BIODEGRADABLE MULTIBLOCK COPOLYMERS FOR DRUG DELIVERY APPLICATIONS

Riemke van Dijkhuizen-Radersma

Biodegradable multiblock copolymers for drug delivery applications
R. van Dijkhuizen-Radersma
Thesis University of Twente, Enschede, The Netherlands
With references- with summary in English and Dutch
ISBN 90-365-2055-X

Financial support by OctoPlus Technologies BV (www.octoplus.nl) and minicamping P90 (www.p90.nl) for the publication of this thesis are gratefully acknowledged.

Cover design: Aart van Apeldoorn
Cover: Histology of subcutaneous injected microspheres in rabbits (Chapter 3).

Press: FEBODRUK BV, Enschede, The Netherlands

BIODEGRADABLE MULTIBLOCK COPOLYMERS FOR DRUG DELIVERY APPLICATIONS

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Twente,
op gezag van de rector magnificus,
prof .dr. F.A. van Vught,
volgens besluit van het College voor Promoties
in het openbaar te verdedigen
op vrijdag 2 juli 2004 om 13.15

door

Riemke van Dijkhuizen-Radersma

geboren op 30 november 1973
te Lelystad

Dit proefschrift is goedgekeurd door:

Promotor:	Prof. Dr. K. de Groot
Promotor:	Prof. Dr. C.A. van Blitterswijk
Assistent promotor:	Dr. Ir. J.M. Bezemer

Contents

Chapter 1	General introduction	1
Chapter 2	Biodegradable multiblock copolymers for drug delivery applications: Literature review	11
Chapter 3	Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation	51
Chapter 4	Control of vitamin B_{12} release from poly(ethylene glycol)/ poly(butylene terephthalate) multiblock copolymers	73
Chapter 5	Stability aspects of salmon calcitonin entrapped in poly(ether-ester) sustained release systems	93
Chapter 6	In-vitro and in-vivo evaluation of salmon calcitonin loaded poly(ether-ester) microspheres	107
Chapter 7	In vitro / in vivo correlation for protein release from poly(ether-ester) microspheres	117
Chapter 8	Succinate substituted poly(ethylene glycol)/poly(butylene terephthalate) copolymers: synthesis and characterization	137
Chapter 9	Succinate substituted poly(ethylene glycol)/poly(butylene terephthalate) copolymers: an in-vivo evaluation	159
Chapter 10	Controlled release of proteins from succinate substituted poly(ethylene glycol)/poly(butylene terephthalate) multiblock copolymers	181
Summary		201
Samenvatting		207
Dankwoord		215
Curriculum vitae		217

Chapter 1

General introduction

Controlled release of proteins and peptides

With rapid advances in genomic research and biotechnology, drug companies are developing new protein and peptide-based compounds for a variety of diseases. In contrast to classical drugs, these new compounds are hydrophilic, larger in size, complicated in structure and relatively unstable. These characteristics make them sensitive towards (enzymatic) degradation and poorly permeable across biological barriers. As a consequence, proteins and peptides are hard to deliver by conventional ways, including oral, transdermal and nasal routes [1-4]. Due to poor adsorption and low bioavailability by these non-injectable routes, proteins and peptides are preferable administered parenterally by subcutaneous or intramuscular injection. However, the half-lives of proteins injected parenterally are only several minutes up to a few hours in most cases, thus requiring frequent injection for therapeutic effectiveness. To overcome the problem of patient compliance, extensive research is performed on the development of controlled release systems. These systems should provide a controlled or sustained release of proteins by parenteral route to obtain a well-defined pharmacokinetic profile.

A promising approach would be to encapsulate the protein or peptide drug within a polymer matrix, to protect the drug from rapid clearance and to provide a sustained release [1-6]. A variety of degradable and non-degradable polymers have been utilized as matrices to incorporate the drugs [1,3,7,8]. However, biologically degradable polymers are preferred for drug delivery applications, as surgical removal of the matrix after depletion of the drug is avoided. The continuous release of drugs from the biodegradable polymer matrix could occur either by diffusion of the drug from the matrix, or by the degradation of the polymer or by a combination of the two mechanisms.

Major concerns for these controlled release applications are the stability of the encapsulated proteins and peptides during processing, release and storage and the safety and biocompatibility of the degradable polymer. Despite various challenges, several controlled release formulations based on biodegradable polymers have been approved and marketed (listed in Table 1). As shown in Table 1, the polymer matrix can be formulated as either microspheres, nanospheres, injectable gel or implant. Spheres and gels are preferred over implants, as no surgical procedure for administration is required and weight-based dosing therapy is possible.

Table 1: Marketed controlled release formulations based on biodegradable polymers [1,3,9,10].

Product	**Drug**	**Polymer**	**Formulation**	**Application**
Atridox®	Doxycycline	PLA	gel	periodontal disease
Gliadel®	Carmustin	Polyanhydride	wafer	brain cancer
Lupron Depot®	Leuprolide acetate	PLGA	microspheres	prostate cancer
Nutropin Depot®	Somatropin	PLGA	microspheres	growth hormone deficiency
Sandostatin LAR®	Octreotide acetate	PLGA-glucose	microspheres	growth hormone suppression
Trelstar^{TM} Depot	Triptorelin	PLGA	microspheres	prostate cancer
Zoladex®	Goserelin acetate	PLGA	rod	prostate cancer

Biodegradable polymer matrices for controlled release applications

To be used as drug delivery matrix, the polymer should satisfy the following criteria: it should be biocompatible, degradable within a reasonable period of time (depending on application) and the resulting degradation products should be non-toxic. Furthermore, the polymer should provide an optimal environment for the encapsulated protein or peptide drug, in order to prevent denaturation, which may cause unwanted immunogenicity when administered. In addition, the release rate of the drug should be tailorable by varying the polymer characteristics in order to obtain optimal pharmacokinetics.

Most of the work on biodegradable polymers for drug delivery has focused on poly(lactic-co-glycolic acid) (PLGA) copolymers, which are therefore highly represented in Table 1. These polymers have been utilized as biodegradable surgical suture materials for several decades and are known to be biocompatible [11,12]. The degradation rate can

be modulated (e.g. by varying the lactide / glycolide ratio) and the degradation products (lactic and glycolic acid) are reduced by the Kreb's cycle to carbon dioxide and water. Despite the success with small peptides, such as luteinizing hormone releasing analogues (Table 1), there is considerable concern about the suitability of PLGA as polymeric carrier for high molecular weight protein drugs [e.g. 13-16]. During storage and release, protein unfolding and aggregation often occur because of the interaction of protein molecules with the hydrophobic polymeric surface. Moreover, the low pH generated during polymer degradation could cause chemical degradation of entrapped proteins. Another major problem with PLGA matrices is the limited possibilities to manipulate protein release rates. Frequently, an initial burst has been reported for protein release from these matrices followed by a plateau and subsequently results in incomplete release [16-18]. To overcome these problems other polymer systems are under investigation.

Amphiphilic block copolymers have gained increasing interest for drug delivery applications [19]. By introducing hydrophilic and hydrophobic blocks a protein friendly environment can be created with tailorable release properties. Recently, a series of poly(ether-ester) multiblock copolymers composed of repeating blocks of hydrophilic poly(ethylene glycol)-terephthalate (PEGT) and hydrophobic poly(butylene terephthalate) (PBT) was introduced as matrix for controlled release systems [20-24]. This copolymer system is currently applied for a wide range of biomedical applications [25-27], including a FDA approved cement restrictor. Many in-vitro and in-vivo studies have shown that PEGT/PBT copolymers are biocompatible and can be made biodegradable [26-34]. Quantitative in-vitro release of fully active lysozyme has been reported from these multiblock copolymers [21,35]. A long time constant release of proteins was obtained by a combination of diffusion and degradation. In addition, varying the copolymer composition could precisely modulate the protein release rate.

Aim of the study

So far, PEGT/PBT copolymers have been shown to be biocompatible and degradable as medical device and suitable as matrix for the in-vitro release of proteins. However, the

development of a controlled release system based on these poly(ether-ester)s requires additional research. The aim of this thesis is to answer the following questions:

What is the tissue response towards PEGT/PBT microspheres? All in-vitro and in-vivo studies up to now investigating the biocompatibility and degradability PEGT/PBT copolymers dealt with larger implants [26-32]. Biocompatibility of biomaterials, however, depends to a large extent on the size and the shape of the implant. For controlled release applications, easily injectable microspheres are preferred. Injection of microspheres results in the implantation of a high surface area at a low volume biomaterial, which may show a more intense tissue response [12].

Can PEGT/PBT copolymers be used as matrix for the sustained release of small molecules? Previously reported research on PEGT/PBT copolymers as matrix for controlled release applications focused on the release of high molecular weight model proteins [21-24]. A lot of pharmaceuticals, however, are small molecules like peptides. Therefore, the suitability of PEGT/PBT matrices for the release of a small water-soluble model should be investigated.

Is the in-vitro release from PEGT/PBT matrices predictive for the in-vivo release? All experiments so far using these poly(ether-ester)s as matrix for controlled drug release have been performed in-vitro. However, for many release systems, the in-vivo release behaviour is very often not predicted by the in-vitro release [17,36-38]. Therefore an in-vitro / in-vivo correlation for the release from PEGT/PBT copolymers should be obtained.

Is the degradation of PEGT/PBT sufficiently fast for controlled release applications and can the degradation rate be modified, with preservation of the release characteristics? Extensive evaluations of the poly(ether-ester)s based on PEGT and PBT as biomaterial have addressed the degradation behavior of these type of polymer systems [26-34]. For controlled release applications requiring frequently repeated injections, the degradation rate of some PEGT/PBT copolymer compositions might be too slow. A known method to increase the degradation rate of aromatic polyesters is by (partial) substitution of the aromatic groups by aliphatic groups [39-41]. The effect of the substitution on the biocompatibility and the release properties has to be evaluated.

Outline of the thesis

A literature overview on the therapeutic potential of biodegradable multiblock copolymers is given in *chapter 2*, including the synthesis and characteristics of these polymer systems and their controlled release applications. In particular, amphiphilic multiblock poly(ether-ester)s based on PEGT and PBT are described in more detail.

In *chapter 3* [42], the in-vivo biocompatibility of PEGT/PBT microspheres of two copolymer compositions is described. The presence of specific cell types indicative for the tissue response is scored. In addition, the cytotoxicity and in-vitro degradation of the microspheres are evaluated.

Chapters 4 to 6 evaluate the suitability of PEGT/PBT copolymers as matrices for small water-soluble molecules, like peptides. In *chapter 4* [43], the effect of the matrix composition on the release of small molecules is investigated. As a model, vitamin B_{12} is incorporated in films of a series of PEGT/PBT copolymers and blends of these copolymers. The development of a controlled release system for a therapeutically active peptide, calcitonin, is studied in *chapter 5* [44]. As this peptide is known for its physical instability, special attention is paid to stabilization of calcitonin in the matrix. In *chapter 6*, the results of in-vitro and in-vivo studies on calcitonin-loaded PEGT/PBT microspheres are reported. The in-vitro study focuses on the encapsulation and calcitonin release, whereas in-vivo the pharmacodynamic effect of the microspheres is investigated.

An direct in-vitro / in-vivo correlation for the sustained release of a protein from poly(ether-ester) microspheres is established in *chapter 7* [45]. Three radiolabelled lysozyme loaded PEGT/PBT microspheres varying in copolymer composition are evaluated for their in-vitro release in a phosphate buffer and in-vivo release in rats.

In developing a polymer system with more suited degradation behavior for controlled release applications requiring frequently repeated injections, the terephthalate units are (partly) replaced by succinate units. In *chapter 8* [46], the synthesis of these new series of poly(ether-ester)s and the characterization for their thermal properties, swelling in water and in-vitro degradation are described. The in-vivo degradation and biocompatibility is addressed in *chapter 9* [47]**.** The effect of certain ions or radicals, present in-vivo, is evaluated by comparing the in-vitro and in-vivo degradation of porous sheets. The application of these new poly(ether-ester) multiblock copolymers as matrices

for the controlled release of proteins is evaluated in *chapter 10* [48]. A mechanism for the release of model proteins, lysozyme and bovine serum albumin, from films is proposed.

References

1. Sinha VR, Trehan A, Biodegradable microspheres for protein delivery. J. Control. Release 90 (2003) 261-280.
2. Cleland JL, Daugherty A, Mrsny R, Emerging protein delivery methods. Current opinion in biotechnology 12 (2001) 212-219.
3. www.drugdel.com
4. www.drugdeliverytech.com
5. Langer R, Polymeric delivery systems for controlled drug release. Chem. Eng. Commun. 6 (1980) 1-48.
6. Schwendeman SP, Costantino HR, Gupta RK, Langer R, Peptide, protein, and vaccine delivery from implantable polymeric systems: progress and challenges. In K. Park, editor, Controlled drug delivery, challenges and strategies, Am. Chem. Soc. Washington, D.C., 1996 pp 229-267.
7. Kumar N, Ravikumar MNV, Domb AJ, Biodegradable block copolymers, Adv. Drug Del. Rev. 53 (2001) 23-44.
8. Kissel T, Li Y, Unger F, ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins. Adv. Drug. Del. Rev. 54 (2002) 99-134.
9. Whitaker MJ, Howdle SM, Shakesheff KM, Polymeric delivery of protein-based drugs. Business Briefing: Pharmatech (2002) 118-123.
10. Okada H, One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv. Drug Del. Rev. 28 (1997) 43-70.
11. Couvreur P, Puisieux F, Nano- and microparticles for the delivery of polypeptides and proteins. Adv. Drug Del. Rev. 10 (1993) 141-162.
12. Anderson JA, Shive MS, Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv. Drug Del. Rev. 28 (1997) 5-24.
13. Lu W, Park TG, Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. J. Pharm. Sci. & Technol. 49 (1995) 13-19.
14. Jiang G, Woo BH, Kang F, Singh J, DeLuca PP, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. J. Control. Release 79 (2002) 137-145.
15. Aubert-Pouëssel A, Bibby DC, Vernier-Julienne MC, Hindré F, Benoît JP, A novel in vitro delivery system for assessing the biological integrity of protein upon release from PLGA microspheres. Pharm. Res. 19 (2002) 1046-1050.

