the same LE of octreotide in PLGA microspheres (33). The LC of the Sandostatin LAR® was 4.1 ± 0.1 which is within the experimental error equal to the load as specified by the supplier (20 mg peptide per 500 mg formulation). SEM analysis

of the different microspheres of table II showed that the particles are spherical and essentially non-porous (Fig. 3) and the average size by SEM corresponded well with that of accusizer measurements (Table II). SEM analysis of Sandostatin LAR® shows particles with a bigger size than the PLHMGA microspheres, in agreement with accusizer measurements (average size around 65 µm, Table II). Besides, SEM analysis also showed the presence of some smaller irregularly shaped particles in Sandostatin LAR® (Fig. 3d).

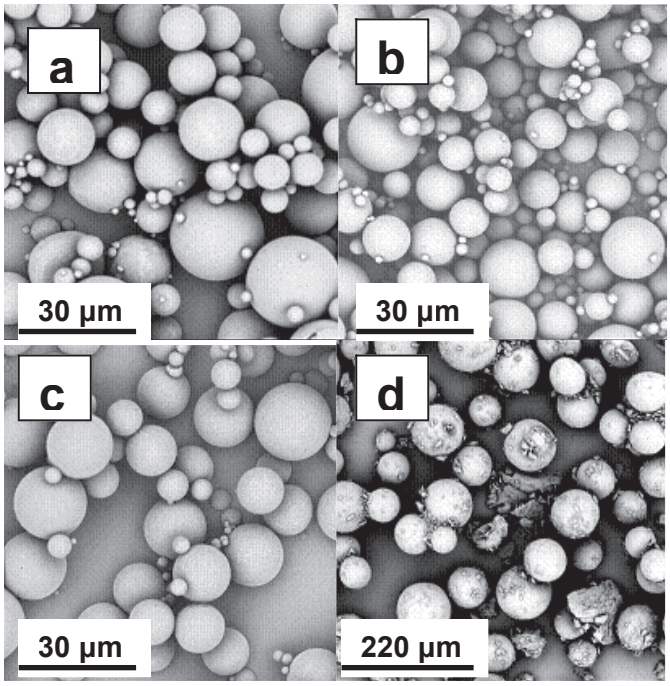


Fig. 3. SEM analysis of microspheres; (a) PLHMGA 75/25, (b) PLHMGA 65/35, (c) PLHMGA 50/50 and (d) Sandostatin LAR® (mark the difference in scale bar between Fig d and Fig a-c).

5.3.3. In vitro release of octreotide-loaded PLGA/PLHMGA microspheres

The HPLC chromatogram of octreotide in PBS (pH 7.4) incubated at 37 °C for 21 days showed a sharp peak with a retention time around 10 min (Fig. 4a). No other peaks were detected demonstrating that octreotide has a good stability under the conditions applied for evaluation of the release of the different octreotide formulations. However, octreotide released from PLHMGA and

PLGA microspheres showed extra peaks with longer retention times (11-12 minutes) originating from acylated octreotide adducts (see MALDI-TOF Analysis section) as also observed by Murty et al. (30). It is remarked that the extent of peptide modification is more pronounced after being released from PLGA than from PLHMGA microspheres (compare Fig. 4b, 4c). At the end of release study when the microspheres were fully degraded (day 95 for PLGA microspheres and day 60 for PLHMGA 65/35 microspheres, respectively), mainly acylated peptide was released from PLGA microspheres, whereas mainly native octreotide was released from PLHMGA microspheres (supplementary information 2 and 3, respectively). It should be stressed that acylated octreotide adducts are formed during degradation of the microspheres and not during their preparation, since only native peptide was detected with HPLC when the loading capacity of the different microspheres was determined (data not shown).

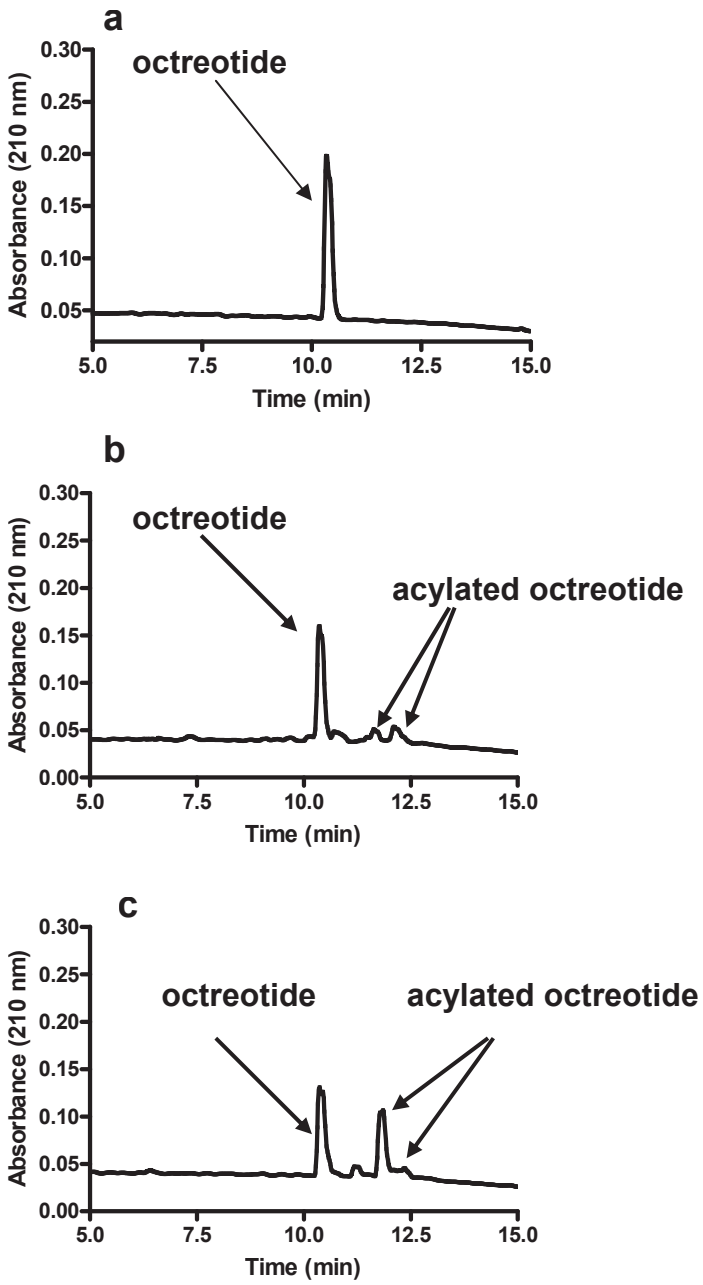


Fig. 4. HPLC chromatograms of octreotide after 21 days incubation in PBS pH 7.4 at 37 °C (a) and after release from microspheres of PLHMGA (65/35, b) and PLGA (c) after 21 days.

Fig. 5 shows the in vitro release of octreotide from PLGA microspheres in PBS pH 7.4 during 95 days. After 95 days the PLGA microspheres were fully degraded, in accordance to what has been reported by Spenlehauer et al. (39). The formulation showed no burst release and after 10-15 days peptide release started which continued for the next 80 days until a plateau value was reached at 85 % of the octreotide loading. The incomplete release can be due to the formation of insoluble peptide residues (not visible with naked eye) or to adsorption of the peptide to surfaces with which it has been brought in contact. HPLC analysis (see Fig. 4) makes it possible to differentiate between octreotide that is released in its native form and in acylated forms. Of the released peptide, 25 % was unmodified octreotide whereas the remaining 75 % were acylated products (Fig. 5, Table III).

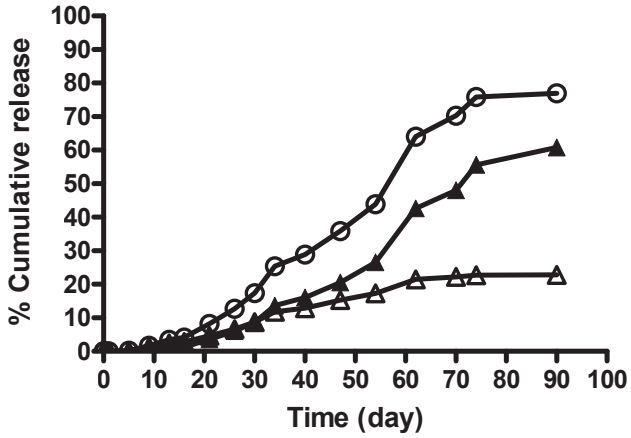


Fig. 5. In vitro release of octreotide from PLGA (the results are from one study); (Δ) native octreotide, (▲) acylated octreotide and (○) total octreotide (native and acylated octreotide adducts). The percentage of release is based on the LC of PLGA microspheres detected by HPLC (Table II).

The release of octreotide from microspheres based on PLHMGA of different hydrophilicity (different copolymer composition (75/25, 65/35 and 50/50 mol/mol D,L-lactide and BMMG (after deprotection: HMG)) was studied. The results are shown in Fig. 6 and in the supplementary information 4; the data are summarized in table III. Fig. 6 shows the release of octreotide from microspheres of PLHMGA 65/35. It appears that after a burst (around 20 % of the loading), which is probably caused by nanopores present in the microspheres that are not detected with SEM, octreotide was released in a sustained manner for 60 days and almost quantitative release (80-90 %) was obtained. Although also acylated forms of the peptide were released, more than 65 % of the released amount was

native octreotide while PLGA microspheres mainly released acylated octreotide (Table III). Importantly, octreotide-loaded PLHMGA microspheres were fully degraded after 35 days, in line with our previous study (23). The explanation for the continuation of the release after visible microsphere degradation will be given below (vide infra).

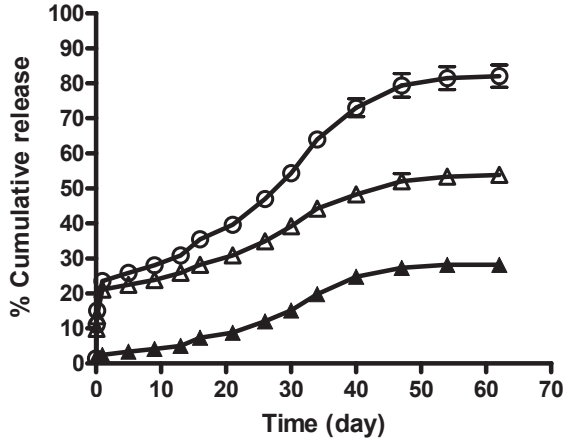


Fig. 6. In vitro release of octreotide from PLHMGA microspheres (65/35) in PBS pH 7.4 at 37 °C; (▲) acylated octreotide, (Δ) native octreotide and (○) total octreotide release (native and acylated octreotide adducts) (n=3).

Table III. Summary of in vitro release of octreotide-loaded PLHMGA microspheres of different composition (n=3) and PLGA microspheres in PBS (pH 7.4) at 37 °C.

Batch	% of the loaded amount released	% burst release [a]	% native octreotide[c]	microspheres degradation (days)	duration of release
PLHMGA 75/25	69.6±7.0	18.5±0.5	74.5±0.3	55	75
PLHMGA 65/35	82.1±5.0	21.1±0.2	65.6±1.5	35	60
PLHMGA 50/50	84.5±4.0	23.7±0.3	68.3±3.2	20	20
[b]PLGA	85	no	25	90	95

[a] defined as the amount released after 24 hours
[b] results are from a single batch
[c] defined with respect to the total octreotide that was released

Microspheres of copolymer 50/50 showed, after a burst (around 20 % of the loaded amount), an almost zero order release of the peptide for 20 days until 85 % of the loaded amount was released (of which 70 % is native octreotide and the remaining 30 % is in the form of acylated peptide; supplementary information 4 and Table III). Complete degradation of the microspheres occurred in 20 days. Octreotide release from microspheres of the more slowly degrading copolymer 75/25 (supplementary information 4) was characterized by a burst (around 20 % of the loaded amount) followed by a sustained release for more than 60 days. In line with the results of PLHMGA 65/35 and 50/50 microspheres around 70 % of the released peptide was native octreotide whereas 30 % were acylated adducts. PLHMGA 75/25 microspheres degraded completely in 55 days. There is a tendency that the total released amount of peptide decreases with increasing degradation time of the particles. This might be due to the presence of polymer-peptide conjugates in the release medium that are not detected with the HPLC method (see below, text accompanying Fig. 7). Also adsorption of the peptide to surfaces to which it has been exposed for longer times with increasing degradation time might be responsible for this observation.

In our previous studies, the release of model proteins lysozyme and BSA from microspheres of copolymer 50/50, 65/35 and 75/25 was reported. It was concluded that the release of these proteins was essentially governed by degradation of the polymer matrices (22,23). The present results show that degradation governs to some extent the release of the peptide as well (the faster the degradation of the particles, the faster its release). Probably also diffusion plays a role, given the observation that, after the burst, the PLHMGA microspheres released the peptide without delay (as opposed to the protein delivery studies). Obviously, the hydroxyl groups of the polymer give sufficient water absorption to release the peptide by diffusion.

In the present study we observed that after complete degradation of the microspheres (at day 35 for the PLHMGA 65/35 microspheres, Fig. 6) the release of octreotide still continued (from 62 % of the loading at day 35 to 80 % at day 60). This can be explained as follows. The reaction of the copolymer and a nucleophile (e.g. lysine residue) of the peptide results in the formation of a peptide-polymer conjugate (step 1, Fig. 7). Likely, these polymer-peptide conjugates have either limited solubility or are not detected by the used HPLC method (30) due to their heterogeneous character. The peptide-polymer conjugates may still be present at the time the microspheres are fully degraded and are slowly converted by hydrolysis during the next days into peptide-LA/HMGA adducts (Fig. 7, step 2), which are detected with HPLC explaining the apparent increase in release after degradation of the microspheres (the apparent increase in total percentage of release is thus due to acylated peptide

adducts). The observation that after full microspheres degradation the amount of released native peptide indeed hardly increased supports the above mentioned explanation.