16. Morita T, Sakamura Y, Horikiri Y, Suzuki T, Yoshino H, Evaluation of in vivo release characteristics of protein-loaded biodegradable microspheres in rats and severe combined immunodeficiency disease mice. J. Control. Release 73 (2001) 213-221.
17. Jiang G, Qiu W, DeLuca PP, Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres. Pharm. Res. 20 (2003) 452-459.
18. Johansen P, Corradin G, Merkle HP, Gander B, Release of tetanus toxoid from adjuvants and PLGA microspheres: How experimental set-up and surface adsorption fool the pattern. J. Control. Release 56 (1998) 209-217.
19. Kumar N, Ravikumar MNV, Domb AJ, Biodegradable block copolymers. Adv. Drug Del. Rev. 53 (2001) 23-44.
20. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, A controlled release system for proteins based on poly(ether–ester) block-copolymers: polymer network characterization. J. Control. Release 62 (1999) 393–405.
21. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA, Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J. Control. Release 64 (2000) 179–192.
22. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 1. Influence of preparation techniques on particle characteristics and protein delivery. J. Control. Release 67 (2000) 233-248.
23. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 2. Modulation of release rate. J. Control. Release 67 (2000) 249-260.
24. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Control of protein delivery from amphiphilic poly(ether ester) multiblock copolymers by varying their water content using emulsification techniques. J. Control. Release 66 (2000) 307-320.
25. Radder AM, Leenders H, Van Blitterswijk CA, Application of PEO/PBT copolymers for bone replacement. J. Biomed. Mater. Res. 30 (1996) 341-351.
26. Beumer GJ, Van Blitterswijk CA, Ponec M, Biocompatibility of degradable matrix induced as a skin substitute: an in vivo evaluation. .J Biomed. Mater. Res. 28 (1994) 545-552.
27. Bakker D, Van Blitterswijk CA, Hesseling SC, Koerten HK, Grote JJ. Biocompatibility of a polyether urethane, polypropylene oxide and a polyether polyester copolymer. A qualitative study of three alloplastic tympanic membrane materials in rat middle ear. J. Biomed. Mater. Res. 24 (1990) 489-515.
28. Van Loon, Thesis University of Leiden, The Netherlands, 1995.
29. Beumer GJ, Van Blitterswijk CA, Ponec M, Degradation behaviour of polymeric matrices in (sub)dermal and muscle tissue of the rat: a quantitative study. Biomaterials 15 (1994) 551-559.
30. Van Blitterswijk CA, Van de Brink J, Leenders H, Bakker D, The effect of PEO ratio on degradation, calcification and bone bonding of PEO/PBT copolymer (PolyActive). Cell and Materials 3 (1993) 23-36.
31. Bakker D, Van Blitterswijk CA, Hesseling SC, Grote JJ, Deams WT, Effect of implantation site on phagocyte/polymer interaction and fibrous capsule formation. Biomaterials 9 (1988) 14-21.

32. Radder AM, Leenders H, Van Blitterswijk CA, Interface reactions to PEO/PBT copolymers (Polyactive®) after implantation in cortical bone. J. Biomed. Mater. Res. 28 (1994) 141-151.
33. Deschamps AA, Claase MB, Sleijster MWJ, De Bruijn JD, Grijpma DW, Feijen J, Design of segmented poly(ether ester) materials and structures for tissue engineering of bone. J. Control. Release 78 (2002) 175-186.
34. Deschamps AA, Van Apeldoorn AA, Hayen H, De Bruijn JD, Karst U, Grijpma DW, Feijen J, In vivo and in vitro degradation of poly(ether-ester) block copolymers based on poly(ethylene glycol) and poly(butylene terephthalate). Biomaterials 25 (2004) 247-258.
35. Van de Weert M, Van Dijkhuizen-Radersma R, Bezemer JM, Hennink WE, Crommelin DJA, Reversible Aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer. Eur. J. Pharm. Biopharm. 54 (2002) 89-93.
36. Machida Y, Onishi H, Kurita A, Hata H, Morikawa A, Machida Y, Pharmacokinetics of prolonged-release of CPT-11-loaded microspheres in rats. J. Control. Release 66 (2000) 159-175.
37. Soriano I, Evora C, Llabrés M, Preparation and evaluation of insulin-loaded poly(DL-lactide) microspheres using an experimental design. Int. J. Pharm. 142 (1996) 135-142.
38. Cleland JL, Johnson OL, Putney S, Jones AJS, Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development. Adv. Drug Del. Rev. 28 (1997) 71-84.
39. Tokiwa Y, Suzuki T, Hydrolysis of copolyesters containing aromatic andaliphatic ester blocks by lipase. J. Appl. Polym. Sci. 26 (1981) 441-448.
40. Heidary S, Gordon III B, Hydrolyzable Poly(ethylene terephthalate). J. Environ. Polym. Degrad. 2 (1994) 19-26.
41. Müller RJ, Kleeberg I, Deckwer WD, Biodegradation of polyesters containing aromatic constituents. J. Biotech. 86 (2001) 87-95.
42. Van Dijkhuizen-Radersma R, Hesseling SC, Kaim PE, De Groot K, Bezemer JM, Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation. Biomaterials 23 (2002) 4719-4729.
43. Van Dijkhuizen-Radersma R, Péters FLAMA, Stienstra NA, Grijpma DW, Feijen J, de Groot K, Bezemer JM, Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers. Biomaterials 23 (2002) 1527-1536.
44. Van Dijkhuizen-Radersma R, Nicolas HM, Van de Weert M, Blom M, De Groot K, Bezemer JM, Stability aspects of salmon calcitonin entrapped in poly(ether-ester) sustained release systems. Int. J. Pharm. 248 (2002) 229-237.
45. Van Dijkhuizen-Radersma R, Wright SJ, Taylor LM, John BA, De Groot K, Bezemer JM, In vitro / in vivo correlation for ^{14}C-methylated lysozyme release from poly(ether-ester) microspheres. Pharm. Res. 21 (2004) 484-491.
46. Van Dijkhuizen-Radersma R, Roosma JR, Kaim P, Métairie S, Péters FLAMA, De Wijn J, Zijlstra PG, De Groot K, Bezemer JM, Biodegradable poly(ether-ester) multiblock copolymers for controlled release applications. J. Biomed. Mater. Res. 67A (2003) 1294-1304.

47. Van Dijkhuizen-Radersma R, Roosma JR, Sohier J, Péters FLAMA, Van den Doel M, Van Blitterswijk CA, De Groot K, Bezemer JM, Biodegradable poly(ether-ester) multiblock copolymers for controlled release applications: an in-vivo evaluation. Submitted.
48. Van Dijkhuizen-Radersma R, Métairie S, Roosma JR, De Groot K, Bezemer JM, Controlled release of proteins from degradable poly(ether-ester) multiblock copolymers. Submitted.

Chapter 2

Biodegradable multiblock copolymers for drug delivery applications: literature review

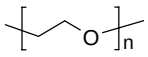
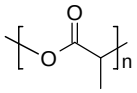
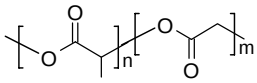
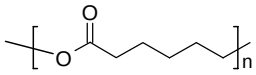
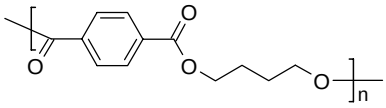
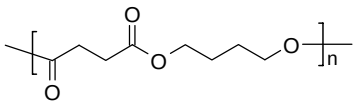
Introduction

The tremendous growth in biotechnology and the recent sequencing of the human genome resulted in an increasing number of pharmaceutical proteins and peptides. However, as discussed in chapter 1, the efficient delivery of these drugs is hampered by their large size and (biological) instability. Drug delivery systems are developed to overcome these difficulties [1-3]. One way to obtain therapeutic drug levels is by encapsulating the protein or peptide into a biodegradable polymer that protect the drug against inactivation and prolong their release over a few weeks. Most of the work on degradable polymers for controlled release applications has focused on matrices composed of poly(lactic-co-glycolic acid) (PLGA) because of their good biocompatibility, low toxicity and biodegradation characteristics [4,5]. Despite the success with small peptides, such as luteinizing hormone releasing analogues [6], there is considerable concern about the suitability of PLGA as polymeric carrier for high molecular weight protein drugs [e.g.7-10]. During storage and release, protein unfolding and aggregation often occur because of the interaction of protein molecules with the hydrophobic polymeric surface. Moreover, the low pH generated during polymer degradation could cause chemical degradation of entrapped proteins. Another major problem with PLGA matrices is the limited possibilities to manipulate protein release rates. Frequently, an initial burst has been reported for protein release from these matrices followed by a plateau and subsequently results in incomplete release [G10-12].

To overcome these problems amphiphilic block copolymers have gained increasing interest for drug delivery applications [13-15]. By introducing hydrophilic

blocks a protein friendly environment can be created. Materials with a broad range of properties can be obtained by varying the type, length or weight ratio of the building blocks. As hydrophilic block, poly(ethylene glycol) (PEG), also referred to as poly(ethylene oxide) (PEO), is most frequently used as it is nontoxic, biocompatible and can be excreted by the kidney [13,16] (Table 1). Indeed, PEG has been cleared by the US Food and Drug administration (FDA) for internal use in the human body [17]. Examples of frequently used hydrophobic blocks are polyesters like poly(lactic acid) (PLA) [15,18-23], poly(lactic-co-glycolic-acid) (PLGA) [24,25], poly(ε-caprolactone) (PCL) [15,16,26-29], poly(butylene terephthalate) (PBT) [30-34] and poly(butylene succinate) (PBS) [35] (see Table 1).

Table 1: Building blocks frequently used in amphiphilic block copolymers

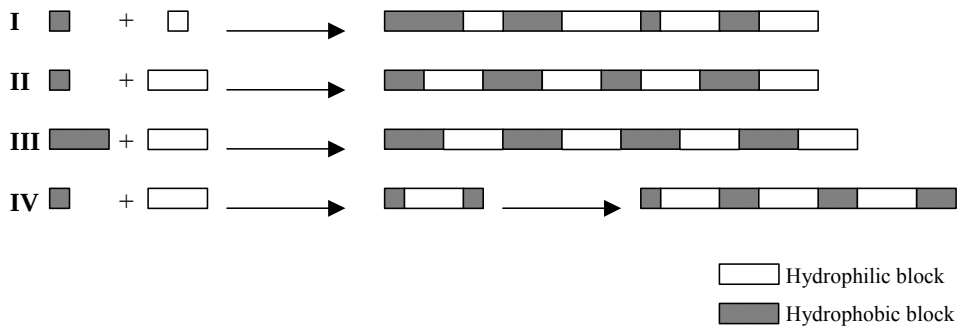
Name	Abbreviation	Formula
Poly(ethylene glycol)	PEG	
Poly(lactic acid)	PLA	
Poly(lactic-co-glycolic acid)	PLGA	
Poly(ε-caprolactone)	PCL	
Poly(butylene terephthalate)	PBT	
Poly(butylene succinate)	PBS	

Amphiphilic block copolymers are classified into di-block copolymers (AB-type), tri-block copolymers (ABA-type), multiblock copolymers ($(AB)_n$-type) and star block copolymers. In this review, we will focus on the multiblock copolymers as matrix

for controlled release applications. An advantage of multiblock copolymers over di- and tri-block copolymers is the wide range of properties due to the variety in molecular weight of the building blocks and the hydrophilic/hydrophobic block ratio. The molecular weights and the hydrophilic/hydrophobic block ratio of di-and tri-block copolymers are limited by the molecular weight of the PEG segment [36-39]. As high molecular weight PEG blocks are likely to accumulate in the body, preferably only short PEG segments are copolymerized [37-39]. For an extensive review of the possibilities of ABA-triblock copolymers for protein delivery systems we refer to Kissel et al. 2002 [14]. An interesting approach of drug delivery, by drug conjugates based on multiblock copolymers [40] is also outside the scope of this review. In this review, the synthesis and characterization of amphiphilic multiblock copolymers as well as their biological and degradation properties will be discussed. Subsequently their use as biodegradable drug delivery devices in the form of microspheres, nanospheres, implants and gels are reviewed.

Synthesis

Several methods have been described for the synthesis of amphiphilic multiblock copolymers. These methods are based on the coupling of either monomers or pre-synthesized blocks or a combination of these two. Scheme 1 shows possible synthesis routes for multiblock copolymers, which are reviewed in more detail below. In general, two mechanisms of copolymerization can be distinguished for the coupling of the monomers and /or oligomers: polycondensation and ring-opening [41]. Polycondensation can be applied for a variety of combinations of monomers, with or without coupling agent, and results in the formation of characteristic linkages such as ester, amide or urethane bonds. In general, polycondensation requires higher temperature and/or longer reaction time to obtain high molecular weight polymers. In addition, polymers obtained by polycondensation show a wide chain length distribution. In contrast, ring-opening polymerization has a restriction on monomers, but it can be carried out under milder reaction conditions to produce high molecular weight polymers in shorter time. Furthermore, recent progress in catalysts and initiators for living polymerization enables the production of polymers with controlled chain lengths [41].



Scheme 1: Routes of synthesis for amphiphilic multiblock copolymers: direct copolymerization (I), block copolymerization (II), block coupling (III) and triblock coupling (IV).

I: Direct copolymerization

Multiblock copolymers may be synthesized by direct copolymerization of the monomers. The success of this method depends on the monomers and the reaction conditions, as also extremes like a random copolymer or the homopolymers can be easily formed. Therefore, careful selection of the catalyst and the reaction conditions is required to regulate the molecular weight and average block length of the multiblock copolymer.