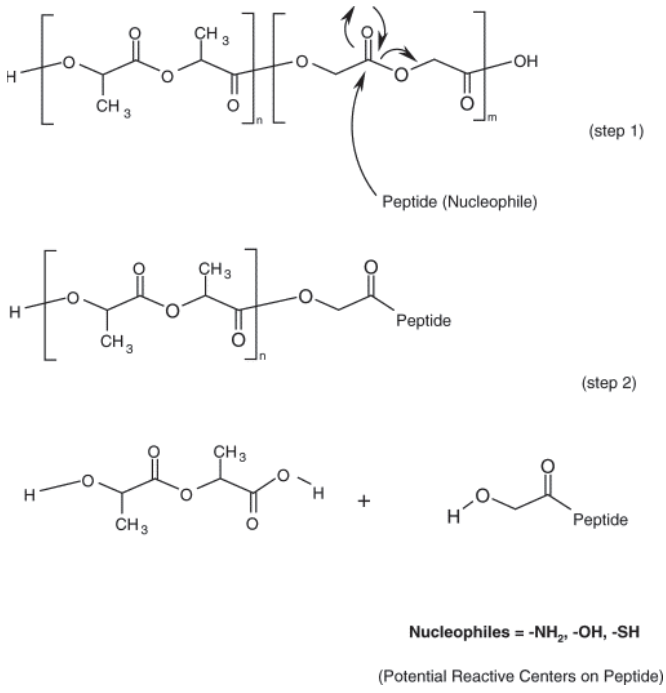


Fig. 7. Proposed mechanism of reaction between peptide and PLGA (18,40); the same mechanism is also valid for the reaction of a peptide with PLHMGA

5.3.4. MALDI-TOF analysis

The structure of the released octreotide adducts was investigated by MALDI-TOF analysis. Fig. 8a shows the mass spectrum of the native octreotide with only peaks corresponding with octreotide H+ (1019 m/z) and that of the Na-adduct (1041 m/z). Fig. 8b and 8c show the mass spectra of octreotide released after 21 days from PLGA and PLHMGA microspheres, respectively. Clearly, besides native octreotide, also peaks with higher masses were detected. In agreement with HPLC analysis (vide supra), more extensive peptide derivatization is seen in octreotide released from PLGA than from PLHMGA microspheres. MALDI-

TOF spectra of the octreotide released from microspheres based on PLHMGA of different copolymer composition were similar.

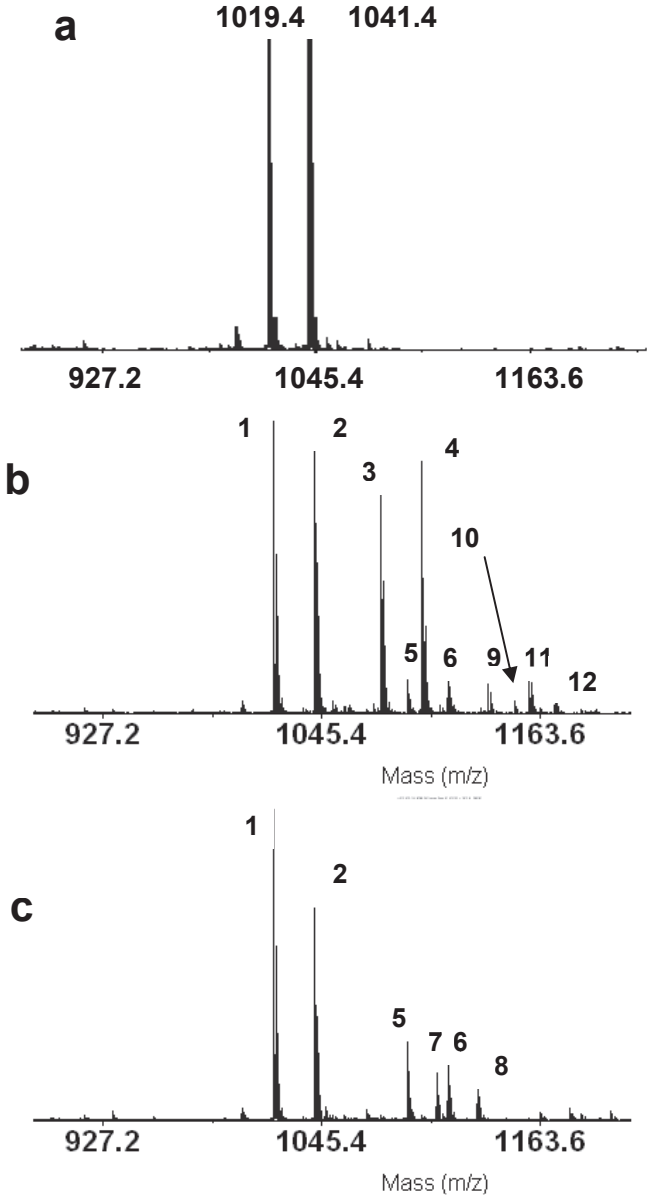


Fig. 8. Mass spectrum of octreotide acetate after incubation of 21 days in PBS at 37 °C (a), after 21 days of release from Sandostatin LAR® (b) and PLHMGA microspheres of copolymer 65/35 (c).

The attribution of the MALDI-TOF MS peaks to different octreotide derivatives is summarized in table IV. The masses of the observed peaks of octreotide released from PLGA microspheres can be fully ascribed to peptide modified with lactic (octreotide-LA) and glycolic acid (octreotide-GA) units. These products are formed as suggested by Na et al.(15) and Zhang et al.(41). The relative amounts of octreotide-GA (peak numbers 3 and 4, Fig. 8b) was substantially greater (height ratio about 7/1) than octreotide-LA (peak numbers 5 and 6, Fig. 8b). This can be explained because the nucleophilic attack is more hindered with lactic acid monomers as compared with glycolic acid monomers (Fig. 9) (8).

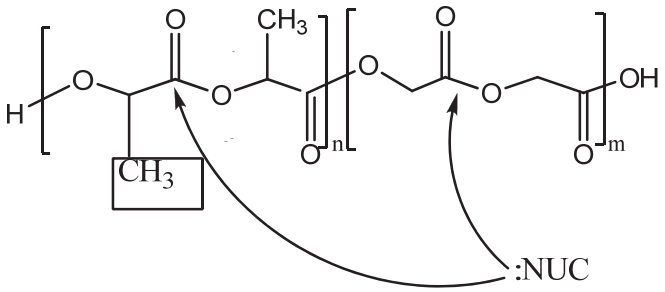


Fig. 9. Proposed explanation of reduced reactivity of nucleophilic species with lactic acid monomers (8).

Table IV. Acylated octreotide adducts detected by MALDI-TOF MS in release samples of PLHMGA and PLGA microspheres (15,41).

Peak number	Observed m/z	Assigned structure (octreotide released from PLGA)	Assigned structure (octreotide released from PLHMGA)
1,2	1019, 1041	Octreotide, Octreotide-Na	Octreotide, Octreotide-Na
3,4	1077, 1099	[a]Octreotide-GA, Octreotide-GA-Na	[c]N.D.
5,6	1091, 1113	[b]Octreotide-LA, Octreotide-Na-LA	Octreotide-LA, Octerotide-Na-LA
7,8	1107, 1129	N.D.	[d]Octreotide-HMGA, Octreotide-HMGA-Na
9,11	1135, 1157	Octreotide-GA-GA, Octreotide-Na-GA-GA	N.D.
10,12	1149, 1171	Octreotide-LA-GA, Octreotide-Na-LA-GA	N.D.

[a]Octreotide-GA = octreotide-glycoyl adduct
[b]Octreotide-LA =octreotide-lactoyl adduct
[c]N.D.= not detected
[d]Octreotide-HMGA = octreotide-hydroxymethyl glycoyl adduct

No octreotide-GA was detected in octreotide released from PLHMGA microspheres due to the lack of glycolic acid units in PLHMGA, while minor amounts of octreotide-HMGA were observed instead. The ratio of octreotide-LA to octreotide-HMGA was 1.6, 1.2 and 1.3 for copolymer 75/25, 65/35 and 50/50, respectively. The 50/50 copolymer contains 75 % lactic acid units and 25 % HMGA units (Fig. 2). This means that if both units are equally reactive with the peptide, a 3/1 ratio octreotide-LA to octreotide-HMGA can be expected (for the peptide released from the other PLHMGA microspheres, this ratio would be even higher). Further it should be mentioned that in time the degrading polymers become richer in lactate content (21,22) which would point to an even higher expected ratio of octreotide-LA to octreotide-HMGA. It is therefore concluded that acylation of octreotide preferentially occurs by reaction with the HMGA

esters. Likely, this monomer unit due to its hydroxyl group is more hydrated than the lactic acid units in the polymer chain, favoring reaction with the peptide.

Based on the results (Fig. 5, peptide release from PLGA, Fig. 6 peptide release from PLHMGA, Fig. 8 and Table IV) it can be concluded that less peptide acylation occurs during release from PLHMGA microspheres compared to that of PLGA microspheres. Three factors might contribute to the lower extent of peptide acylation in degrading PLHMGA microspheres. Firstly, as mentioned above, the absence of the more reactive glycolic acid units in PLHMGA slows down the acylation reaction (9). Secondly, the increased degradation rate promotes the removal of the HMGA units and thus reduces the adduct formation. Thirdly, it has been shown that the low pH generated inside degrading PLGA matrices catalyzes the formation of acylated peptide adducts (40). However, less acidification of PLHMGA matrices occurs during degradation compared to that of PLGA matrices (manuscript in preparation) likely due to the more hydrophilic character of degrading PLHMGA matrices which favors the extraction of formed water-soluble acidic degradation products.

5.4. Conclusions

Our data demonstrate that the release of octreotide from PLHMGA microspheres can be tailored by the copolymer composition and outperformed that of the commercial octreotide formulation Sandostatin LAR®. Importantly, substantially less acylated octreotide adducts were formed in degrading PLHMGA microspheres than in Sandostatin LAR® due to the lack of glycolic acid and less acidification. To further decrease the acylation, PEGylation of octreotide or co-encapsulation of excipients which can prevent the nucleophilic attack of a peptide to the polymer backbone ((17,41)) are logical options that warrant further investigation.

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Chapter 6

Investigation of pH distribution in degrading microspheres of hydrophilic poly(D,L-lactide-co-hydroxymethyl glycolide) and PLGA by confocal laser scanning microscopy

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Abstract

The microclimate pH (μpH) distribution inside microspheres of a hydrophilic polyester, poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA), was investigated quantitatively and compared to that of PLGA microspheres. Lysosensor yellow/blue® dextran was used as a pH sensitive fluorescent probe to study μpH distribution kinetics inside PLHMGA microspheres during incubation under physiological conditions using a ratiometric method by confocal laser scanning microscopy. The effect of hydroxymethyl glycolide (HMG) ratio (35 and 25 %) and concentration of PLHMGA solution used for the preparation of the microspheres were evaluated. Confocal images revealed that PLHMGA microspheres, regardless of copolymer composition, developed less acidic μpH during 4 weeks of incubation compared to PLGA microspheres. Increasing the polymer concentration from 25 % (w/w) to 35 % (w/w) for PLHMGA 75/25 (D,L-lactide/HMG) showed a decrease in μpH during the first two weeks of study and as the degradation continued, the μpH increased to above 5.8 due to the release of acid degradation products. PLGA microspheres showed acidic μpH as low as 4 through the total 4 weeks of study. This study shows that PLHMGA microspheres are less susceptible to acidification during degradation as compared to PLGA. This might be ascribed to the more hydrophilic character of PLHMGA which increases the water-absorbing capacity of microspheres made of the polymer resulting in a faster release of the formed acid degradation products. PLHMGA systems are therefore suitable to release acid labile molecules such as peptide/protein pharmaceuticals.

6.1. Introduction

Poly(D,L-lactic-co-glycolic acid) (PLGA) is a biodegradable and biocompatible aliphatic polyester that has been widely investigated for controlled delivery of peptides, proteins and vaccine antigens (1-7). PLGA degrades in aqueous medium via hydrolysis of ester bounds connecting the monomer units in the polymer chain and the final degradation products are lactic and glycolic acid (8). A major drawback of PLGA systems is the accumulation of acid degradation products inside degrading matrices (9,10) which is associated with a drop in microclimate pH triggering some unwanted chemical reactions such as acylation and oxidation or inducing aggregation of acid labile molecules (e.g. proteins) loaded into PLGA matrices (11,12). The use of alkaline salts such as magnesium carbonate, magnesium hydroxide and zinc carbonate has been investigated to prevent the drop of pH in microspheres of protein-loaded PLGA and to enhance protein stability and release (3,13-16). Although co-encapsulation of these additives has shown to improve protein stability and release kinetics (17), the release of peptide/protein drugs from PLGA systems is still incomplete and difficult to be tailored (18,19). Recently, polyesters with functional pendant hydroxyl groups have been developed and showed attractive degradation and release properties for drug delivery purposes (20-23). Poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA) in particular, showed tailorable degradation kinetics and release of proteins from PLHMGA microspheres was governed by degradation of the microspheres (20,22). The introduction of hydroxyl groups in the backbone of the PLHMGA makes it more hydrophilic than PLGA and as a consequence, PLHMGA microspheres have a higher water absorbing capacity than their PLGA counterparts. This might allow extraction of the formed acid degrading products which in turn will prevent the drop of μpH and subsequently improve the stability of encapsulated peptides/proteins. Therefore, to have a better insight into the μpH distribution of PLHMGA microspheres, in the present study we monitored μpH changes during degradation of the microspheres by confocal laser scanning microscopy. The μpH drop in PLGA degrading microspheres has been shown by several techniques including electron paramagnetic resonance (EPR), using pH-sensitive spin probes (24), potentiometric titration (25) and confocal laser scanning microscopy using pH sensitive fluorescent probes (10). The first two techniques have some limitations. For instance, viscosity changes in the microenvironment can affect the EPR measurements and correlation between pH measured by an electrode and the pH inside of the microspheres is difficult (10). Therefore, in the present study confocal laser scanning microscopy was used as a tool to monitor possible μpH changes in degrading PLHMGA microspheres of different

copolymer composition as well as prepared from solutions of the different polymer concentration and compared to that of PLGA microspheres.