Chen et al. successfully carried out (L)-lactide ((L)-LA)/ethylene oxide (EO) ring-opening copolymerizations by using various catalyst including $AlR_3 \cdot 0.5H_2O$ systems (R = ethyl, isobutyl) and Sn-Al bimetallic catalysts [42]. To remove PEO homopolymers and high EO containing copolymers, the synthesized products were extracted in methanol. ^{1}H NMR (nuclear magnetic resonance spectroscopy) analysis showed that the methanol insoluble copolymer fractions had multiblock structures based on PLA and PEO. The multiblock segment length and the molecular weight of the copolymers varied by changing the reaction temperature, reaction time, reaction medium and catalyst structure. An increase in the reaction temperature resulted in shorter segment block lengths. Bulk reactions at elevated temperatures gave shorter block lengths than those of corresponding polymerizations conducted in solution (xylene). However, adequate control of the average block length and the distribution of the blocks was difficult using this method. At a monomer feed of (L)-LA/EO:1/2, the copolymer composition varied from 20:80 up to 62:38, depending on the reaction conditions (in solution). In addition, the average hydrophobic block varied from 4.7 up to 22 (l)-lactide units. On the other hand, Wen et al. used one-step ring-opening copolymerization for the synthesis of

poly(D,L,lactide-co-ethyl ethylene phosphate) (PLAEEP) [36]. Although not shown, the authors claim that the final molar ratios of PLA and PEEP in the copolymers were in good agreement with the monomer feeding ratios. As could be expected, however, differential scanning calorimetry (DSC) indicated the random nature of the copolymers instead of multiblock copolymers. Based on these two examples, we conclude that direct copolymerization of monomers is not the most suitable synthesis method for the design of tailor-made amphiphilic multiblock copolymers with desired properties.

II. Block copolymerization

To be able to synthesize multiblock copolymers with desired block lengths, pre-synthesized blocks are introduced in the copolymerization process. In most cases the hydrophilic block is pre-synthesized, while the hydrophobic block is polymerized by either polycondensation or ring-opening.

Polymerization of hydrophobic monomers onto pre-synthesized hydrophilic blocks by polycondensation has been described for several hydrophobic compounds, such as butylene terephthalate [43,44], ethylene terephthalate [45-48], butylene succinate [35] and ethylene succinate [49-51]. In general, a two-step melt polycondensation procedure is followed, which involves the hydrophilic blocks (mainly PEG) and (methyl esters of) dicarboxylic acids and diols for the hydrophobic blocks. For examples, the synthesis of multiblock copolymers based on PEG and poly(butylene terephthalate) (PBT) is as follows [43]: The first step involves trans-esterification of dimethyl terephthalate (DMT) with PEG and an excess of 1,4-butanediol at approximately 200^{o}C, as methanol is removed by distillation. Then, polycondensation is performed at about 240^{o}C, using vacuum to facilitate removal of the condensation product 1,4-butanediol. By varying the feed ratio of PEG to 1,4-butanediol, or the molecular weight of the hydrophilic PEG blocks, a family of polymers can be obtained. Various catalysts are used in this process, such as titanium tetrabutoxide [32] or antimony trioxide [45,46]. In addition, often an antioxidant, as Irganox [32] or α-tocopherol [52], is added. Despite of the high reaction temperatures, amphiphilic multiblock copolymers of weight average molecular weights above 100 kg/mole have been reported [30,45] with compositions consistent with the feed [50], using this synthesis method. The reaction mixture in the synthesis of poly(ethylene succinate) (PES)/PEG, however, was extremely sensitive to thermal

degradation during the final vacuum stage, that no high molecular weight copolymer could be obtained. Therefore, Albertsson et al. introduced an extra chain extension step with adipoyl chloride [49,51].

Besides the synthesis of the poly(ether-ester) multiblock copolymers described above, polycondensation is also used to prepare hydrophilic/hydrophobic poly(ether-ester-amides) [52,53]. The synthesis of these multiblock copolymers was similar to the synthesis of PEG/PBT based poly(ether-ester)s. However instead of using the dimethylterephthalate, biester-bisamide monomers were selected. These were prepared from 1,4-diaminobutane and dimethyl adipate [52]. The obtained composition of the resulting copolymers was in good agreement with the polymer composition expected from the feed composition. In conclusion, the block copolymerization by polycondensation is a suitable method for the synthesis of amphiphilic multiblock copolymers. It should, however, be noted that the hydrophobic block length can only be considered as an average block length, which is determined by both the hydrophilic block length and the hydrophobic/hydrophilic ratio.

Copolymerization involving preformed hydrophilic blocks and ring-opening has been described for lactide [54,55], ε-caprolactone [55,56] and a combination of these monomers [39,55]. This method is mainly used in the synthesis of a particular type of multiblock copolymers, namely five-block copolymers. Huang et al. described the syntheses of PLA-PCL-PEG-PCL-PLA copolymers by a two-step bulk ring-opening reaction [39]. In the first step, ring-opening polymerization of ε-caprolactone initiated by dihydroxyl PEG formed an ABA-type triblock copolymer with a central PEG block and two lateral PCL blocks. The triblock copolymer served as macroinitiator and opened the ring of DL-lactide in the second step. Both reactions were catalyzed by zinc metal, which was selected because of its biocompatibility, in contrast to stannous octoate or other catalysts. ^{1}H NMR and SEC (size exclusion chromatography) analysis showed that the molecular weight and the LA/CL/EO molar ratio of the resulted polymers could be tailored by the feed composition. Other five-block copolymer systems are based on pre-synthesized hydrophilic tri-blocks, such as Pluronics (PEO-PPO-PEO), which are extended with hydrophobic blocks [54-56]. Pluronics are commercially available nonionic macromolecular surface-active agents and are used in pharmaceutics [56,57]. Catalyzed by stannous octoate, hydrophobic blocks as PLA [54,55] and PCL [55,56] are

polymerized on the hydrophilic tri-block. The resulting five-blocks, however, are limited in varying their composition as, similar to triblock copolymers, the length of the (non-degradable) hydrophilic segments is restricted. On the other hand, the particular structure of these five-blocks offers specific properties including micelle formation [56] and thermosensitivity [55], which may be useful for controlled release applications (see paragraph 6).

III. Block coupling

Multiblock copolymers containing hydrophobic and hydrophilic segments of controlled length can be obtained by coupling of well-defined prepolymers. Both hydrophobic and hydrophilic blocks are pre-synthesized and subsequently connected by a (degradable) linkage, like e.g. ester [15,22,23,28,29,41,58-62], carbonate [20,24,25,41], amide [26,41,63] or urethane [27,41,64] bonds. As the blocks are, in general, coupled by polycondensation, the starting oligomers must have reactive terminal groups to obtain multiblock copolymers. Various combinations of reactive end-groups have been studied with or without using a coupling or condensing agent. The functionality of the terminal groups of the oligomers may be significant factor affecting the molecular weight of multiblock copolymers [59].

The ester bond as the condensation product of a hydroxy- and a carboxy-termal group is the most frequently used linkage in the block coupling synthesis of multiblock copolymers for biomedical purposes [15,22,23,28,29,58-62]. In most cases, both the pre-synthesized hydrophilic (e.g. PEG) and the hydrophobic (e.g. PCL or PLA) blocks are diols of selected molecular weight. To be able to condensate either the hydrophilic [15,22,29,60] or the hydrophobic [28,59] blocks are transferred into dicarboxylated blocks by reaction of the hydroxyl end-groups with succinic anhydride. Another method to obtain carboxylate termal-groups, is to incorporate succinic acid [23,58] or citric acid [59] during the pre-synthesis of the hydrophobic block. Huh et al. varied the molecular weight of the PLA blocks by changing the feed ratio of L-lactic acid to succinate acid [23]. Subsequently, the dihydroxylated and the dicarboxylated blocks are coupled in the presence of dicyclohexylcarbodiimide (DCC) and N-dimethylaminopyridine (DMAP). DCC is added as condensing agent and formed into dicyclohexylurea precipitate as reaction byproduct. DMAP is an efficient catalyst for the esterification of organic acid

and alcohol with high steric hindrance at ambient temperature [59]. The resulting weight average molecular weight (M_w) of the multiblock copolymers varied from approximately 15 kg/mol [22,23,60] up to 40 kg/mol [23] depending on the composition. The polydispersity (M_w/M_n) was around 2 for all compositions, which is expected for polymers prepared by a polycondensation reaction. Yamaoka et al. used thermal dehydration to prepare amphiphilic multiblock copoly(ether-ester)s consisting of hydrophobic PLA and a hydrophilic Pluronic [61]. As the Pluronic was a diol and the lactide oligomers contained both hydroxyl and carboxyl end-groups, dodecanedioic acid (DDA) was added as a carboxyl/hydroxyl adjusting agent. The reaction conditions were optimised by varying the catalyst, pressure, reaction time and temperature and the amount of solvent. Using stannous oxide as a catalyst at 180^{o}C over 20 hours resulted in multiblock copolymers with a M_w value exceeding 100 kg/mol.

In 1995, Ferruti et al. introduced a chain-extension process leading to high molecular weight poly(ester-carbonates) based on PLGA and PEG [20]. This synthetic process involves the activation of PEG oligomers by means of phosgene, resulting in the introduction of terminal chloroformate groups [20,24,25]. Without isolating, activated PEGs were reacted with PLGA oligomers containing both hydroxyl and carboxyl end-groups. The subsequent chain extension process is a combination of two different reactions. The first one, the reaction of a chloroformate group with an alcoholic group to give a carbonate group with the formation of one molecule of HCl, is common in organic chemistry. The second one, the reaction of a chloroformate group with a carboxylic group to give an ester group with the loss of one molecule of HCl and one molecule of CO_2, is less usual. The M_w values of the synthesized multiblock copolymers varied from 180 kg/mole up to 430 kg/mole, although the authors indicated that the polystyrene standards used in the GPC measurements may not be appropriate [20].

Poly(ether-ester-amide)s synthesized by block coupling have been reported by Barbato et al. [26] (containing PCL) and D'Angelo et al [63] (containing PLA). Both authors used diamines as hydrophilic blocks and activated the dihydroxy terminated hydrophobic blocks with sebacoyl chloride. The obtained multiblock copolymers showed M_w values of approximately 50 kg/mol and a polydispersity index of around 2.

Thermosensitive biodegradable multiblock copolymers coupled by urethane linkages are synthesized by several groups [27,64-66]. Lee et al. described the synthesis

of PEG/PCL multiblock copolymers with various block lengths through one-step condensation copolymerization with hexamethylene diisocyanate (HMDI) as a coupling agent [27]. Poly(ether-ester-urethane)s with narrow polydispersities were obtained (1.3-1.6). In addition, the copolymer compositions were consistent with the feed ratios in all cases.

The last type of multiblock copolymers synthesized by block coupling that will be addressed here are PEG-containing polyanhydrides. Jiang et al. reported the introduction of PEG segments into a polyanhydride main chain by copolymerization of terminal-carboxylated PEG with diacidic monomers, like adipic acid, sebacic acid and dodecanoic acid [67,68]. Melt polymerization of the prepolymers at 180^{o}C for 2 hours under high vacuum resulted in multiblock copolymers with M_w values lower than 10 kg/mol at a yield of more than 80%. When comparing various feeds, it was observed that the compositions were almost equal to the feed composition indicting equal reactivity of the SA prepolymer and the PEG prepolymer. It seems that the prepolymer type does not influence its reactivity, which basically depends on its end-functional groups. The authors indicate that transesterification of the various prepolymers during melt polycondensation may also play an important role.

In conclusion, block coupling is a very suitable method for the synthesis of amphiphilic multiblock copolymers containing blocks of predetermined length. It can, however, not be excluded that in case of coupling of blocks with similar reactive end-groups, the hydrophilic and hydrophobic blocks may not completely alternate along the chain.

IV. Triblock coupling

A synthesis method for the preparation of amphiphilic multiblock copolymers close to the block coupling discussed before, is coupling of pre-synthesized triblocks. These triblocks are usually of the ABA-type in which A are hydrophobic segments and B is a hydrophilic segment. Several groups studied the coupling of tri-block for the formation of multiblock copolymers based on either PCL [69-71] or PLA [19,37,38] and PEG. Firstly, the triblock copolymers were synthesized by ring-opening polymerization of CL or LA, initiated by the hydroxyl end-groups of the PEG chain. Triblocks with different hydrophobic and hydrophilic block lengths could be obtained by using different molecular weight PEG as

initiators and different hydrophobic monomer/PEG molar ratios [38]. Thereafter, the amphiphilic multiblock copolymers were synthesized by coupling of the triblocks. Similar to block coupling, various type of linkages have been evaluated.

Polyester coupling linkages were created with succinic anhydride in the presence of DCC and DMAP by Chen et al. [37,38]. At a yield of 85%, multiblock copolymers with M_w values up to 66 kg/mole were obtained. According to the authors, their method of triblock coupling was simpler and more effective compared to the block coupling method resulting in similar multiblock polymers. Panoyan et al. used adipic chloride as linking agent to couple PLA-PEG-PLA triblocks by ester bonds [19].

Others created urethane bonds for the coupling of triblocks [69-71]. Similar as discussed above for block coupling, Cohn et al. obtained a family of poly(ether-ester-urethane) block copolymers by chain extension of OH-terminated PCL-PEG-PLC triblocks with HMDI [71]. Guan et al. used 1,4-diisocyanatobutane to activate the hydroxy groups and added in a second step putrescine as chain extender to form poly(ether-ester-urethane) urea elastomers [70]. Multiblock copolymers were obtained with M_w values up to 112 kg/mole at a polydispersity varying from 1.4 up to 2.3 depending on the PEG segment length and the molar percentage of PCL.

In conclusion, assuming triblock symmetry, coupling of triblocks is an efficient method to synthesize high molecular weight multiblock copolymers with alternating hydrophilic and hydrophobic blocks along the polymer chain.

Physical properties

Combining hydrophilic and hydrophobic blocks in one polymer chain leads to a whole family of biomaterials with properties differing from the corresponding homopolymers. The amphiphilic multiblock copolymers described above have been characterized with respect to their physical properties using a wide range of methods. In this paragraph, we summarize the physical properties that may be relevant for controlled release applications.

Thermal properties

In block copolymers, the distinctive properties of the different blocks are preserved to a certain extent. Consequently, block copolymers often show multiple melting and glass transition points, which represent the different building blocks. As homopolymers, the hydrophilic and hydrophobic blocks are usually incompatible and would therefore show phase separation upon blending. In case of multiblock copolymers where the different blocks are covalently linked, macrophase separation is impeded. Microphase separation, however, can still occur, depending on the block length and block ratio. An increase in molecular weight of the blocks favors phase separation. In addition, crystallization upon cooling of one of the components is an additional driving force for phase separation.

Fakirov and coworkers investigated the influence of both the block length and the block ratio on the thermal properties of poly(ether-ester)s based on PEG and PBT [72,73]. Using differential scanning calorimetry (DSC), small angle X-ray scattering (SAXS) and wide angle X-ray scattering (WAXS), it was found that domains of four different types may exist in PEGT/PBT copolymers: crystalline PBT, amorphous PBT, amorphous PEG and crystalline PEG. The ratio of the different phases is dependent on the polymerization conditions, copolymer composition and the thermal and mechanical history of the sample [74]. Concerning the effect of the copolymer composition, for polymers with a low PBT content (24 wt%) and consequently short PBT sequences, no crystalline PBT phase was found. The butylene terephthalate units could not aggregate to form crystalline regions, since they were short and present in low concentration. Increasing the molecular weight of the PEG-segments at a constant PEGT/PBT ratio, or increasing the PBT content at a constant PEG segment length will increase the average block length of the PBT segments and thus facilitate PBT crystallization. Higher melting temperatures and an increase in the heat of fusion of the PBT confirmed this. The PBT melting temperatures of the multiblock copolymers (184-224^{o}C), however, were lower than expected from the PBT homopolymer (226^{o}C), indicating the formation of smaller or less crystals due to the presence of PEG [35,75]. In addition, the PBT melting temperatures of the multiblock copolymers were relatively broad due to the random condensation process during the synthesis, which leads to the formation of chains with a distribution of PBT sequences [32]. The distance between the crystalline PBT domains was strongly dependent on the molecular weight of the used PEG segment length,

whereas the PEG/PBT ratio does not alter the long spacing significantly [72,73]. Crystalline PEG domains were only observed in PEGT/PBT multiblock copolymers for PEG segments of 2000 g/mole or larger and low weight fractions of PBT [32,73]. This observation was also reported for other amphiphilic multiblock polymer systems [22,47,58,60].

Multiblock copolymers, such as PEG/PLA copolymers, synthesized by block coupling contain blocks of pre-determined lengths. Similar to the above discussed PEG/PBT system, DSC and X-ray diffractometry of the multiblock copolymer suggested that PLA and PEG blocks were phase separated and the crystallization behavior of one block was markedly affected by the presence of the other block, and in particular the block length [58,60]. The longer the PEG and PLA blocks, respectively, the easier they were able to crystallize [22,37]. When the PLA/PEG copolymer cools down after melting, PLA blocks are solidified and crystallize at higher temperatures. This limits the mobility if the soft PEG segments. Consequently, the crystallization of the PEG is severely hindered by the already solidified PLA chains [23,58]. Often, only one (mediated) glass transition temperature was observed, indicating that the two components got some miscibility in the multiblock copolymer, at least in the amorphous domain [22]. Multiblock copolymers containing relatively short block lengths show a high degree of phase mixing [23].

So far, most research focused on multiblock copolymers crystallized from the melt. However, crystallization behavior from solution may be more relevant for controlled release applications. Lee et al. showed that crystallization of the PLA blocks from solution was much more suppressed than from the melt in the presence of PEG [58]. The crystallization rate of PEG blocks larger than 4000 g/mole was so fast at room temperature that PEG blocks could form the crystalline phase before PLA blocks crystallized. Therefore, PLA blocks could hardly crystallize because of the sudden decrease in the mobility of the PLA chain segment.

Water-uptake

Another material property interesting for controlled release applications is the water-uptake or swelling of the multiblock copolymer. Depending on the degradation mechanism, the swelling will affect the degradation rate. In addition, a higher

hydrophilicity of a biomaterial will, in general, improve its biocompatibility. Most important for drug delivery, however, is the effect of the swelling on the permeability of the matrix for (water-soluble) compounds and on the stability of proteins that are sensitive towards aggregation during incorporation and release.

As expected, the water-uptake of the amphiphilic multiblock copolymer increases with increasing amount of the hydrophilic block [18,20,30,35,37,38,46,48,59,70,71]. In an aqueous environment, the water will penetrate into the hydrophilic domains in the polymer matrix. For example, three molecules of water can be bound to each ethylene glycol unit in PEG [76]. The water-uptake of the polymer matrix, however, also depends on the hydrophobic blocks. Interaction of the PEG segments with the more hydrophobic segments reduces the number of bound water molecules [77,78]. Moreover, physical crosslinks, formed by crystallization and hydrophobic interaction of these blocks, restrict the expansion of the polymer network in water [30,37]. Figure 1 shows the equilibrium swelling ratios of multiblock copolymers based on poly(ethylene glycol) and poly(butylene terephthalate) as function of the amount of hydrophilic PEG blocks for various PEG block lengths.

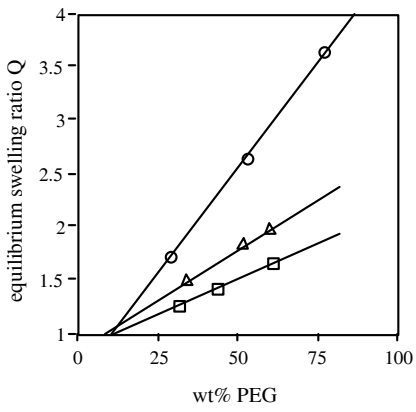


Figure 1: Equilibrium swelling ratio of PEG/PBT copolymers as a function of PEG wt% of the copolymers. Polymers have PEG block lengths of 600 (□), 1000 (Δ) and 4000 (○) g/mole. (n=3; ± s.d.) (from ref. [30]).

At a constant weight percentage of PEG, the swelling ratio increases with increasing PEG segment length. For longer PEG segments, the negative effect of the hydrophobic blocks on the number of bound water molecules in the matrix is less pronounced. In case of a constant PEG block length, the swelling ratio is directly proportional to the PEG content

in the PEG/PBT copolymers. Similar relationships between the swelling and the hydrophilic block content have been observed multiblock copolymer systems based on PEG and PCL [71], PLGA [20], PLA [18,37], PET [46,48,79] and PBS [35], respectively. The equilibrium swelling ratio of the copolymers based on PEG and PBT, however, was less than that of other PEG containing multiblock copolymers with equivalent PEG content and PEG molecular weight. Since swelling of a polymer network in a solvent is dependent on the degree of cross-linking, the lower equilibrium swelling ratio found for the PBT-based hydrogels indicate that the PBT blocks are more efficient in forming physical cross-links than hard segments based on PET and PLGA, for example.

The swelling of the polymer matrix can thus be precisely tailored by varying the multiblock composition. Bezemer et al. reported a correlation between the equilibrium swelling ratio, the mesh size and the permeability of PEGT/PBT copolymers [30]. The mesh size is the space between neighboring chains in the polymer network, and can be considered as an indication for the available space in the matrix for solute diffusion [80]. An adjustable swelling of the matrix may therefore be a powerful tool to control the release of water-soluble compounds.

Thermo-sensitive behavior

Thermo-sensitive behavior has been reported for several amphiphilic multiblock copolymers. For various types of polymers, it has been demonstrated that polymer solubility [23,27,64], water-uptake [15,23,28] and the formation of nanospheres [81] could be triggered by temperature changes.

Lee et al. [27] synthesized a series of PEG/PCL multiblock copolymers. Some multiblock copolymers with optimal molecular weight and compositions displayed a thermoreversible gel-sol transition in water. The polymers could be dissolved in water at high temperatures and formed a hydrogel at low temperatures in case the polymer concentration was above the critical gel concentration (CGC). The phase transition behavior was mainly determined by the PEG/PCL block ratio, the PCL block length and the molecular weight. With increasing PEG/PCL block ratio, the CGC decreased with an elevated sol-gel transition temperature on account of enhanced hydrophobicity. At high molecular weights, the CGC decreased, related to the enhanced aggregation of PCL

blocks and physical crosslinking between PCL block domains due to the increased number of PCL blocks in each molecule. Huh et al. [23] reported comparable results for low molecular weight PEG/PLA multiblock copolymers. Their polymers exhibit clouding at lower critical solution temperatures (LCST) in an aqueous solution. This phase transition was attributed to temperature-dependent hydrogen bonding and hydrophobic interaction of the water-soluble amphiphilic copolymers. By increasing the hydrophobicity of the multiblock copolymer, the LCST increased from 27^{o}C up to 45^{o}C. In particular, copolymers with an LCST around body temperature may offer good potential for applications in drug delivery [23,64].

In case the block copolymers described by Huh et al. had high molecular weights and a high PLA content, they became a water-insoluble, swollen hydrogel. The effect of temperature on the swelling of amphiphilic multiblock copolymers have been subject of several studies [15,28]. The hydrogels showed thermonegative swelling behavior, indicating that higher swelling ratios were obtained at lower temperatures. At low temperature (15^{o}C), the hydrogel swells exclusively by the hydration of the hydrophilic blocks via hydrogen bonding with water. A temperature increase weakens the hydrogen boding, while the hydrophobic interaction within the hydrogel is enhanced [28]. Bae et al. [15] exposed hydrogels based on PEG and PCL or PLA to several temperature cycles. The swelling change of PEG/PCL copolymers was reversible after the first temperature cycle, whereas the PEG/PLA block copolymers showed no reversible swelling even after repeated thermal cycles. Hydrogels based on poly(2-ethyl-2-oxazoline)/PCL copolymers exhibited complete thermally reversible swelling and shrinking behaviors [28]. By adjusting the block composition of the multiblock copolymers, the thermo-sensitive swelling-shrinking capacity could be controlled, which would be a useful feature of thermo-sensitive hydrogels for temperature-sensitive delivery applications.

Another type of thermo-responsive behavior was observed by Kim et al. [81] for nanospheres of five-block copolymers based on Pluronic and PCL. Nanospheres were prepared by dialysis process using Pluronic/PCL block copolymers with different compositions. The copolymeric nanospheres exhibited a change of size depending on the temperature. As the temperature increased, their size gradually decreased. Moreover, this change of size exhibited a reversible tendency according to the repetitive thermal cycles.

Although the exact mechanism is unclear, it seems that interaction of the PPO block (from the Pluronic) with the PCL block influences the reverse tendency of size versus temperature. As the size of the nanospheres may affect the release rate, a thermal-sensitive drug delivery system can be designed.

Degradation

For controlled release applications, biodegradable polymers are preferred because surgical removal of the matrix after treatment is not required. Also no toxicological problems are expected as they degrade in biological fluids to produce biocompatible or nontoxic products, which are removed from the body via normal physiological pathways. In addition, the release rate of the active compound can be tailored by the degradation rate of the matrix.

For most synthetic biodegradable materials, hydrolysis is the most important mode of degradation [82]. There are several factors that influence the velocity of this reaction; such as the type of chemical bond, pH, copolymer composition and water-uptake. The most important parameter for monitoring degradation is molecular weight. The molecular weight of the polymer chains decreases due to chain scission at the hydrolysable bonds. The resulting oligomers undergo either further hydrolysis or leave the polymer matrix. The latter results in mass loss of the polymeric device, which can be monitored, and is referred to as erosion. Besides the degradation rate, polymer erosion depends on many other processes such as swelling, dissolution and diffusion of oligomers and morphological changes [82]. A distinction is made between surface (or heterogeneous) and bulk (or homogeneous) eroding materials. Surface eroding polymers lose material from the surface only and the molecular weight of the residue remains constant [1,82]. Release applications based on these types of polymers depend on the geometric shape of the device, which size decreases in time. Amphiphilic multiblock copolymers, however, undergo mainly bulk erosion. Due to the water-uptake by the hydrophilic blocks, hydrolysis can occur throughout the polymer matrix. Homogeneous degrading polymers therefore show both weight loss and a decrease in molecular weight of the residue. Figure 2 shows the molecular weight and weight loss as function of

degradation time for multiblock copolymers based on PEG and PBT. The PEG segments in these copolymers are kept constant at 1000 g/mole, whereas the PBT content varies from 30 up to 60 wt%. This figure can be considered as a typical plot for the in-vitro degradation of amphiphilic multiblock copolymers, although the time scale is composition dependent.

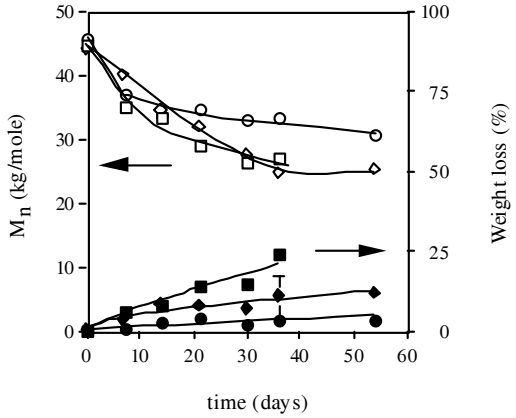


Figure 2: Degradation of 1000PEGT70PBT30 (□), 1000PEGT60PBT40; (◇) and 1000PEGT40PBT60 (O) films in phosphate buffered saline at 37°C. Filled symbols indicate weight loss (n=2 ± s.d.), open symbols represent $\overline{M}_n$ (from ref. [30]).