6.2. Materials and Methods

6.2.1. Materials

Poly(D,L-lactide-co-hydroxymethyl glycolide)s with copolymer ratios of 65/35 and 75/25 (mol D,L-lactide/mol BMMG feed ratio, after deprotection 87.5 mol lactic acid/ 12.5 mol HMG) were synthesized as described before (20,23). Poly(D,L-lactide-co-glycolide), end capped, 50/50 with an inherent viscosity (i.v.) of 0.19 dl/g (M_n=19 kg/mol) was generously provided by Alkermes Inc. (Cambridge, MA). Polyvinyl alcohol (PVA, 9-10 kDa, 80 % hydrolyzed) was from Polysciences (Warrington, PA) and the fluorescent pH sensitive probe Lysosensor yellow/blue® dextran (M_w=10 kg/mol) was purchased from Invitrogen (Eugene, OR). Unless otherwise stated, all chemicals were used as received.

6.2.2. Preparation of microspheres

Dextran conjugated Lysosensor yellow/blue® as an acidic pH-sensitive probe was encapsulated in PLHMGA and PLGA microspheres by a double emulsion evaporation technique as described before (22). Briefly, 125 µl of dye solution (12 mg/ml) was added to a polymer solution with 350 mg of PLHMGA/PLGA in 500 µl DCM (35 % w/w). The mixture was homogenized (Virtis IQ^2, Gardiner, NY) at 20000 rpm for 30s to form the w/o emulsion. Next, 500 µl of an aqueous PVA solution (1 % w/w) was slowly added to the first emulsion and a w/o/w was formed by homogenizing the mixture at 20000 rpm for 30s. The prepared w/o/w was slowly transferred into a 5 ml of an aqueous PVA solution (0.5 % w/w) and stirred at room temperature for 2 hours to extract and evaporate DCM. The formed microspheres were sieved for 20-45 µm size and washed three times with 100 ml reverse osmosis (RO) water and thereafter freeze dried on a Labconco freeze dryer (Kansasa City, Co).

Microspheres of copolymer PLHMGA 75/25 from three different polymer concentrations (25, 30 and 35 % w/w) were also prepared following the same procedure.

6.2.3. Scanning electron microscopy (SEM)

The morphology of the microspheres was studied using a Hitachi S3200 scanning electron microscope (SEM, Hitachi Ltd., Tokyo, Japan). Approximately 1-2 mg of lyophilized microspheres was evenly sprinkled onto a brass stub with double-adhesive conductive tape. Samples were sputter coated with gold under vacuum using a Denton Vacuum DESK II sputter coater (Moorestown, NJ). The images of microspheres were taken at an excitation voltage of 15.0 kV.

6.2.4. Confocal laser scanning microscopy

A ratiometric method was developed as essentially described by Ding et al.(10). A Carl Zeiss LSM 510 confocal laser scanning microscope (CLSM, Carl Zeiss Microimaging, Inc., Thornwood, NY) was equipped with an enterprise UV laser and a Carl Zeiss inverted Axiovert 100 M microscope. The laser power was set at 150 μW and the detection gain was set at 650 nm. The excitation was at 364 nm and two filters (450 nm and 520 nm) were used in combination with a Plan-Neofluar 60x water immersion objectives lens with numerical aperture of 1.2 to build images. The image size was 512x512 pixels and the images were scanned by 8 bit plane mode at a scan speed of 6.40 μs/pixel.

6.2.5. Standard curve of fluorescent intensity ratio vs. pH

Buffers of pH from 2.8 to 5.8 were prepared using 0.1 M citric acid solutions and 0.2 M Na_2HPO_4 solutions. Lysosensor yellow/blue® dextran was dissolved in buffer solutions with concentration of 0.8, 1.2, and 2.0 mg/ml. The standard curve was established by plotting the fluorescent intensity ratio of the dye solutions under two emission wavelengths, 450 nm and 520 nm, versus pH of that solution. The excitation wave length was at 364 nm.

6.2.6. Determination of water-soluble acids in PLHMGA and PLGA

200 mg of PLHMGA or PLGA were dissolved in 1 ml chloroform before adding 5 mL of double-distilled water. After a mild vortex mixing, the two phase system was left for 10 min and then was centrifuged at 4 °C for 5 min. The upper water layer was then quickly removed. The extraction was repeated for 4 times, and finally the water phases were combined and freeze-dried for 48 hours. The dried extracts was then reconstituted in 1 ml double-distilled water, and titrated

with 0.1 M NaOH solution. The electromotive force (EMF) was recorded as a function of the titrant added using a pH meter. The acid amount was determined by the total added titrant amount at the end point, which corresponds to the inflection point of the potentiometric titration curve obtained by taking the first derivative

6.2.7. Microclimate pH distribution kinetics inside microspheres

Around 15 mg of microspheres was suspended into 1 ml phosphate buffer saline (7.74 mM Na_2HPO_4, 2.26 mM NaH_2PO_4, 137 mM NaCl and 3 mM KCl) containing 0.02 % Tween 80 (PBST, pH=7.4, 10 mM) and incubated in a Glas-Col® rotator (Terre Haute, IN) at 37 °C. At predetermined time points, a small amount of microspheres was separated for confocal imaging study. The release media was also removed for pH measurement using a Corning 430 pH meter (Corning, NY), followed by replacing with fresh media. The acquired confocal images were first processed as described by Li et al. to eliminate signal noise (26). The ratio of pixel intensities of two images obtained from two wavelengths (450 nm and 520 nm) was calculated and related to pH from the standard curve independent of dye concentration. In the processed images, each pixel was converted to a color corresponding to pH. The probability of specific pH value inside microspheres was obtained by dividing the amount of pixels corresponding to a specific pH to the total pixels in the images. Pixel ratios that exceeded the limit of standard curve range referred to pH of either above 5.8 or below 2.8. In such cases, their percentages were plotted as the boundary of pH distribution curve. The pH distribution curves were plotted using a Matlab program.

6.3. Results and Discussion

6.3.1. Characteristics of PLHMGA copolymers

Table 1 shows the characteristics of the protected poly(D,L-lactic acid-ran-benzyloxymethyl glycolic acid) (PLBMGA) and deprotected PLHMGA. The molecular weight and thermal behavior of the copolymers were measured and the characteristics are in line as reported in our previous studies (20,22,27).

Table 1. Characteristics of PLBMGA and PLHMGA copolymers

polymer	feed ratio D,L[a]/M[b]	copolymer composition (NMR)[c]	M_n (kg/mol)	M_w (kg/mol)	T_g (°C)
PLBMGA	75/25	78/22 (11)	16	35	36
	65/35	70/30 (15)	24	51	41
PLHMGA	75/25	80/20 (10)	13	30	49
	65/35	69/31 (15.5)	22	45	47

[a]D,L = D,L-lactide
[b]M = BMMG (benzyloxymethyl methyl glycolide)
[c]The numbers in brackets are correspondent to either BMGA or HMGA

6.3.2. Preparation of microspheres loaded with an acidic pH sensitive probe

Microspheres were prepared from PLHMGA copolymers with different copolymer composition (65/35 and 75/25) and PLGA 50/50 using the w/o/w double emulsion solvent evaporation method. Additionally, for PLHMGA 75/25, solutions with different polymer concentration were employed to prepare microspheres. Since the development of microclimate pH depends on the size of microspheres (10), the microspheres used for confocal microscopy imaging were sieved to yield particles with a similar size distribution of 20-45 μm. As can be seen from the scanning electron micrographs (Fig. 1), all microspheres displayed spherical shape and essentially they had non-porous surfaces.

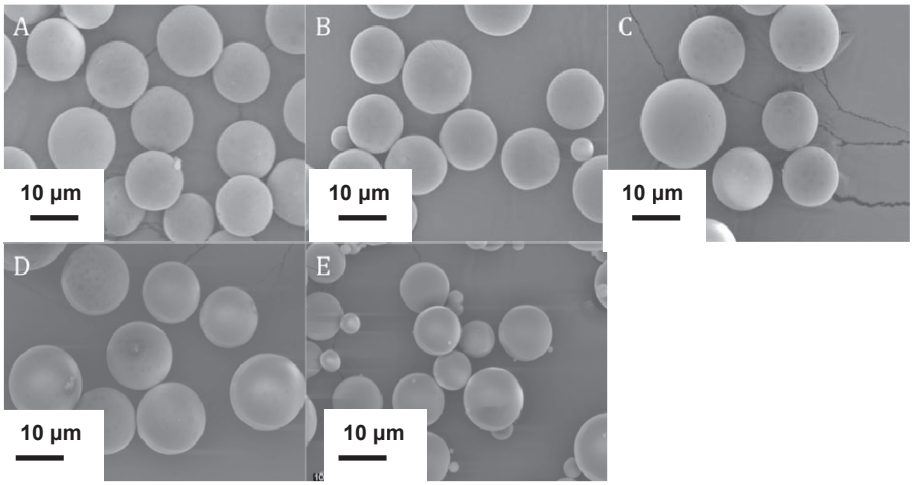

Fig. 1. Scanning electron micrographs of microspheres made from PLHMGA 75/25 with 25 % w/w (A), 30 % w/w (B), 35 % w/w (C) of copolymer concentration, PLHMGA 65/35 (D) and PLGA 50/50 (E) prepared from a 35 % w/w solution concentration.

6.3.3. Microclimate pH distribution inside degrading PLHMGA and PLGA microspheres

The microclimate pH distribution inside PLHMGA and PLGA microspheres was monitored using confocal laser scanning microscopy as previously reported (26). The encapsulated Lysosensor yellow/blue®, is sensitive between pH 2.8 and 5.8. A standard curve of the dye correlating its fluorescence intensity ratio at wavelength of 450 nm and 520 nm and pH from 2.8 to 5.8 was established and fitted into a third order polynomial function (r^2=0.999), as shown in Fig. 2. This figure shows that the pH sensitivity of the dye is concentration independent as previously reported (10) which ensures that the standard curve is not affected even though the dye concentration in the degrading microspheres might decrease during incubation. Some important instrument parameters (detection gain, pinhole, laser power, etc.) were adjusted so that within the concentration range from 0.8 mg/ml to 2.0 mg/ml, the images of dye solutions gave fluorescence intensity from 10 to 255. In the microspheres images, any value below 10 was regarded as background and the value could not exceed 255 since the images were saturated in that case. Intensities staying within the range of 10 to 255 indicated the existence of entrapped dye. The blank regions in the processed images suggested the release out of the dye from the microspheres.

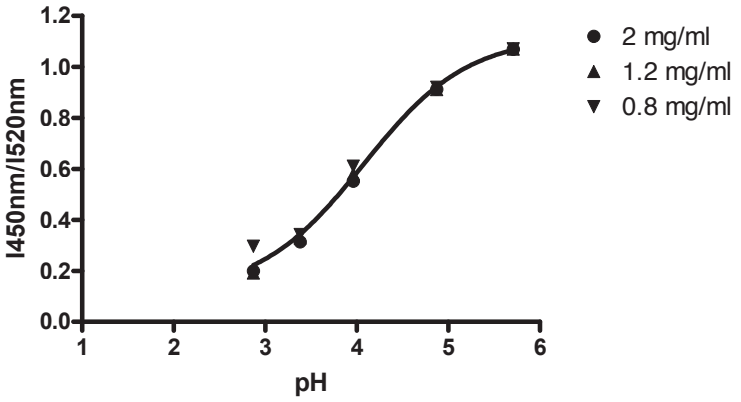

Fig. 2. The pH sensitivity and concentration-independence of Lysosensor yellow/blue®. conjugated to dextran. The third-order polynomial curve fitting the data was $Y=-0.0582x^3+0.7221x^2-2.5676x+3.0213$, where $Y=I_{450nm}/I_{520nm}$; x=pH, $r^2=0.9988$. The excitation was at 364 nm.

6.3.3.1. Effect of polymer composition on μpH distribution kinetics

μpH changes were compared in degrading microspheres made from PLHMGA of different composition (65/35 and 75/25) and PLGA 50/50 during incubation in PBST at 37 °C for four weeks, as shown in processed confocal images (Fig. 3) and μpH distribution curves (Fig. 4).

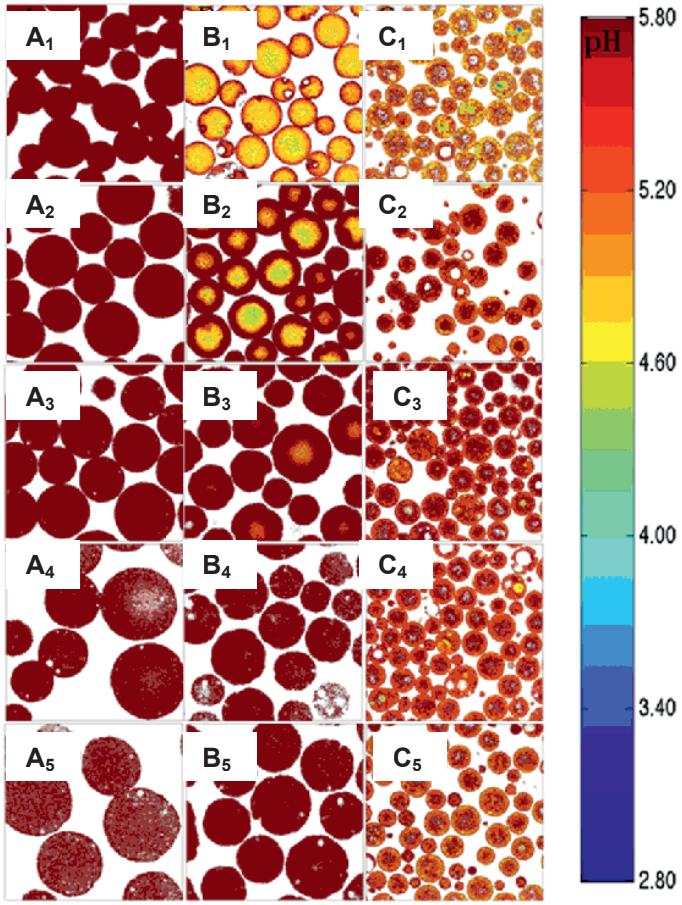

Fig. 3. Processed confocal images of (A) PLHMGA 65/35, (B) PLHMGA 75/25 and (C) PLGA 50/50 microspheres during incubation in PBST at 37 °C for 4 weeks. Images were taken at 1 (A_1-C_1), 7 (A_2-C_2), 14 (A_3-C_3), 21 (A_4-C_4) and 28 (A_5-C_5) days.

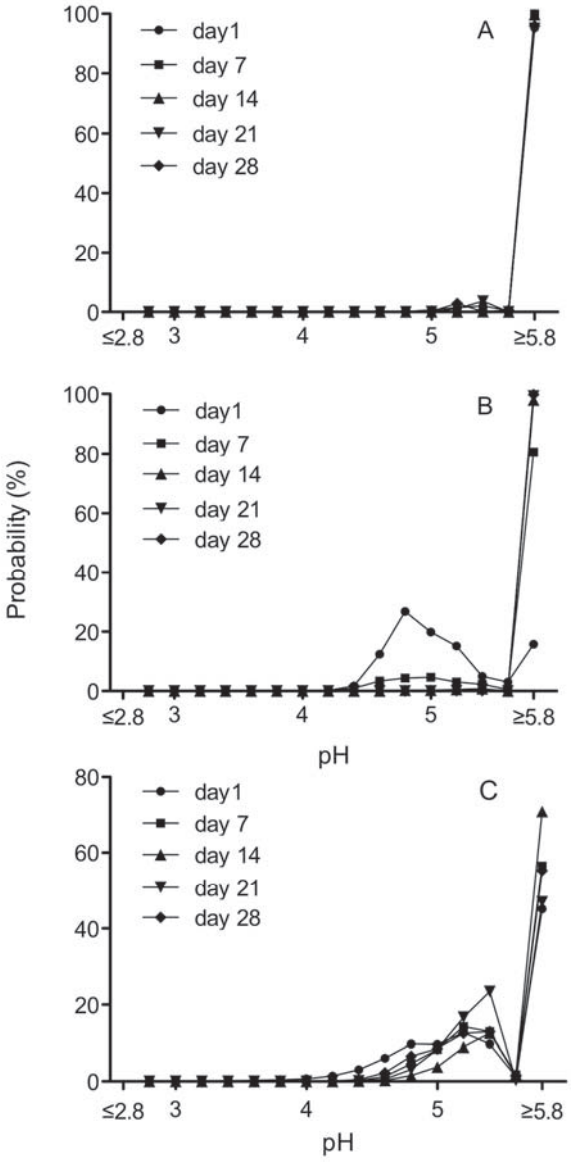

Fig. 4. μpH distribution kinetics inside (A) PLHMGA 65/35, (B) PLHMGA 75/25 and (C) PLGA 50/50 microspheres during incubation at 37 °C in PBST for 4 weeks.

Within four weeks incubation of microspheres prepared from PLHMGA 65/35, more than 95 % of pixels in the images gave fluorescence ratio

corresponding to pH out of detection range (pH>5.8), suggesting they did not develop detectable acidity during that time (Fig. 4A). For PLHMGA 75/25 microspheres, acidity in most aqueous pores with average μpH of 4.8 was observed after 1 day incubation, however, the acidity decreased with increasing incubation time and almost disappeared after 14 days incubation (Fig. 4B). This initial low pH might be explained by the presence of acidic impurities in the polymer (Table 2). Although the 65/35 copolymer also contains acidic impurities (Table 2), these are probably extracted out of the microspheres during their preparation due to the higher hydrophilicity of the polymer matrix.

Table 2. Total acid impurities in PLGA and PLHMGA

Polymer	PLGA 5050	PLHMGA 6535	PLHMGA 7525
total acid (μmol/mg)	0.062	0.048	0.058

In comparison, PLGA microspheres displayed detectable acidity through all the course of incubation. The μpH is most acidic after 1 day incubation, with around 55 % of pixels domains giving pH below 5.8, and then it kept rising until 2 weeks incubation before decreasing again. Meanwhile, the pH in the release media of different polymers was monitored using a pH meter. The pH dropped with the progression of incubation for all polymers, with that in the release medium of PLGA microspheres highest, followed by PLHMGA 75/25 and PLHMGA 65/35 (Fig. 5).

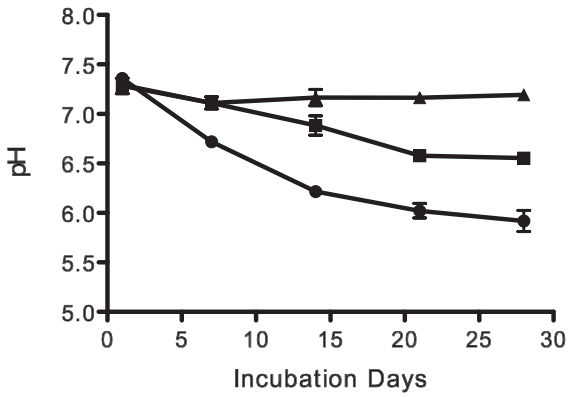

Fig. 5. pH kinetics of release media of PLHMGA 65/35 (●), PLHMGA 75/25 (■) and PLGA 50/50 (▲) microspheres during incubation in PBST at 37 °C for 4 weeks. The buffer was changed each week and the pH was measured again after one week of incubation.

As compared to PLGA, PLHMGA's are more hydrophilic due to the pendant hydroxyl groups on polymer backbone. Therefore, it can be expected that PLHMGA microspheres have a higher water-absorbing capacity than PLGA microspheres, which can dilute acid concentration in polymer matrices. Moreover, the water-soluble acid degradation products, which are responsible for the drop of microclimate pH, can be released faster from the more hydrated PLHMGA microspheres, as evidenced by the lower pH in the release media of PLHMGA copolymers. Admittedly, since the degradation times of this hydrophilic polyester were much shorter than those of PLGA (20), the production of acids should be faster. Since those acids were quickly released, they did not accumulate in the polymer, leading to an overall less acidic microenvironment in PLHMGA than in PLGA microspheres. Also a more evenly distributed μpH was observed in PLHMGA microspheres because of their hydrophilic properties, as can be seen from the more homogeneous distribution of dye emission in PLHMGA microspheres (Fig. 3). Increasing the feed ratio of BMMG from 25 % to 35 %, increases the hydrophilicity of PLHMGA which can further facilitate diffusion of acid degradation products out of the polymer, causing a more neutral μpH inside PLHMGA 65/35 microspheres than in PLHMGA 75/25 and more acidic pH in the corresponding release media. The acidity in PLHMGA 75/25 microspheres after 1 day incubation can be possibly due to the acid impurities existing in the polymer. However, as the incubation proceeds, those acids were gradually released, giving rise to a μpH increase.

6.3.3.2. Effect of polymer concentration used for preparation of microspheres on μpH distribution kinetics

To investigate the effect of polymer concentration on μpH distribution kinetics, microspheres were prepared using DCM solutions of PLHMGA 75/25 of three different polymer concentrations (25, 30 and 35 % w/w). The incubation time was prolonged to 6 weeks in which complete degradation of the microspheres is achieved (20). As shown in the processed confocal images (Fig. 6) and μpH distribution curves (Fig. 7), increasing the polymer concentration decreased the initial μpH after 1 day incubation which can be rationalized by the fact that microspheres made from solutions with higher polymer concentration possessed more acidic impurities. This was confirmed by measuring the amount of water-soluble acids of different polymers determined by titration (Table 2). It is clearly shown that PLHMGA 75/25 contained more acid products than the 65/35 copolymer assuming that negligible copolymer hydrolysis occurred during extraction. Additionally, microspheres prepared from copolymer solutions with

higher concentration usually have denser structures (28) which impede the liberation of acid degradation products. As the incubation continued, the pH inside of the microspheres increased due to the release of acid degradation products into the incubation medium (Fig. 7). The μpH is usually more acidic in the center of microspheres than the peripheral regions, due to the relatively shorter diffusion length of acids around the microsphere surfaces. However, the pH declined again after 28 days incubation for all microspheres (Fig. 7), with microspheres made from solution of higher polymer concentration developing relatively lower μpH. A possible explanation for this is after 28 days, due to the bulk erosion, the rate of acids production exceeded the rate of acids release, thereby generating lower pH in some aqueous pores.

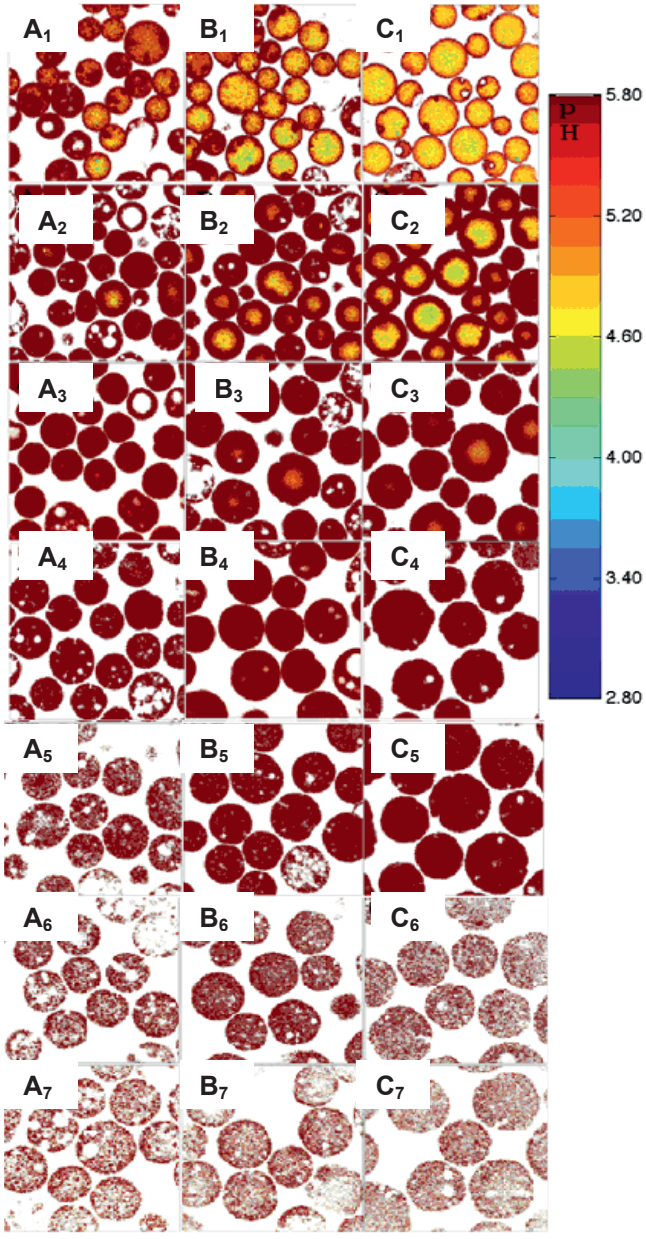

Fig. 6. Processed confocal images of PLHMGA 75/25 microspheres made from (A) 25% w/w (B) 30% w/w (C) 35% w/w of polymer concentration during incubation in PBST at 37 °C for 6 weeks. Images were taken at 1 (A_1-C_1), 7 (A_2-C_2), 14 (A_3-C_3), 21 (A_4-C_4), 28 (A_5-C_5), 35 (A_6-C_6), and 42 (A_7-C_7) days.

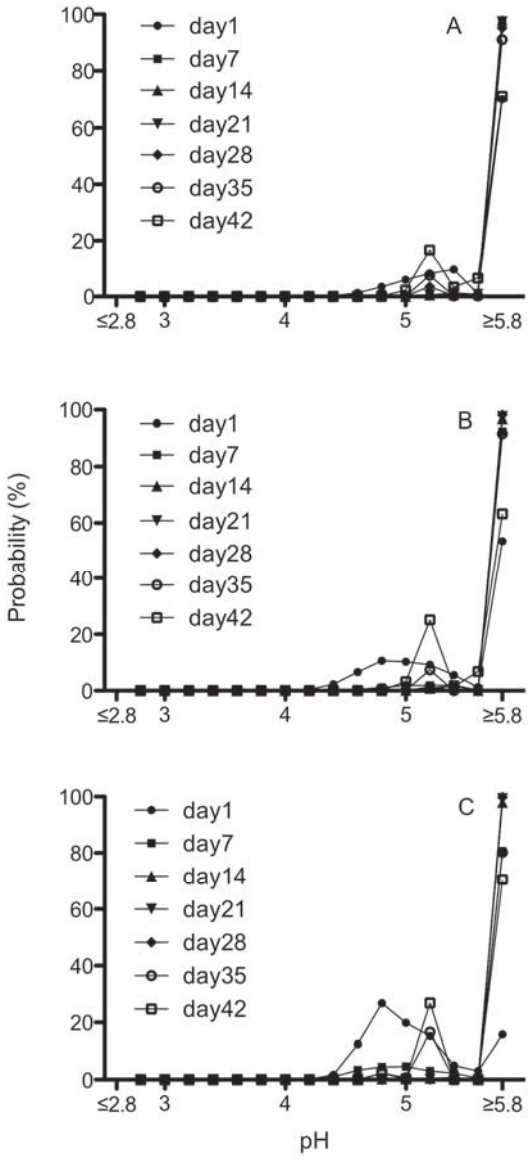

Fig. 7. μpH distribution kinetics inside PLHMGA 75/25 microspheres made from (A) 25 % w/w (B) 30 % w/w (C) 35 % w/w of polymer concentration during incubation in PBST at 37 °C for 6 weeks.

6.4.

Conclusions

The microclimate pH (μpH) inside degrading microspheres prepared from a novel hydroxylated aliphatic polyester; poly(lactic-co-hydroxymethyl glycolic acid) (PLHMGA) was measured based on a quantitative ratiometric method by confocal laser scanning microscopy during incubation under physiologic conditions. The results showed that PLHMGA microspheres made from copolymer 65/35 and 75/25 displayed less acidic pH during one-month incubation compared to that of PLGA microspheres with similar molecular weight. The μpH inside PLHMGA microspheres made from copolymer 75/25 decreased with increasing polymer concentration. Although microspheres of copolymer 75/25 showed an average pH of 4.8 on the first day of study, the pH increased in time to above detection limit (pH> 5.8) as the degradation of the microspheres proceeded. In contrast, PLGA microspheres developed μpH as low as 4 during 28 days of study and maintained acidic μpH despite the degradation of the microspheres continued. This study showed that PLHMGA microspheres are potential carriers for controlled delivery of acid labile molecules such as peptides/proteins and preventing structural changes due to the acidic environment.