The molecular weight decreases exponentially in time due to chain scission at the hydrolysable linkages. The introduction of hydrophilic (PEG) blocks in the polymer results into a faster degradation compared to the hydrophobic homopolymers [21,59,65]. Furthermore, independent of the type of hydrophobic block, the degradation rate increases with increasing PEG content in the copolymer [21,29,30,32,44,52,65,70,71,83]. As suggested by Reed and Gilding for PEG/PET block copolymers [83], primary cleavage will be at the linkage between the hydrophilic and the hydrophobic segments, since they are located in the most hydrophilic environment. The number of these bonds as well as the swelling increases with increasing PEG content in the copolymer, resulting in a faster hydrolysis [21,30,35,83]. Besides the PEG content, the degradation rate also depends on the length of the blocks [21,29,35,44,70,71]. This relationship, however, is more complicated as with increasing hydrophilic block length the number of easy hydrolysable bonds decreases, whereas the swelling increases. The resulting effect of these two factors on the degradation rate depends on the copolymer composition. After a certain time period, the decrease of the molecular weight levels off (Fig. 2) due to

solubilization of the low molecular weight oligomers [30,49]. The plateau value as well as the time period to reach this plateau depends on the degradation rate of the copolymer and the dissolution and diffusion of the oligomers.

For controlled release applications requiring frequent injection, weight loss of the polymer matrix is the most important parameter of degradation. To avoid accumulation of polymer in the body, the matrix should disappear within a reasonable period of time (depending on the application). As can be seen from Figure 2, a higher PEG content in the polymer matrix results in a faster weight loss. This phenomenon is, similar to the molecular weight decrease, independent of the type of hydrophobic block [21,30,35,44,52,71,83]. The time scale of mass loss strongly depends on the copolymer composition. For example, complete dissolution within several days has been reported for copolymers based on PEG and PLGA [21,25], whereas only 2 wt% of mass loss was observed after 48 weeks for polyurethanes based on PCL and a Pluronic [66]. In contrast to hydrophobic degradable polymers like PLGA, which show a delayed mass loss profile, continuous loss of weight has been reported from the beginning for amphiphilic multiblock copolymers [21,30,35,52,59,70,83]. Due to the presence of hydrophilic (PEG) blocks, relatively high molecular weight hydrophobic segments can be brought in solution by attached PEG segments [21,44,59]. The preferential release of PEG-rich oligomers, however, may change the copolymer composition during degradation. NMR analysis showed indeed showed a small decrease in PEG content for several copolymer systems [29,30,44,83,84], but no direct indication was given for a remaining residue containing only (inert) hydrophobic segments.

The degradation results discussed so far were obtained from in-vitro experiments. However, as reported for several polymer systems, the in-vitro degradation can differ considerably from the in-vivo degradation. In most cases the in-vivo degradation is faster than the in-vitro degradation [85-91]. This is attributed to the presence of certain ions or radicals, produced by inflammatory cells [90-92], enzymes [93] and lipids [87] in the body fluid, which may affect the chain cleavage mechanism as well as the dissolution of the oligomers [94]. To our best knowledge, the only degradable amphiphilic multiblock copolymers that have been evaluated extensively in-vivo are based on PEG and PBT [33,34,44,95-97]. PEG/PBT matrices varying in copolymer composition, shape, size and porosity have been implanted at different sites in the body.

Van Blitterswijk et al. [33] evaluated the effect of the PEG/PBT proportion in porous films during subcutaneous and intrabony implantation in rats. The in-vivo degradation was followed by light microscopy and reported as extension and rate of fragmentation. Implants of 1000PEG/PBT copolymers with a high percentage of PBT (≥ 60 wt%) did not show any signs of degradation after one year of implantation. Fragmentation only occurred with implants containing a higher PEG content (55-70%). This trend is in agreement with observations of Van Loon at al. [97], who showed that an increasing PEG content resulted in a faster drop in molecular weight of PEG/PBT plugs implanted intramuscularly in goats. After 26 weeks of implantation, the molecular weight of the copolymer with a hydrophilic segment / hydrophobic segment ratio of 70/30 decreased over 60%. For the same copolymer composition, Deschamps et al. [44] found over 50% mass loss for melt-pressed discs after 24 weeks of subcutaneous implantation in rats. Besides dissolution of the oligomers, also active transport of the polymer fragments from the implantation site may occur. Several studies using Transmission Electron Microscopy (TEM) showed phagocytes with intracellular polymeric particles near the implant surface [33,34,96,97].

In conclusion, varying the copolymer composition can modulate the degradation rate. For medical treatments requiring repeated injection, repeated the matrix should disappear within a reasonable period of time to avoid accumulation of polymer in the body. For the optimal copolymer composition for a specific controlled release application one should therefore consider both the release profile and the degradation rate. As the release profile may be affected by the degradation rate, conflicting arguments may complicate the selection.

Biocompatibility

Knowledge of the biocompatibility, expressed by cellular and tissue responses, of materials intended for use in the human body, is important for the design of new polymeric drug delivery systems. Therefore, the amphiphilic multiblock copolymers should be investigated extensively, both under in-vitro and in-vivo conditions, according to the guidelines of the International Standardization Organisation (ISO) [98].

The in-vitro biocompatibility is generally evaluated by using a cell culture assay. The cytotoxicity of the material is determined towards a certain cell line, depending on the ultimate application. The cells are exposed to the polymer material directly or to an extract of the material for a certain time period and subsequently evaluated for their growth, morphology and metabolism. Panoyan et al. evaluated the cytotoxicity of multiblock copolymers based on PEG and PLA towards mice melanoma cells [19]. An MTT (dimethylthiazol dephenyltetrazolium bromide) assay was used for the assessment of cell proliferation since it measures tetrazolium ring cleavage in active mitochondria, so that the reaction only occurs in active cells. The copolymer and its starting materials did not influence cell growth, neither did the empty nanospheres based on this copolymer. Moreover, rhodamine enclosed in the nanospheres did not inhibit cell growth, while rhodamine alone exerted 25% inhibition. Kim et al [81] obtained comparable results for the cytotoxicity of amphiphilic five-block copolymers based on Pluronic and PCL towards human fibroblast cells. The free model drug indomethacin exhibited higher cytotoxic activity than Pluronic/PCL block copolymeric nanospheres containing indomethacin at the same drug concentration. D'Angelo et al [63] evaluated the cytotoxicity of poly(ether-ester-amide)s (PEEAs) based on PEG and PLA by culturing Caco-2 cells on polymer films. Caco-2 cells were selected as they play a fundamental role as barrier between potentially dangerous substances ingested and internal organs. Six days after cell plating, the cell number and viability were evaluated. Regardless of the copolymer composition, no difference in reaching confluence and cell viability was observed in the cells grown on the films compared with the control. To investigate whether the poly(ether-ester-amide)s release toxic agents, films of different compositions were extracted in a cell culture medium for 24 hours. Caco-2 cells were exposed to the extracts and after 24 hours, cell growth and viability were tested. The results indicated that evaluated copolymer compositions did not release compounds, which are toxic for Caco-2 cells. Van Loon et al. [97] evaluated extensively the in-vitro biocompatibility of multiblock copolymers based on PEG and PBT: Melt-pressed films of copolymers varying in PEG/PBT ratio were put in direct contact with human skin fibroblasts. Cell counting after several time periods upto 21 days showed some delay in proliferation of cells cultured on top of copolymers with PEG/PBT ratios of 55/45, 60/40 and 70/30. This was attributed to the hydrophilic nature of these polymers. Extracts of the films prepared

in pseudo extracellular fluid at 37°C for 24 hours did not show any significant difference in proliferation, viability and cellular activity as compared to control cultures.

The results of short-term extraction indicate the absence of toxic leachables, but do not exclude the formation of toxic degradation products during the implantation of the polymer matrix. To simulate long term in-vivo degradation, extracts of films with different PEG/PBT compositions were made at 37°C during 1, 4, 26 and 52 weeks. The extraction media were exposed to human fibroblast cultures and screened for their cytotoxicity by evaluating cell proliferation, cell morphology by light microscopy and viability by MTT assay. It was concluded from this study that none of the evaluation tests showed any sign of toxic response from the extract media. These results indicate that no toxic degradation products are released from the evaluated polymer compositions. Guan et al. [70] found similar results for poly(ether-ester-urethane)urea (PEEUU) elastomers based on PCL and PEG. Endothelial cell viability on tissue culture polystyrene with culture medium supplemented with 4-week degradation solution of various PEEUU compositions was statistically equivalent to unsupplemented medium controls.

The absence of in-vitro cytotoxicity for the evaluated multiblock copolymers is no guarantee for in-vivo biocompatibility. Therefore, various implantation studies are required before a copolymer can be approved for use in the human body. Although several in-vivo biocompatibility studies have been reported on amphiphilic triblock copolymers [14 for a review], only limited data are published for multiblock copolymers. A small study was performed by Wen et al. [36] who injected microspheres of poly(D,L-lactide-co-ethyl ethylene phosphate) into the mouse brain. Histology after 5 days showed no significant difference in terms of inflammatory reaction between the microspheres and the saline injection as control. The only degradable amphiphilic multiblock copolymers that have been evaluated extensively for their in-vivo biocompatibility are based on PEG and PBT [33,34,95-97,99]. All studies showed that the PEG/PBT copolymers exhibit satisfactory biocompatibility properties. In general, the early post-operative results showed an inflammatory response, which resembled postoperative trauma without an implant [95-97]. In the first weeks after subcutaneous implantation, macrophages surrounded by fibrous tissue covered the implant surface. No chronic inflammation, abscess formation or tissue necrosis was observed in tissues adjacent to the implants. Polymers which did not show any sign of degradation after one year implantation gave

rise to a non-specific tissue response due to the physical presence of the implant. This response resulted in the formation of a thin fibrous capsule. Beumer et al. [95] implanted large areas of porous sheets (20 cm^2 per animal) subcutaneously in rats. To investigate local or systemic effects, the weight development of the complete animal and their hearts, kidneys, lungs, livers and spleens as well as the macroscopic and histologic appearance of the implants and organs were monitored. A one-year follow-up showed no significant differences between PEG/PBT implants and animals, which received a sham operation. No systemic effects of the implants on the organs or on the animal as a whole were observed after one-year implantation.

To evaluate the in-vivo toxicity of the poly(ether ester) degradation products extracts of PEG/PBT films were injected into the peritoneal cavity of mice [97]. An advantage of this intraperitoneal injection model is that interfering wound reaction, caused by an implantation procedure with more invasive surgery, is largely omitted. The degradation products of different copolymers (PEG/PBT weight ratios of 30/70, 60/40 80/20 and PEG of molecular weight of 1000 g/mole) were obtained by extracting dense films in PBS at 80°C for 60 days. Injection of the degradation products caused a mild effect in comparison with the positive control. 14 days after injection, however, the compositions of the peritoneal cell populations had almost turned back to the initial composition, indicating the absence of a chronic stimulus. Since PEG/PBT degradation products are water-soluble, distribution throughout the body by passive diffusion or by active circulatory transport may potentially cause problems concerning the systemic toxicity of the material. Therefore, after injection of the extracts of PEG/PBT films into the peritoneal cavity of mice, the morphology of liver, spleen and kidneys was determined, as well as the function of the liver and kidneys by measuring the activity of related enzymes in the blood serum. None of the injected extracts caused adverse physical symptoms, except for the positive control (Brewer thioglycolate). No changes in the morphology of the organs on light microscopy level were found for the injected extracts. Furthermore, no adverse effects on the function of the liver and spleen were found.

Thus, from the in-vivo biocompatibility studies, it can be concluded that PEG/PBT copolymers are well-tolerated and do not cause adverse tissue or systemic effects. It should, however, be noted that all of these studies dealt with larger implants.

For controlled release applications, easily injectable microspheres may be preferred. Injection of microspheres results in the implantation of a high surface area at a low volume of biomaterial, which may show a more intense tissue response [5]. In order to assess the feasibility of multiblock copolymer microspheres for drug delivery applications, studies should address the in vivo behavior of microspheres.

Controlled release applications

To protect a peptide or protein drug from rapid clearance and to provide a sustained release, the drug can be encapsulate within an amphiphilic multiblock copolymer. The polymer matrix can be formulated as microspheres, nanospheres, injectable gel or implant. Spheres and gels are preferred over implants, as no surgical procedure for administration is required and weight-based dosing therapy is possible. A drawback of spheres and gels as compared to implants is that they cannot be retrieved easily, in case of an adverse reaction for example. The drug delivery systems are administered subcutaneously or intramuscularly and release their bioactive compound in the tissue or general circulation for a certain time period.

Gels

Polymeric gels for controlled release can be divided in solvent-based and thermosensitive gels. In solvent-based gels, the polymer is dissolved in a suitable solvent containing the dissolved or dispersed drug. When the polymer solution is injected into the body and is exposed to body fluids, the solvent diffuses away from the polymer-drug mixture and water diffuses in. As a result, a solid polymer implant is formed in which the drug is encapsulated. Although solution-based gel is an interesting approach, no data on these systems have been reported for multiblock copolymers so far. As described above, specific multiblock copolymers compositions show reverse thermal gelation. This behaviour can be used in thermosensitive gel systems. The polymer is dissolved in an aqueous solution containing dissolved or dispersed drug. Prior to use, the liquid is maintained at a temperature below the reverse gelation temperature of the polymer. The liquid is than administered into the body by intramuscular or subcutaneous injection.

Subsequently, the liquid forms a gel depot of the drug and the polymer as the temperature of the liquid is raised by the body temperature. Among other poly(ether-ester) block copolymers, Cha et al. describe the use of multiblock copolymers based on PEG and poly(ethylene carbonate) (PEC) [64]. The authors mention the release of insulin from these multiblock copolymers, but unfortunately no release data are shown. For other thermosensitive polymer systems, several authors suggest the use in drug delivery applications, but no release data are reported [15,23,27]. Thus, the use of amphiphilic multiblock copolymers in gel systems requires more research before one can draw a conclusion on the suitability of this application.

Microspheres

Drug delivery applications in the form of microspheres are extensively investigated for various polymer systems. Microspheres are defined as spherical devices with diameters in the range of 1–100 μm to allow subcutaneous or intramuscular injection in suspension using conventional syringes. Various microencapsulation techniques have been used for the preparation of drug-loaded microspheres; such as spray-drying methods, phase separation methods and emulsion techniques. The release of (model) drugs from microspheres of different multiblock copolymers is reviewed below.