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Chapter 7

Summary & Perspectives

7.1. Summary

Pharmaceutical peptide/proteins have proven to be potent molecules for the treatment of a great variety of chronic and life threatening diseases. An increasing number of approved products and related revenues demonstrate their significance for the pharmaceutical industry. Besides, advances in molecular biology will ultimately lead to more potential peptide/protein based drug candidates for the treatment of diseases. These molecules however demand a suitable formulation for their successful delivery.

For formulation and delivery of such molecules the parenteral route is the preferred one, as other routes of administration (e.g. oral, rectal, lung) have been shown to be inefficient. Another characteristic of many peptides and proteins is that they have a short plasma and tissue half live. This leads to frequent injection schemes to maintain a therapeutic drug level. Consequently, sustained release systems would lead to less frequent injections and an improved pharmacokinetic profile as the blood and tissue levels will show fewer fluctuations over time. The development of a new family of biodegradable polymer-based sustained release systems for injection is the subject of this thesis.

Chapter 1 provides a general overview of different methods commonly used for the preparation of prolonged and sustained release of pharmaceutical peptide/proteins to circumvent frequent injections and increase patient compliance. In particular, microspheres based on biodegradable polyesters such as PLGA have been shown to be suitable carrier systems. These microspheres are ideally able to gradually release their payload upon degradation leading to prolonged action and consequently to less frequent injections. However, in many cases the release of peptide/proteins from these hydrophobic PLGA systems (e.g. from nano/micrsopheres, implants) is incomplete and difficult to tailor due to the unwanted reactions between the peptide/protein and the polymer, the drop of the microenvironment pH in PLGA systems and the complex erosion of the polymer matrix forming basis of the microspheres. Efforts have been made to improve release profiles and stability of biotherapeutics from PLGA systems. For example, PEGylation of peptide/protein and/or co-encapsulation of excipients improved the release behavior from PLGA systems. However, these approaches are not considered a general solution to compensate for the disadvantages of these polyesters. Therefore, hydrophilic polyesters have been developed. Within our Department, a new hydrophilic polyester, poly(lactide-co-hydroxymethyl glycolide) (PLHMGA) was synthesized and characterized, and preliminary results showed tailorable degradation of films made from these polyesters.

The aim of this thesis is to investigate the feasibility of applying PLHMGA in the form of microspheres for controlled delivery of pharmaceutical peptides/proteins.

Chapter 2 reviews the major synthesis routes for (hydrophilic) polyesters including polycondensation, enzymatic polymerization and ring opening polymerization (ROP). Step-growth polymerization (polycondensation) refers to the condensation of hydroxyl-acids or mixtures of diacids and diols. The major drawbacks of this method are the high temperatures and long reaction times which may lead to side reactions, such as racemization. Moreover, only a low degree of polymerization can be reached resulting in relatively low molecular weight polymers. Enzymatic polymerization is an efficient method for the synthesis of aliphatic polyesters which can be obtained under mild reaction conditions without using toxic reagents. Also unprotected monomers can be used. Therefore, enzymatic polymerization has a large potential as an environmentally friendly synthetic process. However, the major drawback of the enzymatic synthesis of polyesters is the relatively low molecular weights of the obtained polymers. High molecular weight aliphatic polyesters can be routinely synthesized by ROP and more importantly, limited or absent side reactions such as racemization occur during polymerization. At the end of this chapter application of functionalized aliphatic polyesters for protein delivery, tissue engineering and gene delivery are discussed.

In **chapter 3** we investigated the encapsulation and release of macromolecular hydrophilic Dextran Blue and the model protein lysozyme from PLHMGA microspheres and results were compared to the data obtained for PLGA microspheres. Microspheres were prepared with a double emulsion evaporation method and the effect of PLHMGA concentration in the organic phase used for the preparation of the microspheres on release and degradation of the microspheres was studied. Dextran Blue and lysozyme were successfully encapsulated in PLHMGA microspheres and the loading efficiency enhanced by increasing the PLHMGA concentration used for the preparation of microspheres. A complete release of Dextran Blue was measured after 27 days from PLHMHA microspheres while only 65 % of Dextran Blue and 11 % of lysozyme was released from PLGA microspheres after 65 days. Lysozyme was released from PLHMGA microspheres with a burst caused by the porosity of microspheres. The porosity and subsequent burst release could be diminished by decreasing the rate of solvent removal and increasing the PLHMGA concentration in the volatile organic solvent (DCM) used for the preparation of the microspheres. Increasing the polymer concentration did not have effect on the degradation of

the microspheres. The gradual decrease in mass loss and M_n demonstrated that the degradation of PLHMGA microspheres is characterized by a combination of surface erosion and bulk degradation. Although the secondary and tertiary structure of released lysozyme and its enzymatic activity were fully preserved, lysozyme release was incomplete (50-70 %) and some insoluble residues remained comprising of protein-polymer mixture (FTIR-analysis). NMR analysis of the insoluble residues further showed that they were mainly oligomers of L-lactic acid which are resistant to degradation due to their crystallinity. Likely, due to the hydration of the hydroxyl groups of HMG units, hydrolysis preferentially occurs at the hydroxyl enriched sites in the polymer, leaving oligomers of L-lactic acid as insoluble products.

To prevent the formation of crystalline oligomers of L-lactic acid and achieve a complete polymer degradation, in **chapter 4** D,L-lactide was used for copolymerization with BMMG to yield amorphous D,L-lactic acid oligomers as degradation products which are more susceptible for degradation than crystalline L-lactic acid oligomers. We also studied the relation between degradation kinetics of the microspheres and release of a model protein, bovine serum albumin (BSA), from PLHMGA microspheres made from copolymers with different composition (feed ratios of 50/50, 65/35 and 75/25 D,L-lactide/BMMG) and the effect of PLHMGA molecular weight on BSA release from PLHMGA microspheres was investigated. It was shown that microspheres of PLHMGA fully degraded in a timeframe of 2 weeks to 2 months depending on the copolymer composition. Consequently, *in vitro* release studies showed a quantitative release of BSA from microspheres from 2 weeks to 2 months, which demonstrates that the release is essentially governed by the degradation of the microspheres. This release behavior provides the opportunity to deliver protein pharmaceuticals in a desired timeframe. One of the major challenges of protein delivery by using microspheres is prevention of the burst release which is caused by the porous structure of the microspheres. Microspheres prepared from PLHMGA with a higher molecular weight showed a substantially lower burst than those with a lower molecular weight. The release kinetics after this burst was essentially independent of the molecular weight. Importantly, the structural integrity of the released/soluble protein was fully preserved as demonstrated by fluorescence spectroscopy. Size exclusion chromatography analysis showed that the monomer/dimer ratio in released samples was the same as in freshly dissolved BSA.

The application of PLHMGA microspheres for delivery of a clinically used therapeutic peptide, octreotide, has been addressed in **chapter 5** and the results

were compared to those obtained with the commercial formulation, Sandostatin LAR®, made with a PLGA-glucose star polymer. Octreotide-loaded PLHMGA microspheres were prepared from copolymers with different composition of D,L-lactide and HMG (feed ratios of 50/50, 65/35 and 75/25 D,L-lactide/BMMG). The loading efficiency increased by neutralizing the continuous phase which likely prevents ionization and subsequent migration of the soluble peptide from the dispersed phase into the continuous phase. SEM analysis showed that PLGA and PLHMGA microspheres were essentially non-porous. Octreotide release from PLHMGA microspheres was characterized by a small burst (10-20 %) followed by a sustained release of the peptide for 20 to 60 days, depending on the hydrophilicity of the polymer used for the preparation of the microspheres. The commercial formulation did not show a detectable burst release and sustained release of octreotide was measured after a 10-day lag phase over the next 90 days. Although the total octreotide release both from PLHMGA and PLGA microspheres was almost the same, quantitative HPLC analysis showed that mainly native octreotide was released from PLHMGA microspheres while for PLGA microspheres mainly acylated peptide adducts were released. MALDI-TOF MS analysis showed that the relative amount of peptide modified with glycolic acid units (octreotide-GA) was substantially greater than modified peptide with lactic acid units (octreotide-LA). Likely, nucleophilic attack of the primary amine groups of the peptide more readily occurs on glycolic acid units rather than on lactic acid units in PLGA. No octreotide-GA was detected in octreotide released from PLHMGA microspheres due to the lack of glycolic acid units in this polymer. Instead, some acylation with HMG was however observed. Three factors might contribute to the reduced extent of peptide acylation that occurs during release from PLHMGA microspheres compared to that of PLGA microspheres: **1)** lack of more reactive glycolic acid units in PLHMGA **2)** the shorter degradation times of the PLHMGA microspheres reduces the exposure time of the peptide to the polymer **3)** less acidification of PLHMGA matrices occurs during degradation compared to that of PLGA matrices. It has been reported that peptide acylation can be catalyzed at low pH microenvironment hence the acylation can be slowed down in degrading PLHMGA matrices which indeed have higher microclimate pH as demonstrated in **chapter 6**.

One of the major challenges with PLGA microspheres is prevention of the drop of pH inside degrading microspheres which is detrimental for the integrity of acid labile molecules such as peptides and proteins. We studied the microclimate pH (μpH) of degrading PLHMGA microspheres in **chapter 6** and the results were compared with that of PLGA. The effect of copolymer composition and polymer concentration used for preparation of PLHMGA on

μpH was studied. A ratiometric method by confocal scanning microscopy was exploited and Lysosensor yellow/blue® dextran was used as the fluorescent pH sensitive probe to study μpH distribution kinetics inside PLHMGA and PLGA microspheres degraded under physiological conditions. PLHMGA microspheres prepared from copolymers with 35 and 25 % BMMG in the feed did not show changes in pH during incubation except during the first day of incubation for copolymer prepared from 25 % BMMG when the copolymer concentration in DCM increased from 25 % to 35 %. As the degradation of the microspheres progressed, the acid degradation products (lactic and hydroxymethyl glycolic acid and their oligomers) which are responsible for pH drop, were released into the degradation medium as was confirmed by a decrease in pH of the surrounding medium. On the contrary, confocal images of PLGA microspheres during incubation showed that these microspheres developed a low pH during degradation. This means that the formed acid degradation products were not released in the medium as also evidenced from the observation that the pH of the degradation medium remained constant at 7.4.

In conclusion, this thesis describes the application of novel biodegradable microspheres made from a hydrophilic polyester, poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA) for controlled delivery of peptide and protein pharmaceuticals. PLHMGA microspheres showed superior properties over their PLGA counterpart regarding the control over degradation and release rate. The release of biopharmaceuticals and degradation of the microspheres could be successfully tailored by synthesis of copolymers differing in copolymer composition. The results reported in this thesis highlight the great potential of PLHMGA microspheres for biopharmaceutical applications.

7.2. Discussion and future perspectives

In this thesis the preparation and characteristics of peptide/protein containing PLHMGA-microspheres were discussed. Most of the work dealt with the *in vitro* characterization; the *in vivo* evaluation of the microspheres has just started. The intended use for these microspheres is the controlled release of the encapsulated drug in patients. The route of administration is by injection, either subcutaneously or intramuscularly. The microspheres do not move from the injection site and ‘targeted’ delivery is possible by direct injection into the target site.

In the following paragraphs the perspectives of PLHMGA microspheres are discussed. These topics include:

- 7.2.1. – 7.2.4. What scientific information still has to be collected before the PLHMGA microsphere system may be applied in patients?
- 7.2.5.1. Is their use limited to the controlled release of proteins or peptides or are low molecular weight drugs good candidates as well?
- 7.2.5.2. Can these microspheres be used for drug targeting purposes in addition to their use for controlled release?
- 7.2.5.3. What about other potential areas e.g. tissue engineering?
- 7.2.6. Quality improvement of the polymer: can the impurity profile be improved?

7.2.1. Injectability

Short half-lives (in blood or tissue) of peptides/proteins make frequent injection schemes inevitable. To alleviate the pain caused by these injections and to improve patient compliance, depot formulations of biodegradable polymers such as PLHMGA may turn out to be of great value. PLHMGA microspheres are easy to prepare and have a high loading capacity for peptides and proteins. Lyophilization is a common method to dry microspheres and prolong the storage time. Several lyophilized products have received FDA approval for marketing (e.g. Sandostatin LAR®). Introductory experiments have shown that lyophilized microspheres of PLHMGA can be easily reconstituted with water or any aqueous solution. Their relatively high surface hydrophilicity, because of the hydroxyl groups present in the polymer backbone, make them more readily resuspendable than PLGA microspheres and in that respect, better candidates for im or sc injection. The PLHMGA microspheres are large enough (5-15 μm) to be secured at the site of injection and will not be eliminated by macrophages or other elements of the immune or lymphatic system. Moreover, PLHMGA microspheres are spherical and not irregularly shaped which prevents them clump together during injection. However, further studies are needed to make sure microspheres are not broken and retain their spherical shape after being injected.

7.2.2. Peptide/protein release

The preparation method (w/o/w) appeared to be compatible with the peptides and proteins used in our studies: the physicochemical characteristics and the enzymatic activity (lysozyme) were fully preserved. However, the *in vivo* efficacy of these protein drug loaded delivery systems should be further studied to prove the release of active protein. The release rate of proteins (lysozyme and BSA) could be tailored by varying the copolymer composition and the release

was in all cases to a large extent mediated by the degradation of microspheres. However, the release of lysozyme was incomplete even if the copolymer was fully degradable when D,L-lactide was used instead of L-lactide for the synthesis of the copolymer. Several attempts were made to achieve a complete release of lysozyme by reducing protein-polymer contact including co-encapsulation of neutral PEG, polycationic trimethyl chitosan, thermogelling p(HPMAm-lactide)-PEG-p(HPMAm-lactide) and poloxamer 188. Nonetheless, these efforts did not render complete release of lysozyme (results not shown in this thesis). In contrast, a quantitative release of BSA from PLHMGA microspheres was observed. This shows the importance of suitable physico-chemical characteristics and sufficient stability for achieving successful protein delivery. Another general issue with microsphere systems is the common burst release of proteins which was also observed for PLHMGA microspheres. We successfully minimized the burst release by increasing the polymer concentration in the preparation process. This decreases the porosity of the microspheres and the burst release. Burst release can also be reduced by applying a proper washing procedure after preparation of the microspheres.