Mallardé et al. [24] evaluated the release of teverelix from microspheres based on PLGA/PEG multiblock copolymers. Teverelix is a hydrophobic LHRH antagonist consisting of ten amino acids (1459 g/mole). Teverelix loaded microspheres were produced by a 'phase separation' or 'coacervation' method. In this technique, the reduction of polymer solubility following the addition of a polymer-non-solvent leads to microparticles. Per copolymer composition, a stability window was determined to obtain the optimal solvent (dichloromethane) and non-solvent (silicone oil). Increasing amounts of silicone oil had to be added to the polymer solution to reach the stability window when the weight-average molecular weight decreased. No direct correlation was found between PEG content and the width of the stability window. The authors studied the effect of the teverelix loading and polymer type on the microspheres characteristics and the in-vitro release. All encapsulation yields were above 80%, independent of the initial loading (9 – 34 %). The particle size, however, depended strongly on the amount of peptide. Increasing the amounts of peptide to be encapsulated resulted in increasing particle size.

The microspheres characteristics were not affected by the copolymer composition except for the in-vitro release. The PEG/PLGA copolymer containing 20% PEG showed the fastest release of teverelix: 60% in 15 days. Decreasing the PEG percentage in the copolymer decreased the release rate. Although no data on the integrity of the released peptide were reported, the authors concluded that this new family of bioerodable polymers may constitute a useful alternative to PLGAs for the encapsulation of peptides and proteins.

Wen et al. introduced multiblock copolymers based on PLA and poly(ethylethylene phosphate) (PEEP) as matrix for controlled release applications [36]. Microspheres of the copolymer with LA molar content of 90 % containing bovine serum albumin (BSA) were prepared by a water-in-oil-in-water double emulsion technique. The microspheres displayed a spherical porous surface morphology and the average particle size was 13.5 μm. The BSA encapsulation efficiency was 75% with a loading level of 4.3%. A confocal image of FITC-BSA encapsulated microsphere showed a homogeneous distribution of the protein in the microsphere. The in-vitro release of BSA showed an initial burst of 9% in the first day, followed by only 4% release in the following 40 days. Afterwards, a near zero order release was observed until 91% protein was released. The loss of bioactivity of proteins can happen in the microsphere preparation, storage and release process, as harsh conditions may result in irreversible denaturation, particularly aggregation and degradation of the protein. SDS-PAGE was used to investigate the molecular weight change of BSA. There were no additional bands observed to indicate the presences of aggregation (molecular weight > 67 kDa) or fragments produced by degradation (molecular weight < 67 kDa). This result suggested that the structural integrity of BSA was not significantly affected in the preparation, storage and release process. Therefore, the authors concluded that the PLA/PEEP copolymers show promise as a drug carrier. In addition, they assume diffusion as the dominant mechanism of the release, although no proof is given and only one copolymer composition has been evaluated for its release behavior.

The mechanism of release has been extensively investigated for PEG/PBT copolymers by Bezemer et al. [31,100-102]. Protein-containing PEG/PBT matrices (films or microspheres) were prepared by an emulsion method [31]. Polymer solutions in chloroform or dichloromethane were mixed with aqueous protein solutions to create a

water-in-oil (W/O) emulsion. The emulsions were either cast onto a glass plate to form a film, or dispersed in an aqueous solution to produce microspheres by a water-in-oil-in-water (W/O/W) emulsion method. As demonstrated for a model protein (lysozyme), entrapped in films (approximately 100 μm in thickness) the release rate increased with increasing PEGT content of the copolymer and with an increase in the molecular weight of the PEG segments (Figure 3). This was explained by the difference in degree of swelling in water of the copolymers [31]. Swelling, and consequently the permeation of solutes through the hydrogel, increased as the molecular weight of the PEG segments or the PEG content increased [30].

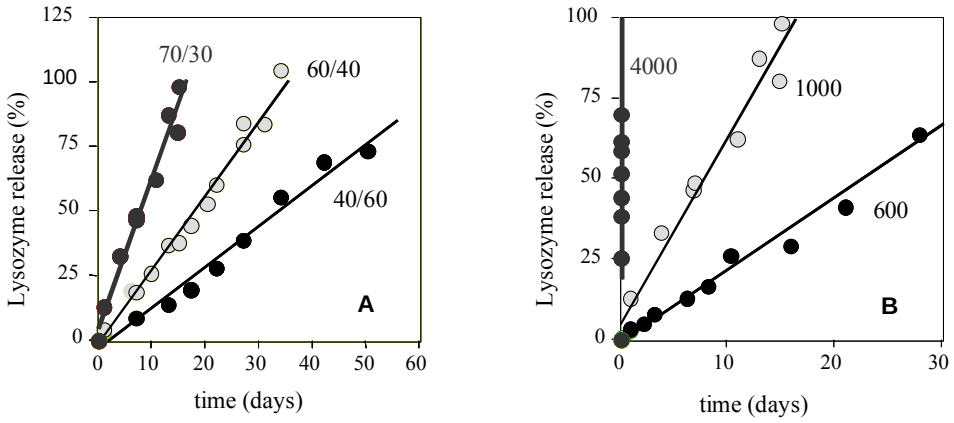


Figure 3: Cumulative release of lysozyme from PEG/PBT films in PBS (pH 7.4) at 37 °C. (A) M_w of the PEG segment is 1000 g/mole and PEGT/PBT ratio is 70/30; 60/40 or 40/60; (B) PEGT wt% is fixed between 70 and 80 % and M_w of the PEG segment is 600, 1000 or 4000 g/mole (adapted from [31]).

For highly swollen copolymers, release of lysozyme was complete within one day, and followed first order kinetics. Polymers that were swollen to a lesser extent released lysozyme with an almost constant rate (near zero order release). This release behavior was attributed to a combination of protein diffusion and polymer degradation [31]. However, the mass loss observed during the in vitro release periods was very limited [30]. Therefore, the effect of polymer chain scission on protein diffusion was studied. Polymers of different molecular weights were obtained by incubating 1000PEGT70PBT30 in phosphate buffered saline (PBS) solutions at 37 °C for various time periods. Subsequently, the pre-degraded polymers were used to prepare protein-

containing films. The lysozyme release rate of films increased with decreasing molecular weight of the polymers. Figure 4 shows the diffusion coefficient of lysozyme (D), calculated from the release curves, as a function of the polymer molecular weight (M_n). A linear relationship was observed between D and M_n^2. Taking into account the polymer degradation during release, M_n can be written as a function of time, resulting in a time-dependent diffusion coefficient. The new equation for the time-dependent diffusion coefficient was incorporated into solutions of the diffusion equation, which could adequately describe lysozyme release form poly(ether ester) copolymers [31]. The release mechanism was explained by assuming that chain scission decreased the amount of effective cross-links or entanglements present in the swollen copolymer matrix. Thereby, the resistance against diffusion of proteins through the copolymer hydrogels was reduced. In case of the observed zero order release, the increasing permeability of the matrix in time may have compensated for the decline in release rate caused by the reduced protein concentration in the matrix, to yield a constant release rate [31].

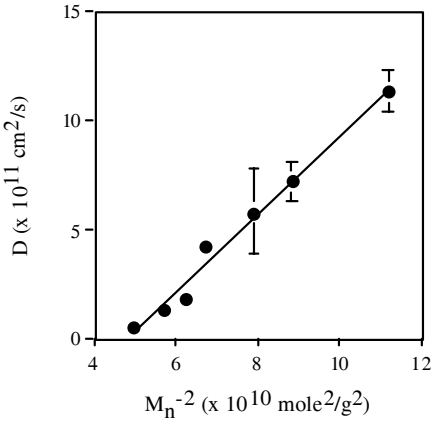


Figure 4: Effect of polymer molecular weight on lysozyme diffusion coefficient (1000PEGT70PBT30) (from [31]).

Protein containing PEG/PBT microspheres were prepared by the well known double emulsion method [100,101]. In contrast to what is often observed for hydrophobic polymers like PLGA, dense poly(ether ester) microspheres were obtained. This was explained by the amphiphilic character of the polymers, creating very stable emulsions. The latter is considered as a prerequisite for the formation of non-porous microspheres. A drawback of applying the W/O/W emulsion technique for producing protein-loaded

microspheres is related to the entrapment efficiency. In case of polymers with a relatively low degree of swelling, (e.g. less than 100% swelling) entrapment efficiencies close to 100% were observed for lysozyme. However, for highly swollen microspheres, only 10% of the lysozyme was effectively entrapped within the microspheres. This was ascribed to premature release of lysozyme during the two hours solvent evaporation in PBS. The release during preparation increased with increasing equilibrium water content of the matrix due to a greater diffusivity. In order to encapsulate small hydrophilic drug in highly swollen PEG/PBT microspheres, other methods should be applied, that avoid in-water hardening of the spheres.

In general, microspheres released proteins and peptides in a similar fashion as films [31,100,101]. A continuous release could be obtained, without an initial burst, for periods ranging from days to months. Besides by changing the copolymer composition, the release profile could be altered by variation of the W/O emulsion composition [101,102]. Increasing the amount of polymer solvent lowered the viscosity of the emulsion and thereby reduced the resulting microsphere size. A linear relationship was found between the particle size and polymer concentration [101]. With decreasing microsphere size, a considerable increase in release rate of lysozyme was observed. In addition, decreasing the average microsphere size from 108 to 29 μm causes the release kinetics to change gradually from zero order release (cumulative release proportional to time) to release controlled by Fickian diffusion (cumulative release proportional to square root of time) [101]. Increasing the water content of the emulsion (the water/polymer ratio, w/p) increased the water uptake of the microspheres [102]. At low w/p, this had no effect on the release rate. However, when the w/p was above a critical value of 1-1.5 ml/g, release kinetics was altered. Figure 5 shows that the rate of bovine serum albumin (BSA) release from microspheres increased with increasing w/p. Interestingly, a delayed release was observed for the formulation with the lowest w/p [102]. This may be of interest when designing vaccine formulations with intrinsic booster effects.

Besides the ability to modulate the release rate, the integrity of the released protein is a major factor for a successful controlled release application. To investigate protein stability problems with respect to PEG/PBT systems, the enzymatic activity of release lysozyme was determined [31]. No decrease in activity was found, indicating that this protein was not damages during the formulation, storage and release periods. Infrared

spectroscopic analysis of the dried films indicated the presence of non-covalent lysozyme aggregates in the polymer matrix [103]. Surprisingly, quantitative in-vitro release of fully active, non-aggregated lysozyme was observed, indicating that lysozyme forms reversible aggregates during encapsulation in PEGT/PBT films. It was therefore concluded that multiblock copolymers based on PEG and PBT are a promising matrix for controlled release applications, although it has to be investigated whether less stable therapeutically relevant proteins can be incorporated without loss of activity.

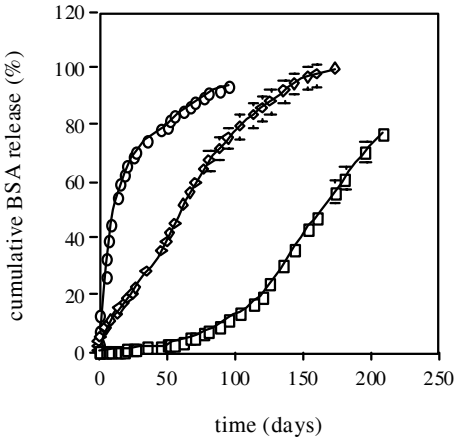


Figure 5: Effect of emulsion w/p on the release of BSA from 1000PEGT70PBT30 microspheres in PBS at 37°C. Matrices were prepared from emulsions with a w/p of 1.75 ml/g (○), 1.5 ml/g (◇) and 1.25 ml/g (□) (from [102]).

A complete different approach of controlled release was followed by Barbato et al. [26], who prepared microspheres based on poly(ether-ester-amide)s for oral drug delivery. Three model compounds with different pK_a and solubilities - diclofenac, nicardipine and dicumarol - were encapsulated by an emulsion-solvent evaporation technique. The drugs were dissolved in the polymer solution, which was subsequently emulsified into an aqueous water phase containing PVA as stabilizer. After evaporation of the organic solvent, the microspheres were collected by centrifugation, washed and freeze-dried. The obtained microparticles with an average size of 6 mm were considered to be suitable for oral delivery of antigens and particle uptake across the gastro-intestinal tract. The encapsulation efficiency of microspheres ranged between 11 and 94% depending on the type of drug. During the emulsification step and prior to polymer precipitation, drugs can diffuse into the continuous aqueous phase according to their

solubilities. The release of diclofenac from the microspheres was very rapid and complete within 2 hours. Instead, the release of nicardipine was better controlled and characterized by an initial rapid release in the first 8 hours, followed by a slower phase. The slowest release was observed for dicumarol (10% in 50 days). The authors attributed the drug release to diffusion of the entrapped material and degradation of the polymer matrix. One of the two mechanisms plays a major role in the release process, depending on the properties of the polymer, morphology of the microspheres and solubility and diffusion properties of the entrapped compound.

Nanospheres

In contrast to microspheres, nanospheres allow direct injection into the bloodstream as their size ranges from 10 to 1000 nm. Intravenous injection offers the possibility of dose reduction as a consequence of targeting. A drawback, however, is the elimination by the reticuloendothelial system (RES) within minutes. Avoidance of recognition by the RES is therefore considered to be a major challenge for drug delivery via nanospheres.

Panoyan et al. developed nanospheres of multiblock copolymers based on PLA and PEG [19]. The nanospheres were prepared under optimized conditions of a modified emulsion-solvent evaporation technique in a continuous flow process using rhodamine B as a drug model. Sperical and non-porous nanospheres with mean size less than 800 nm could be produced. The average yield was approximately 70% with 7% rhodamine loading. The release pattern was biphasic: a burst of 20% was observed after 5 hours, 50% was released after 10 days and slow release continued over 27 days. The zeta potential of the nanospheres was neutral, which should reduce particle blood clearance and recognition by phagocytic cells.

Kim et al. produced thermosensitive nanospheres based on five block copolymers of Pluronic and PCL [81]. Nanospheres with core-shell structure, prepared by dialysis method, showed the average diameter of 116 – 196 nm depending on the type of copolymer. Indomethacin (IMC) loaded Pluronic/PCL block copolymeric nanospheres showed a temperature dependent release of less than 30% in 100 hours. The amount of IMC released from the nanospheres decreased with increasing temperature. The sustained release behavior was attributed to the binding affinity of the drug and the polymer.