Beside protein drugs, demands for effective delivery systems for peptide drugs are increasing due to the increasing number of therapeutic peptides that become available to fight diseases and treat patients. As shown, the released octreotide from the commercially available PLGA formulation mainly comprised of modified (acylated) peptide. Although these modified peptides might keep their therapeutic efficacy, these compounds can be recognized by the immune system as exogenous materials which might trigger highly unwanted severe immunogenic reactions. Moreover, there was a lag-phase of around 10 days before the peptide started to be released from PLGA microspheres. In contrast, PLHMGA microspheres released octreotide mainly in its native form which is definitely preferred when considering to apply for marketing approval by the regulatory authorities. Moreover, by using microspheres with different degradation characteristics, we showed better control over peptide release kinetics and in contrast to the PLGA formulation, the PLHMGA microspheres did not show a lag phase. However, the octreotide release from PLHMGA microspheres was accompanied by a burst. Therefore, in future studies, some measures should be considered to decrease or prevent the burst as pointed out above (polymer concentration, implementation of proper washing procedures in the preparation process of the microspheres). The acylation of peptide may be completely prevented by PEGylation of the peptide or co-encapsulation of excipients that decrease the peptide-polymer contact (1, 2).

7.2.3. Biodegradation

The biodegradability of polymer carriers is an important issue for potential drug delivery applications. Biodegradation can occur through either chemical pathways and/or by enzymatic actions. It has been shown that aliphatic polyesters like PLGA degrade via chemical hydrolysis of the ester bonds present in the polymer (3). *In vitro* studies on the degradability of microspheres based on PLHMGA were performed (**chapter 3** and **4)**. On exposure to water, the copolymer undergoes random chain scission by simple hydrolysis of ester bonds in the polymer chain and the final degradation products are lactic and hydroxymethyl glycolic acid and there is consequently no need for removal of the empty PLHMGA microspheres.

7.2.4. Biocompatibility

One of the beneficial characteristics of PLHMGA microspheres is their degradation products which are (oligomers of) lactic and hydroxymethyl glycolic acid. Lactic acid is processed via physiological metabolic pathways and can also be excreted via glomerular filtration. Hydroxymethyl glycolic acid (glyceric acid) is an endogenous intermediate which exist in phosphorylated form and produced during glycolysis (sequence of reactions that converts glucose into pyruvate with the concomitant production of ATP). It can be expected that it is safe and this highly water-soluble compound can be easily excreted. But ‘the proof is in eating the pudding’: *in vivo* studies are necessary to investigate the biocompatibility of these polymers present in microspheres and their degradation products.

7.2.5. Other (potential) applications of PLHMGA

7.2.5.1. PLHMGA microspheres for small molecule drug delivery

In a recent study, PLHMGA microspheres have been used for encapsulation and release of gentamicin sulfate and various formulation parameters on particles characteristics (size, loading and release) were investigated (Fig. 1) (4).

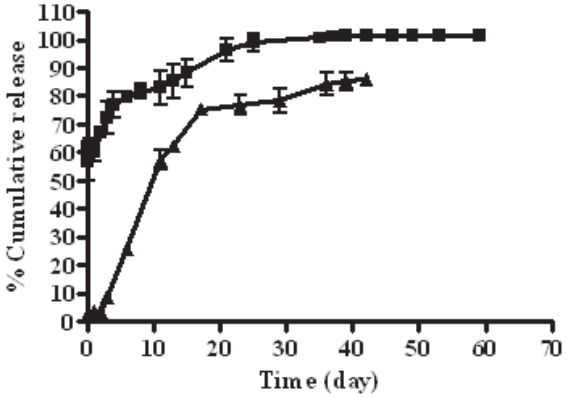

Fig. 1. Release of gentamicin from PLHMGA microparticles in PBS pH 7 at 37 °C prepared with solutions of different polymer concentrations; (■) 15 % and (▲) 25 % polymer solution.

Burst release could be successfully minimized by increasing the polymer concentration of the DCM solution used for the preparation of microspheres. The PLHMGA microspheres also showed better release kinetics than those based on PLGA. These findings show that PLHMGA microspheres are not only suitable for sustained delivery of peptides and proteins, but also can be used for encapsulation and delivery of other low molecular drugs which need a constant plasma concentration to achieve the desired therapeutic effects.

7.2.5.2. Tumor targeting

The microspheres discussed in this thesis are in the micrometer range (5 – 15 um) and were developed for intramuscular or subcutaneous administration. On the other hand, intravenous injection is the preferred route for targeted delivery of the encapsulated drugs. But then, *nano*particles in the sub-micrometer range should be used. Recently, PLHMGA nanospheres for peptide/protein delivery and future drug targeting purposes have been successfully prepared in our Department.

Successful examples of targeting of nanoparticles in animal models have been described in the literature. Tumor targeting by surface modification of PLGA nanoparticles showed promising results for enhanced accumulation of particles and the loaded drug in the tumor (5). Targeted delivery of therapeutics has the potential to localize the therapeutic agents at the site of action and simultaneously decreasing possible side effects. Conjugation with alendronate (6), tetanus toxin C fragment for targeted drug delivery to neurons (7) and conjugation with folate for target-specific cellular uptake (8) are methods to

modify PLGA nanospheres for targeted drug delivery that have been investigated. PLHMGA offers suitable chemical 'handles' (particularly the presence of hydroxyl groups) for conjugation, preferably via degradable linkers, with other molecules for drug targeting. Conjugation can also prevent the burst release of drug load molecules like doxorubicin.

7.2.5.3. Tissue engineering

The application of functionalized aliphatic polyesters has gone further than merely for drug delivery. Scaffolds of copolymers of hydroxymethyl glycolide (HMG) with caprolactone (poly(hydroxymethyl glycolide-co-ε-caprolactone)) showed promising results for the adhesion and subsequent proliferation of human mesenchymal stem cells (9). Three-dimensional printed scaffolds of these functionalized polyesters resulted in porous structures which later were filled by cells. Those cells displayed increased metabolic activity when compared with cells cultured in PCL scaffolds (10). These findings show the bright future of these polymers for tissue engineering.

7.2.6. Polymer quality

One of the issues for further development of PLHMGA that need additional investigation is optimization of the BMMG synthesis. Ring closure of alpha-hydroxy acid using racemic 2-bromopropionyl bromide yields a mixture of two diastereoisomers (S,S and S,R). We were only able to use the (S,S) isomer for polymerization which can be obtained as a white solid after flash column chromatography and can be purified by crystallization. Such highly pure monomers are required to tailor e.g. the molecular weight of the polymers by ROP. New synthetic routes to synthesize BMMG which also allow scaling up are presently exploited in our Department.

7.2.7. Conclusion

In conclusion, this thesis describes novel hydrophilic biodegradable polyesters for peptide/protein delivery. These polyesters have great potentials to enter the pharmaceutical market and make an impact on patients' health. Some preclinical and clinical issues need to be tackled before reaching these goals.

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Appendices

Appendix A: Supporting information chapter 4

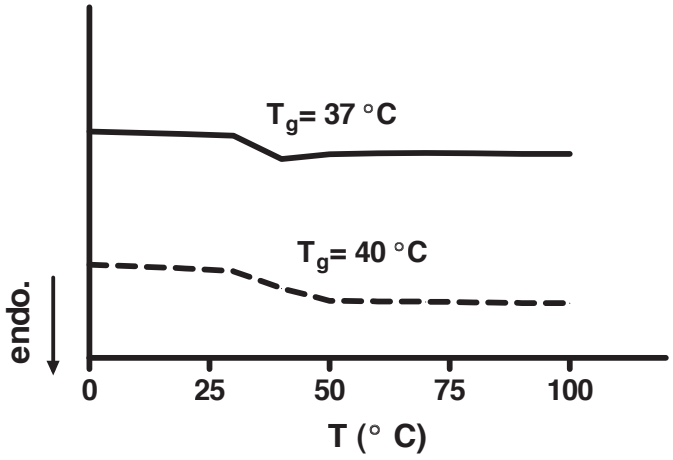


Fig S_1. DSC thermograms of PLBMGA (top) and PLHMGA (bottom) of copolymer 35/65 (Table I; P_3 and P_{11}).

Supporting information 2. **Recrystallization of BMMG.** BMMG was recrystallized as follows. Around 8 grams of BMMG was dissolved in 200 ml t-methyl-butyl ether at 40 °C. Around 50 ml of solvent was removed under vacuum and BMMG crystals were slowly formed in time in this super saturated solution. After 5 hours at room temperature the solution was kept for 16 hours at -20 °C. The crystals were collected by filtration and dried under vacuum at room temperature for 2 hours. The yield of BMMG was about 6 g.

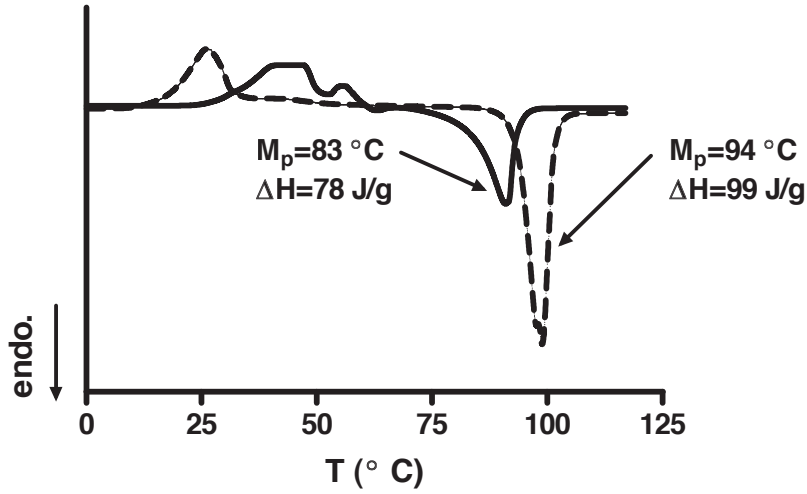


Fig. S_3. DSC thermograms of BMMG before (—) and after (**---)** recrystallization.

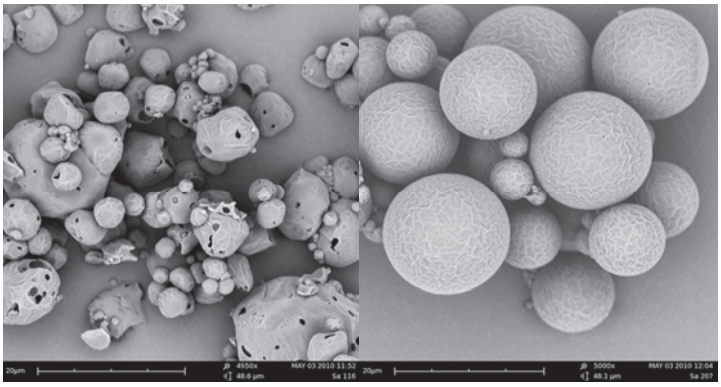


Fig S_4. Scanning electron micrographs of the PLHMGA microspheres (Table II) of F_7 (left) and F_8 (right).

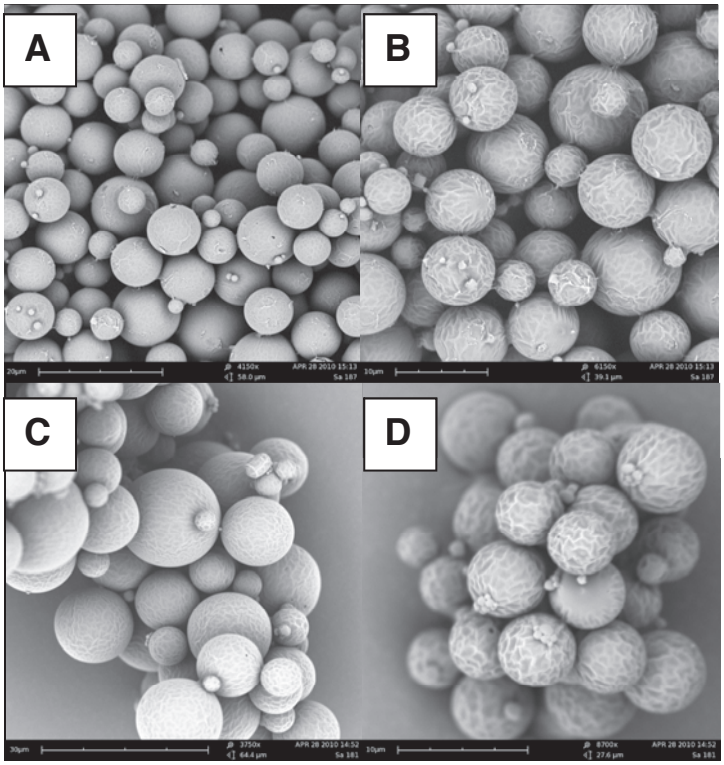


Fig S_5. Scanning electron micrographs of PLHMGA microspheres (Table II) of F_5 (A, B) and F_6 (C, D).

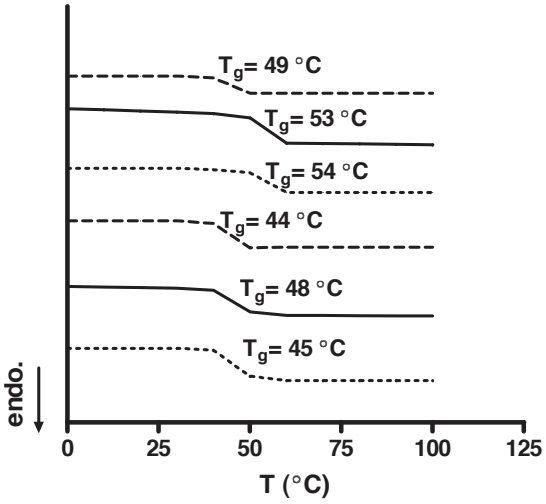


Fig. S_6. DSC thermogarms of PLHMGA microspheres made of P_{10}, P_{12} and P_{16} copolymers. The upper three are the thermograms before degradation and the lowest three are the thermograms of the microspheres after the degradation; (**---**) F_2, (—) F4, (····) F_8.