Implants/devices

Implants or devices for drug delivery are mostly applied in long-term treatment or in tissue engineering. In tissue engineering applications, the polymer implant serves both as matrix for the drug and as scaffold for the new formed cells. A drawback of implants is the surgical procedure required for implantation. On the other hand, the advantage is the possibility of their removal in case of adverse events.

At present, various groups extensively study application of PEG/PBT copolymers as scaffolds for tissue engineering [104-106]. Controlled release of growth factors from such scaffolds could be of major interest to enhance the cell migration, proliferation and differentiation. In order to prepare porous protein-loaded polymer scaffolds, prefabricated PEG/PBT scaffolds were used, of which the inner scaffold pores were coated with a protein-loaded polymeric film [107]. To create the coating, a water-in-oil emulsion was conducted through the pores of the scaffold by applying a vacuum. After freeze-drying, the resulting scaffolds contained a 10 – 40 μm coating, distributed over the scaffold pores. The release of a model protein (lysozyme) could be tailored by the emulsion composition. By decreasing the water content of the emulsion, the release rates could be varied from a complete release within a couple of days up to over two months. In addition, the coating process did not reduce the enzymatic activity of the lysozyme [107].

Chen et al. evaluated the release of hydrophilic 5-flurouracil (5-Fu) from tablets of PEG/PLA multiblock copolymers [37]. 5-Fu powder was dispersed in a polymer solution in methylene chloride. After solvent evaporation and further drying under vacuum, the polymeric film containing 5-Fu was thermopressed into tablets. The drug release rate was enhanced with the increase of PEG content in the multiblock copolymers and was complete within 3 days for all compositions tested.

Bae et al. applied PEG/PLA multiblock copolymers as wound healing material [15]. Therefore, polymer films loaded with basic fibroblast growth factor (bFGF) were prepared by solvent casting. Although bFGF is known to be potent and beneficial in stimulating cell growth and wound healing process, in-vivo studies are hampered by its instability. To evaluate a PEG/PLA multiblock hydrogel formulation for its effectiveness in preserving the healing properties of bFGF, a rat study was performed. Wound sites at the rat skin surface were covered with a bFGF loaded PEG/PLA film. As control

experiments, wounds were covered with a commercial wound covering ointment, an unloaded PEG/PLA film and without treatment. Wounds that had been covered with the bFGF film showed complete recovery after 10 days, while the control wound sites were only partially healed. This result proved that the PEG/PLA film released active bFGF at the wound site. Although no quantitative release of bFGF was determined, this limited study was the only in-vivo release study reported for amphiphilic multiblock copolymers.

Concluding remarks

Biodegradable amphiphilic multiblock copolymers as described in this review have great potential as matrix for drug delivery applications. The presence of hydrophilic and hydrophobic blocks offers the possibility to tailor material properties like swelling and degradation rate, which are important factors for drug release. Complete release within several minutes up to months can be obtained for molecules of various sizes depending on the copolymer composition of the matrix. In addition, the reviewed multiblock copolymers show good biocompatibility, although additional studies are necessary to evaluate the in-vivo degradation and in-vivo biocompatibility of the polymer release systems. The release data published so far for multiblock copolymers are mainly obtained from in-vitro experiments with model drugs. Further development of amphiphilic multiblock copolymer as controlled release matrix, therefore, requires extensive in-vivo release studies using therapeutically relevant drugs.

References

1. Sinha VR, Trehan A, Biodegradable microspheres for protein delivery. J. Control. Release 90 (2003) 261-280.
2. Cleland JL, Daugherty A, Mrsny R, Emerging protein delivery methods. Current opinion in biotechnology 12 (2001) 212-219.
3. Crommelin DJA, Storm G, Verrijk R, De Leede L, Jiskoot W, Hennink WE, Shifting paradigms: biopharmaceuticals versus low molecular weight drugs. Int. J. Pharm. 266 (2003) 3-16.

4. Couvreur P, Puisieux F, Nano- and microparticles for the delivery of polypeptides and proteins. Adv. Drug Del. Rev. 10 (1993) 141-162.
5. Anderson JA, Shive MS, Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv. Drug Del. Rev. 28 (1997) 5-24.
6. Okada H, One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv. Drug Del. Rev. 28 (1997) 43-70.
7. Lu W, Park TG, Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. J. Pharm. Sci. & Technol. 49 (1995) 13-19.
8. Jiang G, Woo BH, Kang F, Singh J, DeLuca PP, Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. J. Control. Release 79 (2002) 137-145.
9. Aubert-Pouëssel A, Bibby DC, Vernier-Julienne MC, Hindré F, Benoît JP, A novel in vitro delivery system for assessing the biological integrity of protein upon release from PLGA microspheres. Pharm. Res. 19 (2002) 1046-1050.
10. Morita T, Sakamura Y, Horikiri Y, Suzuki T, Yoshino H, Evaluation of in vivo release characteristics of protein-loaded biodegradable microspheres in rats and severe combined immunodeficiency disease mice. J. Control. Release 73 (2001) 213-221.
11. Jiang G, Qiu W, DeLuca PP, Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres. Pharm. Res. 20 (2003) 452-459.
12. Johansen P, Corradin G, Merkle HP, Gander B, Release of tetanus toxoid from adjuvants and PLGA microspheres: How experimental set-up and surface adsorption fool the pattern. J. Control. Release 56 (1998) 209-217.
13. Kumar N, Ravikumar MNV, Domb AJ. Biodegradable block copolymers. Adv. Drug. Del. Rev. 53 (2001) 23-44.
14. Kissel T, Li Y, Unger F, ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins. Adv. Drug. Del. Rev. 54 (2002) 99-134.
15. Bae YH, Huh KM, Kim Y, Park KH, Biodegradable amphiphilic multiblock copolymers and their implications for biomedical applications. J. Control. Release 64 (2000) 3-13.
16. Zhou S, Deng X, Yang H, Biodegradable poly(ε-caprolactone)-poly(ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system. Biomaterials 24 (2003) 3563-3570.
17. Zhu KJ, Lin XZ, Yang SL, Preparation, characterization and properties of polylactide-poly(ethylene glycol) copolymers: a potential drug carrier. J. Appl.Polym. Sci. 39 (1990) 1-9.
18. Cohn D, Younes H, Biodegradable PEO/PLA block copolymers. J. Biomed. Mater. Res. 22 (1988) 993-1009.
19. Panoyan A, Quesnel R, Hildgen P, Injectable nanospheres from a novel multiblock copolymer: cytocompatibility, degradation and in vitro release studies. J. Microencapsulation 20 (2003) 745-758.

20. Ferruti P, Penco M, D'Addato P, Ranucci E, Deghenghi R, Synthesis and properties of novel block copolymers containing poly(lactic-glycolic acid) and poly(ethyleneglycol) segments. Biomaterials 16 (1995) 1423-1428.
21. Penco M, Marcioni S, Ferruti P, D'Antone S, Deghenghi R, Degradation behaviour of block copolymers containing poly(lactic-glycolic acid) and poly(ethylene glycol segments. Biomaterials 17 (1996) 1583-1590.
22. Luo W, Li S, Bei J, Wang S, Dependence of morphology on compositions of poly(L-lactide)-poly(ethylene glycol) multiblock copolymers. Polym. Adv. Technol. 13 (2002) 233-238.
23. Huh KM, Bae YH, Synthesis and characterization of poly(ethylene glycol)/poly(L-lactic acid) alternating multiblock copolymers. Polymer 40 (1999) 6147-6155.
24. Mallardé D, Boutignon F, Moine F, Barré E, David S, Touchet H, Ferruti P, Deghenghi R, PLGA-PEG microspheres of teverelix: influence of polymer type on microsphere characteristics and on teverelix in vitro release. Int. J. Pharm. 261 (2003) 69-80.
25. Penco M, Bignotti F, Sartore L, D'Antone S, D'Amore A, Multiblock copolymers based on segments of poly(D,L-lactic glycolic acid) and poly(ethylene glycol) or poly(ε-caprolactone): A comparison of their thermal properties and degradation behavior. J. Appl. Polym. Sci. 78 (2000) 1721-1728.
26. Barbato F, Immacolata La Rotonda M, Maglio G, Palumbo R, Quaglia F, Biodegradable microspheres of novel segmented poly(ether-ester-amide)s based on poly(ε-caprolactone) for the delivery of bioactive compounds. Biomaterials 22 (2001) 1371-1378.
27. Lee JW, Hua FJ, Lee DS, Thermoreversible gelation of biodegradable poly(ε-caprolactone) and poly(ethylene glycol) multiblock copolymers in aqueous solutions. J. Control. Release 73 (2001) 315-327.
28. Lee SC, Kang SW, Kim C, Kwon IC, Jeong SY, Synthesis and characterization of amphiphilic poly(2-ethyl-2-oxazoline)/poly(ε-caprolactone) alternating multiblock copolymers. Polymer 41 (2000) 7091-7097.
29. Li S, Garreu H, Vert M, Petrova T, Manolova N, Rashkov I, Hydrolytic degradation of poly(oxyethylene)-poly-(ε-caprolactone) multiblock copolymers. J. Appl. Polym. Sci. 68 (1998) 989-998.
30. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, A controlled release system for proteins based on poly(ether ester) block-copolymers: polymer network characterization. J. Control. Release 62 (1999) 393-405.
31. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA, Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J. Control. Release 64 (2000) 179–192.
32. Deschamps AA, Grijpma DW, Feijen J, Poly(ethylene oxide)/poly(butylene terephthalate) segmented block copolymers: the effect of copolymer composition on physical properties and degradation behavior. Polymer 42 (2001) 9335-9345.
33. Van Blitterswijk CA, Van de Brink J, Leenders H, Bakker D, The effect of PEO ratio on degradation, calcification and bone bonding of PEO/PBT copolymer (PolyActive). Cell and Matererials 3 (1993) 23-36.

34. Beumer GJ, Van Blitterswijk CA, Ponec M, Degradation behaviour of polymeric matrices in (sub)dermal and muscle tissue of the rat: a quantitative study. Biomaterials 15 (1994) 551-559.
35. Nagata M, Kiyotsukuri T, Takeuchi S, Tsutsumi N, Sakai W, Hydrolytic degradation of aliphatic polyesters copolymerized with poly(ethylene glycol)s. Polymer International 42 (1997) 33-38.
36. Wen J, Kim GJA, Leong KW, Poly(dD,L,actide-co-ethyl ethylene phosphate)s as new drug carriers. J. Control. Release 92 (2003) 39-48.
37. Chen W, Luo W, Wang S, Bei J, Synthesis and properties of poly(L-lactide)-poly(ethylene glycol) multiblock copolymers by coupling triblock copolymers. Polym. Adv. Technol. 14 (2003) 245-253.
38. Wan Y, Chen W, Yang J, Bei J, Wang S, biodegradable poly(L-lactide)-poly(ethylene glycol) multiblock copolymer: synthesis and evaluation of cell affinity. Biomaterials 24 (2003) 2195-2203.
39. Huang MH, Li S, Coudane J, Vert M, Synthesis and characterization of block copolymers of ε-caprolactone and DL- lactide initiated by ethylene glycol of poly(ethylene glycol). Macromol. Chem. Phys. 204 (2003) 1994-2001.
40. Pechar M, Ulbrich K, Šubr V, Seymour LW, Schacht EH, Poly(ethylene glycol) multiblock copolymer as carrier of anti-cancer drug doxorubicin. Bioconjugate Chem. 11 (2000)131-139.
41. Okada M, Chemical syntheses of biodegradable polymers. Prog. Polym.Sci. 27 (2002) 87-133.
42. Chen X, McCarthy SP, Gross RA, Synthesis and characterization of [L]-lactide-ethylene oxide multiblock copolymers. Macromolecules 30 (1997) 4295-4301.
43. G.K. Hoeschele, Thermostabile polyester-block-copolymere. Die Angew. Makromol. Chem. 58/59 (1977) 299-319.
44. Deschamps AA, Van Apeldoorn AA, Hayen H, De Bruijn JD, Karst U, Grijpma DW, Feijen J, In vivo and in vitro degradation of poly(ether-ester) block copolymers based on poly(ethylene glycol) and poly(butylene terephthalate). Biomaterials 25 (2004) 247-258.
45. Reed AM, Gilding DK, Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:1. Polymer 20 (1979) 1454-1458.
46. Nagata M, Kiyotsukuri T, Susumu M, Tsutsumi N, Sakai W, Enzymatic degradation of poly(ethylene terephthalate) copolymers with aliphatic dicarboxylic acids and/or poly(ethylene glycol). Eur. Polym. 33 (1997) 1701-1705.
47. Wang M, Zhang L, Ma D, Degree of microphase separation in segmented copolymers based on (poly(ethylene oxide) and poly(ethylene terephthalate). Eur. Polymer J. 35 (1999) 1335-1343.
48. Kiyotsukuri T, Nagata M, Tsutsumi N, Sakai W, Poly(ethylene terephthalate) copolymers with a smaller amount of poly(ethylene glycol)s and poly(butylenes glycol)s. Polymer 36 (1995) 2629-2635.
49. Albertsson A, Degradable Polymers. II. Synthesis, Characterisation, and Degradation of an Aliphatic Thermoplastic Block Copolyester. J. Macromol. Sci.Chem., A23(3) (1986) 411-422.
50. Albertsson A, Degradable Polymers. III. Synthesis, Characterisation, and Degradation of an Aliphatic Thermoplastic Block Copolyester. J. Macromol. Sci.Chem., A24(8) (1987) 997-990.
51. Albertsson AC, Ljungquist O, Degradable Polymers as Biomaterials. Acta Polymerica 39 (1988) 95-104.
52. Bezemer JM, Oude Weme P, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Amphiphilic poly(ether ester amide) multiblock copolymers as biodegradable matrices for the controlled release of proteins. J. Biomed. Mater. Res. 52 (2000) 8-17.