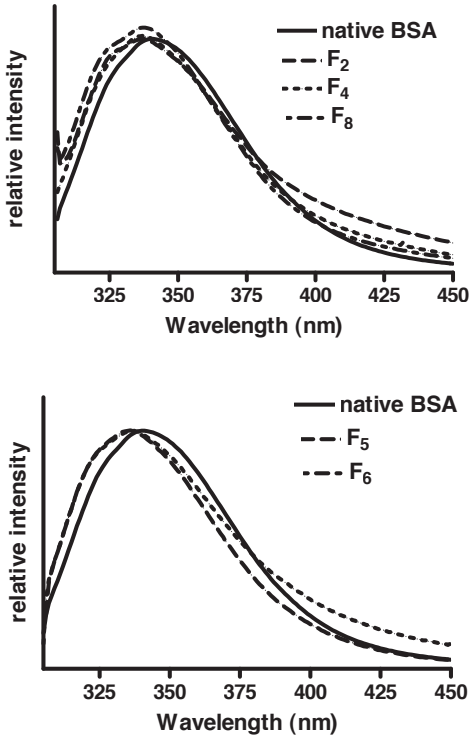


Fig. S7. Fluorescence emission spectra of native and released BSA at day 20 for F_2 and F_4 and day 50 for F_8 (top) and after 20 days for F_5 and F_6 (bottom).

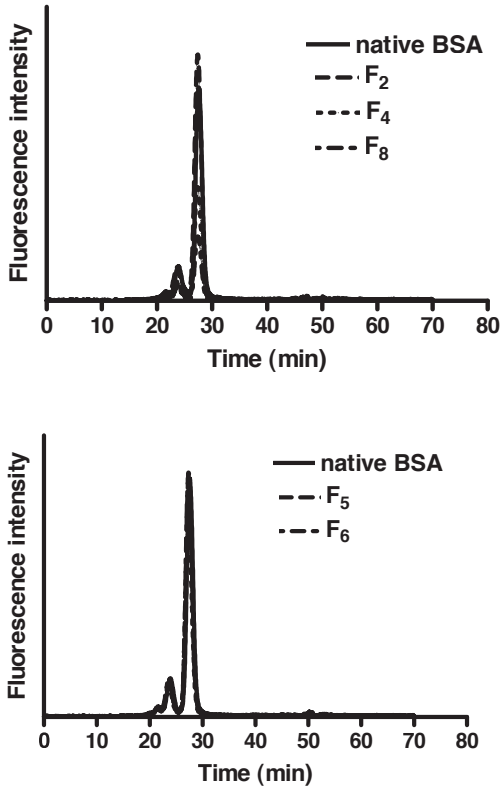


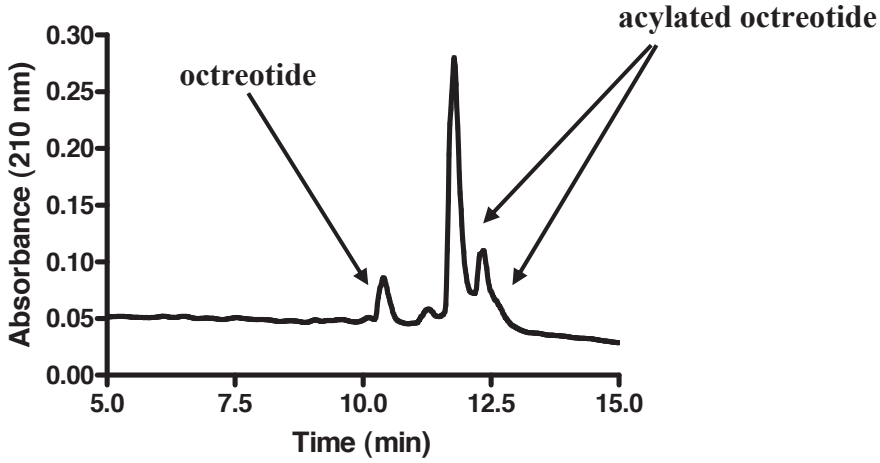
Fig. S8. SEC of native and released BSA at day 20 for F_2 and F_4 and at day 50 for F_8 (Top) and after 20 days for F_5 and F_6 (bottom).

Supplementary information 1: Characteristics of octreotide-loaded PLHMGA microspheres

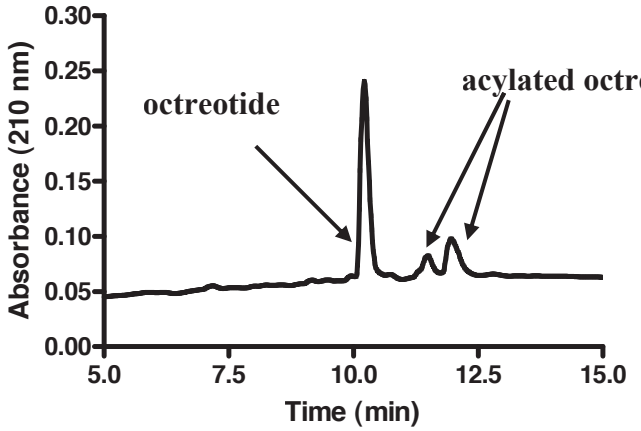
Polymer concentration (% w/w)	Method	pH of continuous phase	NaCl in continuous phase	[a]V_p/V_o	[b]V_{dp}/V_{cp}	LE%
25	w/o/w	3.5	No	1/5	1/10	25
25	w/o/w	3.5	1 M	1/10	1/10	23
25	w/o/w	3.5	1 M	1/10	1/50	35
35	w/o/w	4.0	1 M	1/10	1/10	35
25	o/w	4.0	1 M	N/A	1/10	15
25	w/o/w	3.5	3 M	1/50	1/10	30

[a] V_p/V_o = volume of protein solution/volume of oil phase
[b] V_{dp}/V_{cp} = volume of dispersed phase/volume of continuous phase

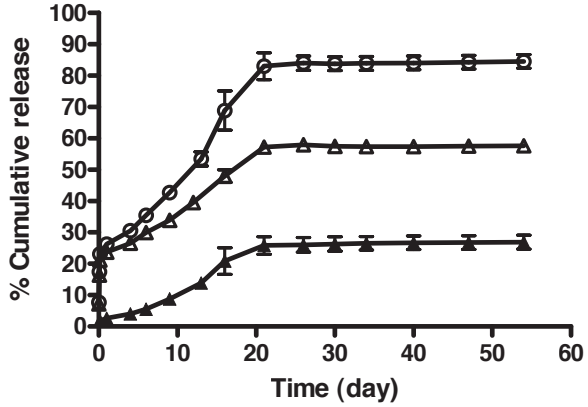
Supplementary information 2: octreotide release from PLGA microspheres after 95 day release in PBS pH 7.4 at 37 °C.



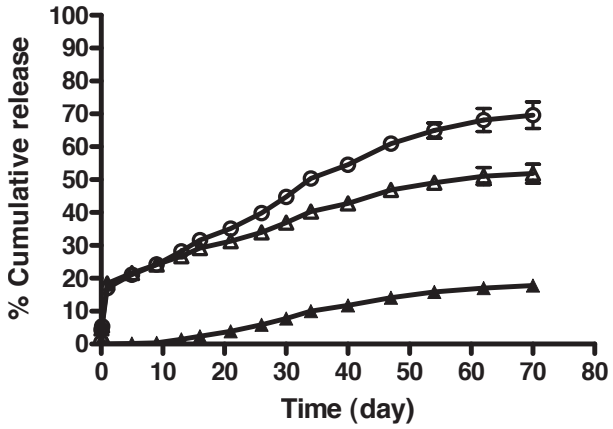
Supplementary information 3: octreotide release from PLHMGA microspheres after 60 day release in PBS pH 7.4 at 37 °C.



Supplementary information 4: Octreotide release from PLHMGA 50/50 microspheres; (Δ) native octreotide, (▲) acylated octreotide and (○) total octreotide release.



Supplementary information 5: Octreotide release from PLHMGA 75/25 microspheres; (Δ) native octreotide, (▲) acylated octreotide and (○) total octreotide release.



Nederlandse samenvatting

Farmaceutische peptiden/eiwitten zijn potentieel geschikt als geneesmiddel voor de behandeling van een groot aantal chronische en levensbedreigende ziekten. Het toenemend aantal op de markt zijnde producten, gebaseerd op peptiden/eiwitten, toont zowel het therapeutische belang voor de patiënt als het financiële belang voor de farmaceutische industrie aan. De vooruitgang in de moleculaire biologie zal vermoedelijk uiteindelijk leiden tot nog meer op peptide/eiwit gebaseerde geneesmiddelen voor de behandeling van ziekten. Voordat deze stoffen als geneesmiddel toegepast kunnen worden, moet een geschikte formulering ontwikkeld worden, waarbij na toediening de werkzaamheid gehandhaafd blijft.

De parenterale toediening (injectie) van deze stoffen blijkt noodzakelijk om de werkzaamheid te behouden; andere routes (b.v. via de mond, longen of rectaal) geven verminderde werkzaamheid. Veel peptiden/eiwitten hebben een korte halfwaarde tijd in bloed en weefsel. Dit geeft aanleiding tot het veelvuldig moeten toedienen van deze stoffen om therapeutische bloed- en weefselconcentraties te handhaven. Wanneer het mogelijk is een gecontroleerde toedieningsvorm te ontwerpen, dan zou dit aanleiding kunnen geven tot een lagere injectie-frequentie en een verbeterd farmacokinetisch profiel, doordat de bloed- en weefselconcentraties mogelijk minder variëren. Het onderwerp van dit proefschrift is het ontwikkelen van een nieuwe klasse biodegradeerbare, op polymeren gebaseerde, vertraagde afgiftesystemen voor farmaceutische eiwitten die per injectie toegediend kunnen worden.

Hoofdstuk 1 geeft een overzicht van de verschillende methoden die momenteel gebruikt worden om toedieningsvormen te vervaardigen om verlengde en gereguleerde afgifte van farmaceutische peptiden/eiwitten te bereiken ten einde veelvuldige injecties te voorkomen en therapietrouw te bevorderen. Vooral microbolletjes,gebaseerd op biodegradeerbare polyesters zoals PLGA, hebben aangetoond geschikte dragersystemen te zijn. Deze microbolletjes zijn in het ideale geval in staat om geleidelijk hun inhoud vrij te geven terwijl ze degraderen, met als effect verlengde werking waardoor minder vaak injecties gegeven hoeven worden. In de praktijk blijkt echter dat de afgifte van peptiden/eiwitten vanuit deze hydrofobe PLGA systemen (b.v. vanuit nano/microbolletjes, implantaten) onvolledig en bovendien moeilijk te sturen is. Dit is het gevolg van de ongewenste reacties tussen de peptiden/eiwitten en het polymeer van het dragersysteem, het dalen van de pH in PLGA-systemen en de

ingewikkelde wijze waarop de polymeermatrix afbreekt. Er is getracht om het afgiftepatroon en de stabiliteit van biotherapeutica die afgegeven worden vanuit PLGA systemen te verbeteren. Dit is onderzocht door bijvoorbeeld PEGylering van het peptide/eiwit en/of door het toevoegen en insluiten van hulpstoffen die het degradatiegedrag van PLGA beïnvloeden. In de praktijk blijkt dat deze benaderingen geen aanleiding geven tot volledige compensatie van de nadelen van deze polyesters. Om deze reden zijn hydrofiele polyesters ontwikkeld. Zo is in onze onderzoekgroep een nieuw hydrofiel polyester, poly(lactide-co-hydroxymethyl glycolide) (PLHMGA) gesynthetiseerd en gekarakteriseerd, en experimenten hebben aangetoond dat de degradatie van films van deze polymeren inderdaad gestuurd kan worden.
Het doel van dit proefschrift is om verder te onderzoeken of PLHMGA in de vorm van microbolletjes toegepast kan worden voor de gecontroleerde afgifte van farmaceutische peptiden/eiwitten.

In **Hoofdstuk 2** wordt een overzicht gegeven van de belangrijkste syntheseroutes voor hydrofiele polyesters, waaronder polycondensatie, enzymatische polymerisatie en ringopening polymerisatie (ROP). Polycondensatie betreft het koppelen van hydroxyzuren of van mengsels van dubbelzuren en diolen door een condensatiereactie via esterbindingen. De belangrijkste schaduwzijden van deze methode zijn de hoge temperaturen en de lange reactietijden, die aanleiding kunnen geven tot nevenreacties, zoals racemisatie. Belangrijker is misschien nog wel, dat alleen een lage polymerisatiegraad bereikt kan worden met als resultaat relatief lage molecuulgewichten. Enzymatische polymerisatie is een effectieve methode voor de synthese van alifatische polyesters onder milde reactie-omstandigheden zonder gebruik te maken van toxische reagentia. Ook onbeschermde monomeren kunnen hiervoor gebruikt worden. Daardoor geeft enzymatische polymerisatie een belangrijk mogelijkheid voor een milieuvriendelijk syntheseproces. De belangrijkste schaduwzijde van de enzymatische synthese van polyesters is dat alleen relatief lage molecuulgewichten bereikt kunnen worden. Alifatische polyesters met een hoog molecuul gewicht kunnen routinematig gesynthetiseerd worden met de ROP methode. Een belangrijk voordeel van ROP is dat geen of bijna geen nevenreacties zoals racemisatie plaatsvinden. Aan het eind van het hoofdstuk worden toepassingen van gefunctionaliseerde alifatische polyesters voor de afgifte van eiwitten, weefsel-engineering en gen- ‘delivery’ besproken.

In **hoofdstuk 3** onderzochten we hoeveel geneesmiddel in de bolletjes opgenomen kon worden en de afgifte van hoogmoleculair hydrofiel Dextran Blue en het modeleiwit lysozyme uit PLHMGA microbolletjes. De gevonden

resultaten zijn vergeleken met de afgifte van dezelfde stoffen uit PLGA microbolletjes. De microbolletjes werden bereid met behulp van een dubbel emulsie verdampingsmethode. Het effect van de PLHMGA concentratie in de organische fase gedurende bereiding van de microbolletjes op afgifte en afbraak werd bestudeerd. Dextran Blue en lysozyme konden opgenomen worden in PLHMGA microbolletjes en de beladingsgraad verbeterde door de PLHMGA concentratie bij de bereiding van de microbolletjes te verhogen. Dextran Blue was na 27 dagen volledig afgegeven uit de PLHMGA microbolletjes, terwijl na 65 dagen slechts 65% van het Dextran Blue en 11% van het lysozyme was afgegeven uit de PLGA microbolletjes. Lysozyme vertoonde een hoge initiële afgifte ('burst') uit PLHMGA, die veroorzaakt wordt door de porositeit van de microbolletjes. De porositeit en de dientengevolge hoge initiële afgifte konden verminderd worden door de snelheid van het verdampen van het oplosmiddel tijdens de bereiding te verlagen en/of de concentratie van het PLHMGA te verhogen in het vluchtige organische oplosmiddel (DCM). Aangetoond werd dat het verhogen van de polymeerconcentratie geen effect heeft op de afbraaksnelheid van de microbolletjes. Het langzame verlies van massa en M_n toonde aan dat de afbraak van PLHMGA microbolletjes het gevolg is van een combinatie van oppervlakte-erosie en bulkdegradatie. De secondaire en tertiaire structuur van het afgegeven lysozyme en de enzymatische activiteit van dit eiwit werden volledig behouden, maar de afgifte van het eiwit was onvolledig (50-70%) en een kleine hoeveelheid onoplosbaar residu bestaande uit een eiwit/polymeer mengsel (FTIR-analyse) bleef achter. NMR analyse van het onoplosbare residu maakte duidelijk dat dit voornamelijk bestond uit L-melkzuur oligomeren die echter moeilijk afbreken ten gevolge van hun kristalliniteit. Vermoedelijk treedt hydrolyse, ten gevolge van de hydratatie van de hydroxylgroepen van de HMG eenheden, preferentieel op in de hydroxylgroepen rijke gebieden in het polymeer, waardoor oligomeren van L-melkzuur als onoplosbaar residu achter blijven.

Om het ontstaan van kristallijne oligomeren van L-melkzuur te voorkomen en aldus een totale afbraak van het polymeer te bewerkstellingen, wordt in **hoofdstuk 4** D,L-melkzuur gebruikt voor de copolymerisatie met BMMG dat amorf D,L-melkzuur oligomeren geeft als afbraakproducten, die meer gevoelig zijn voor afbraak dan de kristallijne L-melkzuur oligomeren. Het verband tussen de afbraaksnelheid van de microbolletjes en de afgifte van het modeleiwit (runder serum albumine, BSA) werd bestudeerd gebruikmakend van PLHMGA microbolletjes vervaardigd met copolymeren met verschillende samenstellingen (aangeboden verhouding tijdens polymerisatie van 50/50, 65/35 en 75/25 D,L lactide/BMMG). Daarnaast werd het effect van het molecuulgewicht van

PLHMGA op de BSA afgiftesnelheid vanuit de microbolletjes bestudeerd. De microbolletjes van PLHMGA degradeerden volledig in een tijdsperiode van 2 weken tot 2 maanden afhankelijk van de copolymeersamenstelling. De hierna uitgevoerde in vitro afgiftestudies lieten een volledige afgifte van BSA uit de microbolletjes in een periode van 2 weken tot 2 maanden zien. Hieruit blijkt dat de afgifte van BSA voornamelijk optreedt door de afbraak van de microbolletjes en maakt het mogelijk om eiwitafgifte te sturen. Eén van de grootste uitdagingen van eiwitafgifte door middel van microbolletjes is het voorkomen van initieel grote hoeveelheden afgegeven eiwit ('burst') die veroorzaakt wordt door de poreuze structuur van de microbolletjes. Microbolletjes gemaakt van PLHMGA met een hoog molecuulgewicht toonden een veel lagere 'burst' dan die, die bereid waren met een polymeer met een lager molecuulgewicht. De afgiftesnelheid na de 'burst' was onafhankelijk van het molecuulgewicht van het polymeer dat gebruikt werd voor de vervaardiging de microbolletjes. Belangrijk is dat de oorspronkelijke structuur van het afgegeven eiwit zoals gemeten met fluorescentie-spectroscopie volledig behouden bleef. Chromatografische analyse met 'size exclusion' chromatografie toonde aan dat de verhouding tussen monomeer en dimeer na afgifte hetzelfde was als in een vers bereid BSA monster.

De toepasbaarheid van PLHMGA microbolletjes voor de afgifte van het in de kliniek gebruikte therapeutische peptide, octreotide, is onderzocht in **hoofdstuk 5** en de resultaten zijn vergeleken met die van een commercieel verkrijgbare formulering, Sandostatin LAR®, gemaakt met een glucose sterpolymeer. Octreotide beladen PLHMGA microbolletjes werden bereid met copolymeren met verschillende samenstelling D,L melkzuur en HMG (aangeboden verhouding tijdens polymerisatie van 50/50, 65/35 en 75/25 D,L-lactide/BMMG). De beladingsefficiëntie verbeterde door de continue fase te neutraliseren, waardoor vermoedelijk ionisatie en daardoor migratie van het opgeloste peptide van de disperse fase naar de continue fase voorkomen werd. SEM-analyse toonde aan dat PLGA en PLHMGA microbolletjes nauwelijks poreus waren. De afgifte van octreotide uit PLHMGA microbolletjes wordt gekenmerkt door een korte hoge initiële afgifte ('burst') van 10-20% gevolgd door een gereguleerde afgifte van het peptide gedurende 20 tot 60 dagen, afhankelijk van het hydrofiele karakter van het polymeer dat gebruikt werd voor de bereiding van de microbolletjes. De commerciële formulering vertoonde geen 'burst' en een gereguleerde afgifte van het octreotide gedurende 90 dagen, nadat in de eerste 10 dagen nauwelijks peptide afgegeven werd. De totale hoeveelheid afgegeven octreotide uit PLHMGA en PLGA microbolletjes was ongeveer gelijk. Kwantitatieve HPLC analyse toonde echter aan dat voor het grootste deel het natieve octreotide

afgegeven was uit PLHMGA microbolletjes, terwijl PLGA microbolletjes voornamelijk geacetyleerde peptideverbindingen afgaven. MALDI-TOF analyse toonde aan dat de hoeveelheid peptide gemodificeerd met glycolzure eenheden (octreotide-GA) aanzienlijk groter was dan met melkzure eenheden (octreotide-LA). Waarschijnlijk vindt een nucleofiele aanval van de primaire amine groepen van het peptide gemakkelijker plaats op de glycolzure eenheden dan op de melkzure eenheden in het PLGA. Geen octreotide-GA werd aangetroffen in het octreotide dat afgegeven was uit PLHMGA microbolletjes omdat glycolzure eenheden ontbreken in dit polymeer. Wel werd een geringe mate van acylering met HMG gevonden. Drie factoren worden verondersteld bij te dragen aan de geringe mate van peptide acylering die plaatsvindt bij afgifte vanuit PLHMGA microbolletjes vergeleken met het peptide dat afgegeven wordt vanuit de PLGA microbolletjes: 1) het ontbreken van de meer reactieve glycolzure eenheden in het PLHMGA, 2) de kortere afbraaktijd van de PLHMGA microbolletjes vermindert de blootstellingtijd van het eiwit aan het polymeer 3) het veel mindere zure milieu in de PLHMGA omgeving gedurende de afbraak. Uit literatuur blijkt dat peptide acylering bevorderd kan worden door een lage pH in de directe reactie-omgeving en vandaar dat de acylering langzamer verloopt in een ontledende PLHMGA omgeving die een hogere pH heeft dan die van een ontledende PLGA matrix, zoals aangetoond is in **hoofdstuk 6.**

Een grote uitdaging is om een pH verlaging in degraderende PLGA microbolletjes te voorkomen, die ongunstig is voor de integriteit van zuur-labiele moleculen zoals peptiden en eiwitten.. We bestudeerden de pH van ontledende PLHMGA microbolletjes (µpH) in **hoofdstuk 6** en de resultaten werden vergeleken met die van de µpH van degraderende PLGA bolletjes. Het effect van de copolymeersamenstelling en polymeerconcentratie die gebruikt werden voor het maken van het PLHMGA op de µpH werd bestudeerd. Er werd gebruik gemaakt van confocale scanning microscopie en Lysosensor geel/blauw® dextran als fluorescent pH gevoelig molecuul om de mate en snelheid van de verandering van de pH in degraderende PLHMGA en PLGA microbolletjes te bestuderen. PLHMGA microbolletjes vervaardigd van copolymeren met 17.5 en 12.5% HMG vertoonden geen pH verandering in het bolletje tijdens incubatie in een bufferoplossing van pH en bij 37 °C (met uitzondering van de eerste dag van incubatie voor de microbolletjes vervaardigd met een copolymeer met 12.5% HMG en een concentratie in DCM 35%). Gedurende de afbraak van de microbolletjes worden de zure ontledingsproducten (melkzuur en hydroxymethyl glycolzuur en hun oligomeren) vrijgegeven in het incubatiemedium (PBS, 10 mM). Dit werd bevestigd door de dalende pH in het incubatiemedium. Daarentegen toonden de confocale beelden van degraderende PLGA microbolletjes dat in de bolletjes een lage pH wordt ontwikkeld. Dit betekent dat

de zure degradatieproducten niet afgegeven worden wat wordt bevestigd door de waarneming dat de pH van het incubatiemedium constant bleef op pH 7.4

Concluderend, dit proefschrift beschrijft de toepassing van nieuwe biodegradeerbare microbolletjes gemaakt van een hydrofiel polyester, poly(D,L-lactide-co-hydroxymethyl glycolide) (PLHMGA) voor de gecontroleerde afgifte van peptide- en eiwitgeneesmiddelen. PLHMGA microbolletjes vertonen superieure eigenschappen ten opzichte van bestaande PLGA systemen betreffende de mogelijkheid voor het sturen van de afbraak- en afgiftesnelheid. De afgifte van biofarmaceutica en de afbraak van de microbolletjes konden succesvol gestuurd en voorspeld worden door gebruik van copolymeren die in samenstelling verschilden. De uitkomsten van het onderzoek in dit proefschrift tonen aan dat PLHMGA microbolletjes potentieel heel geschikt zijn voor biofarmaceutische toepassingen.

List of abbreviations:

ACN	acetonitril
AIBN	azobisisobutyronitrile
$Al(O^iPr)_3$	aluminium isopropoxide
ATRP	atom transfer radical polymerization
BHMG	benzyl-protected hydroxymethyl glycolide
BMMG	benzyl-protected hydroxymethyl methyl glycolide
BnOH	benzyl alcohol
Bis-MPA	2,2'-bis(hydroxymethyl)propionic acid
BSA	bovine serum albumin
CD	circular dichroism
$CDCl_3$	deuterated chloroform
ε-CL	ε-caprolactone
CLSM	confocal laser scanning microscopy
CMC	critical micelle concentration
DCM	dichloromethane
DHO	6,7-dihydro-2(5H)-oxepinone
DMAP	N,N'-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
DXO	1,5-dioxepan-2-one
EM	enzyme-activated monomer
FTIR	fourier transform infrared spectroscopy
GA	glycolide
GPC	gel permeation chromatography
HMGA	hydroxymethyl glycolic acid
hMSCs	human mesenchymal stem cells
HPLC	high performance liquid chromatography
LA	lactide
LC	loading capacity
LDA	lithium diisopropyl amide
LE	loading efficiency
LHRH	luteinizing hormone releasing hormone
LiCl	lithium chloride
mCPBA	m-chloroperoxy benzoic acid
M_n	number average molecular weight
M_w	weight average molecular weight

MTBE	methyl-tert-butyl ether
NaN_3	sodium azide
NMR	nuclear magnetic resonance spectroscopy
PAGA	poly(α-(4-aminobutyl)-L-glycolic acid)
PBS	phosphate buffer saline
PCL	poly(ε-caprolactone)
Pd/C	palladium on carbon
PDI	polydispersity index
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLAL	poly(lactic acid-co-lysine)
PLBMGA	poly(lactic acid-ran-benzyloxymethyl glycolic acid)
PLHMGA	poly(lactic-co-hydroxymethyl glycolic acid)
PLGA	poly(lactic-co-glycolic acid)
PVA	polyvinyl alcohol
RGD	Arg-Gly-Asp
RO	reverse osmosis
ROP	ring opening polymerization
SCVP	self-condensing vinyl polymerization
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
$SnOct_2$	stannous octoate
TBD	1,5,7-triazabicyclo[4.4.0]dec-5-ene
THF	tetrahydrofuran
TFA	trifluoro acetic acid
T_g	glass transition state
T_m	melting temperature
TMC	trimethyl carbonate
UPLC	ultra performance liquid chromatography
UV	ultra violet
V_{cp}	volume of continuous phase
V_{dp}	volume of disperse phase
β-BL	β-butyrolactone
γBrCL	γ-bromo-ε-caprolactone
γEt_3SiOCL	γ-triethylsiloxy-ε-caprolactone

Curriculum Vitae

Amir Hossein Ghassemi was born on July 13th 1979 in Tehran, Iran. After graduating from high school, he passed the National Academic Entrance Exam for Medical Sciences. In September 1997, he started to study pharmacy at Tehran University of Medical Sciences. After obtaining his pharmacist degree (Pharm.D) in July 2003, he worked in public and hospital pharmacies as well as Ministry of Health as a regulatory affair officer and inspector. He worked as a guest researcher at the Department of Pharmaceutical Sciences, Utrecht, the Nethrlands from January 2007 till October 2007. In October 2007 he started his PhD research program at the same university under the supervision of prof. dr. ir. W.E. Hennink and prof. dr. D.J.A. Crommelin. The results of his work are presented in this thesis.

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Patent application

Amir H. Ghassemi, Cornelus F. van Nostrum, Wim E. Hennink
System for controlled drug delivery comprising biodegradable polymer microparticles (WO/2010/123349**).**

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