53. Deschamps AA, Van Apeldoorn AA, De Bruijn JD, Grijpma DW, Feijen J, Poly(ether ester amide)s for tissue engineering. Biomaterials. 24 (2003) 2643-2652.
54. Yamaoka T, Takahashi Y, Fujisato T, Lee CW, Tsuji T, Ohta T, Murakami A, Kimura Y, Novel adhesion prevention membrane based on a bioresorbable copoly(ester-ether) comprised of poly-L-lactide and Pluronic: in vitro and in vivo evaluations. J. Biomed. Mater. Res.54 (2001) 470-479.
55. Pathak CP, Barman SP, Sawhney AS, Coury AJ, Avilla LZ, Kieras MT,Multiblock biodegradable hydrogels for drug delivery and tissue treatment. Patent US 6,639,014 B2 (2003).
56. Ha JC, Kim SY, Lee YM, Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (Pluronic)/poly(ε-caprolactone) (PCL amphiphilic block copolymeric nanospheres I. Preparation and characterization. J. Control. Release 62 (1999) 381-392.
57. Ash M, Ash I, editors: Handbook of Pharmaceutical Additives, second edition. Synapse Information Resources, Inc, 2002. 731-732.
58. Lee SY, Chin IJ, Jung JS, Crystallization behavior of poly(L-lactide)-poly(ethyleneglycol) multiblock copolymers. Eur. Polym. J. 35 (1999) 2147-2153.
59. Yao F, Bai Y, Zhou Y, Liu C, Wang H, Yao K, Synthesis and characterization of multiblock copolymers based on L-lactic acid, citric acid, and poly(ethylene glycol). J. Polym. Sci. Part A: Polym. Chem. 41 (2003) 2073-3081.
60. Petrova T, Manolova N, Rashkov I, Li S, Vert M, Synthesis and characterization of poly(oxyethylene)-poly(caprolactone) multiblock copolymers. Polym. Int. 45 (1998) 419-426.
61. Yamaoka T, Takahashi Y, Ohta T, Miyamoto M, Murakami A, Kimura Y, Synthesis and properties of multiblock copolymers consisting of poly(L-lactic acid) and poly(oxypropylene-co-oxyethylene) prepared by direct polycondensation. J. Polym. Sci. A: Polym. Chem. 37 (1999) 1513-1521.
62. Qian ZY, Li S, He Y, Liu XB, Synthesis and in vitro degradation study of poly(ethylene terephthalate)/poly(ethylene glycol) (PET/PEG) multiblock copolymers. Polym. Degrad. Stab. 83 (2004) 93-100.
63. D'Angelo S, Galletti P, Maglio G, Malinconico M, Morelli P, Palumbo R, Vignola MC, Segmented poly(ether-ester-amide)s based on poly(L,L-lactide) macromers. Polymer 42 (2001)3383-3392.
64. Cha Y, Choi YK, Bae YH, Thermosensitive biodegradable polymers based on poly(ether-ester)block copolymers. Uniotes States Patent: 5,702,717 (1997).
65. Lee SI, Yu SC, Lee YS, Degradable polyurethanes containing poly(butylene succinate) and poly(ethylene glycol), Polymer Degradation and Stability 72 (2001) 81-87.
66. Gorna K, Gogolewski S, In vitro degradation of novel medical biodegradable aliphatic polyurethanes based on ε-caprolactone and Pluronics with various hydrophilicities. Polymer Degradation and Stability 75 (2002) 113-122
67. Jiang HL, Zhu KJ, Preparation, characterization and degradation characteristics of polyanhydrides containing poly(ethylene glycol). Polym. Int. 48 (1999) 47-52.
68. Albertsson, AC, Synthesis, Characterisation and Degradation of Aliphatic Polyanhydrides, British Polymer Journal, Vol.23, 1990, pp.205-212

69. Yen MS, Kuo SC, PCL-PEG-PCL Triblock copolydiol-based waterborne polyurethane.I. Effects of the soft-segment composition on the structure and physical properties, J. Appli. Polym. Sci. 65 (1997) 883-892.
70. Guan J, Sacks MS, Beckman EJ, Wagner WR, Biodegradable poly(ether ester urethane)urea elastomers based on poly(ether ester) triblock copolymers and putrescine: synthesis, characterization and cytocompatibility. Biomaterials 25 (2004) 85-96.
71. Cohn D, Stern T, Fernanda González M, Epstein J, Biodegradable poly(ethylene oxide)/poly(ε-caprolactone) multiblock copolymers. J. Biomed. Mater. Res. 59 (2002) 273-281.
72. Fakirov S, Gogeva T, Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 1. Poly(ether/ester)s with various polyester : polyether ratios. Makromol. Chem. 191 (1990) 603-614.
73. Fakirov S, Gogeva T, Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 2. Effect of polyether length. Makromol. Chem. 191 (1990) 615-624.
74. Adams RK, Hoeschele GK, Witsiepe WK, Thermoplastic polyether ester elastomers. In: Holden G, Legge NR, Quirk R and Schroeder HE, editors. Thermoplastic elastomers, 2nd edition. Munich: Hansen Publishers, 1996. p. 191-225.
75. Hiemenz PC. Polymer Chemistry. New York: Marcel Dekker; 1984. p 204-219.
76. Liu KJ, Parsons JL, Solvent effects on the preferred conformation of poly(ethylene glycols). Macromolecules 2 (1969) 529-533.
77. Yui N, Katanaoke K, Yamada A, Sakurai Y, Novel design of microreservoir-dispersed matrices for drug delivery formulations: Regulative drug release from poly(ethylene oxide)- and poly(tetramethylene oxide)-based segmented polyurethanes. J. Control. Release 6 (1987) 329-342.
78. Schneider NS, Illinger JL, Karasz FE, The interaction of water with polyurethanes containing block copolymer soft segments. J. App. Polymer Sci. 47 (1993) 1419-1425.
79. Gilding KD, Reed AM, Biodegradable polymers for use in surgery - poly(ethylene oxide) poly(ethylene terephthalate) (PEO/PET) copolymers: 1. Polymer 20 (1979) 1454-1458.
80. Canal T, Peppas NA, Correlation between mesh size and equilibrium degree of swelling of polymeric networks. J. Biomed. Mater. Res. 23 (1989) 1183-1193.
81. Kim SY, Ha JC, Lee YM, Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) / poly(ε-caprolactone) (PCL) amphiphilic block copolymeric nanospheres II. Thermo-responsive drug release behaviors. J. Control. Release 65 (2000) 345–358.
82. Göpferich A, Mechanisms of polymer degradation and erosion . Biomaterials17 (1996) 103-114
83. Reed AM, Gilding DK, Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:2 In vitro degradation. Polymer 22 (1981) 499-504.
84. M. Kellomäki, S. Paasimaa, D. W. Grijpma, K. Kolppo and P. Törmälä, In vitro degradation of Polyactive® 1000PEOT70PBT30 devices, Biomaterials, Volume 23, Issue 1, 1 January 2002, Pages 283-295.
85. Tracy M, Ward KL, Firouzabadian L, Wang Y, Dong N, Qian R, Zhang Y, Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in-vitro. Biomaterials 20 (1999) 1057-1062.

86. Spenlehauer G, Vert M, Benoit JP, Boddaert A, In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials 10 (1989) 557-563.
87. Menei P, Daniel V, Montero-Menei C, Brouillard M, Poulard-Barthelaix A, Benoit JP, Biodegradation and brain tissue reaction to poly(D,L-lactide-co-glycolide) microspheres. Biomaterials 14 (1993) 470-478.
88. Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamade JA, Uyama S, Vacanti JP, Langer R, Mikos AG, In vitro and in vivo degradation of porous poly(DL-lactic-co-glycolic acid) foams. Biomaterials 21 (2000) 1873-1845.
89. Mainil-Varlet P, Curtis R, Gogolewski S, Effect of in vivo and in vitro degradation on molecular and mechanical properties of various low-molecular-weight polylactides. J. Biomed. Mater. Res. 36 (1997) 360-380.
90. Stoll GH, Nimmerfall F, Acemoglu M, Bodmer D, Bantle S, Müller I, Mahl A, Kolopp M, Tullberg K, Poly(ethylene carbonate)s, part II: degradation mechanisms and parenteral delivery of bioactive agents. J. Control. Release 76 (2001) 209-225.
91. Fulzele SV, Satturwar PM, Dorle AK, Study of the biodegradation and in vivo biocompatibility of novel biomaterials. Eur. J. Pharm. Sci. 2003; in press.
92. Ali SAM, Doherty PJ, Williams DF, Molecular biointeractions of biomedical polymers with extracellular exudates ad inflammatory cells and their effects on the biocompatibility, in vivo. Biomaterials 15 (1994) 779-785.
93. Gogolewski S, Janovic M, Perren SM, Dillon JG, Hughes MK. Tissue response and in vivo degradation of selected polyhydroxy acids: Polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate –co-hydroxyvalerate) (PHB/VA). J. Biomed. Mater. Res. 27 (1993) 1135-1148.
94. Therin M, Christel P, Li S, Garreau H, Vert M, In vivo degradation of massive poly(α-hydoxy acids): validation of in vitro findings. Biomaterials 13 (1992) 594-600.
95. Beumer GJ, Van Blitterswijk CA, Ponec M, Biocompatibility of degradable matrix induced as a skin substitute: an in vivo evaluation. J. Biomed. Mater. Res. 28 (1994) 545-552.
96. Bakker D, Van Blitterswijk CA, Hesseling SC, Grote JJ, Deams WT, Effect of implantation site on phagocyte/polymer interaction and fibrous capsule formation. Biomaterials 9 (1988) 14-21.
97. Van Loon JA, Thesis University of Leiden, The Netherlands, 1995.
98. ISO 10993, Biological Evaluation of Medical devices-Part5: Tests for in-vitro cytotoxicity, International Standardization Organisation, 1999.
99. Radder AM, Leenders H, Van Blitterswijk CA, Interface reactions to PEO/PBT copolymers (Polyactive®) after implantation in cortical bone. J. Biomed. Mater. Res. 28 (1994) 141-151.
100. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 1. Influence of preparation techniques on particle characteristics and protein delivery. J. Control. Release 67 (2000) 233-248.
101. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 2. Modulation of release rate. J. Control. Release 67 (2000) 249-260.

102. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J, Control of protein delivery from amphiphilic poly(ether ester) multiblock copolymers by varying their water content using emulsification techniques. J. Control. Release 66 (2000) 307-320.
103. Van de Weert M, Van Dijkhuizen-Radersma R, Bezemer JM, Hennink WE, Crommelin DJA, Reversible Aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer. Eur. J. Pharm. Biopharm. 54 (2002) 89-93.
104. Woodfield TBF, Bezemer JM, Pieper JS, Van Blitterswijk CA, Riesle, J. Scaffolds for tissue engineering of cartilage. Crit Rev Eukaryotic Gene Expression 2002 12:209-236.
105. Deschamps AA, Claase MB, Sleijster MWJ, De Bruijn JD, Grijpma DW Feijen J, Design of segmented poly(ether ester) materials and structures for tissue engineering of bone. J Control Rel 2002; 78: 175–186.
106. Claase MB, Grijpma DW, Mendes SC, De Bruijn JD, Feijen F, Porous PEOT/PBT scaffolds for bone tissue engineering: preparation, characterization and in-vitro bone marrow stromal cell culturing. J. Biomed. Mater Res. 64A (2003), 291-300.
107. Sohier J, Haan RE, De Groot K, Bezemer JM. A novel method to obtain protein release from porous polymer scaffolds: emulsion coating, J Control Rel 2003; 87: 57-68.

Chapter 3

Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation

R. van Dijkhuizen-Radersma, S.C. Hesseling, P.E. Kaim,
K. de Groot, J.M. Bezemer

Abstract

Microspheres of a hydrophobic and a hydrophilic poly(ether-ester) copolymer were evaluated for their in-vitro and in-vivo biocompatibility and degradation. The microspheres prior to and after sterilization were tested for in-vitro cytotoxicity. The in-vivo biocompatibility of the poly(ethylene glycol) terephthalate and poly(butylene terephthalate) (PEGT/PBT) microspheres was evaluated subcutaneously and intramuscularly for 24 weeks in rabbits. The in-vivo degradation of the microspheres was studied microscopically and compared to the in-vitro degradation. The in-vitro and in-vivo studies showed the biocompatibility of the microspheres of both the hydrophobic and the hydrophilic PEGT/PBT copolymer. Extracts of these microspheres showed no cytotoxic reactivity in the in-vitro cytotoxicity test. Sterilization of the microspheres by gamma irradiation did not affect the cytotoxicity. PEGT/PBT microspheres injected subcutaneously and intramuscularly in rabbits showed a mild tissue response in vivo, in terms of the inflammatory response, the foreign body reaction and the granulation tissue response. Although an in-vitro degradation experiment showed a decrease in molecular weight due to hydrolysis, the in-vivo degradation of the microspheres was slower than previously published.

Introduction

Polymeric microspheres have been widely investigated as controlled release system for proteins and peptides [1]. A critical aspect in the application of these microspheres is their biocompatibility [2]. Recently, a series of poly (ether-ester) multiblock copolymers composed of poly(ethylene glycol) terephthalate and poly(butylene terephthalate) (PEGT/PBT) was introduced as matrix for controlled release systems [3,4]. This copolymer system is currently applied for a wide range of biomedical applications [5-7], including a FDA approved cement restrictor. Although many in-vivo and in-vitro studies have shown that PEGT/PBT copolymers are biocompatible and can be made biodegradable [6-13], all of these studies dealt with larger implants. Biocompatibility of biomaterials, however, depends to a large extent on the size and the shape of the implant [2]. For controlled release applications, easily injectable microspheres are preferred. Injection of microspheres results in the implantation of a high surface area at a low volume biomaterial, which may show a more intense tissue response [2].

In this publication, we report for the first time results of an in-vivo biocompatibility study on PEGT/PBT microspheres. Two different types of poly (ether-ester) copolymers were selected; a hydrophobic copolymer as a model matrix for the release of peptides [3] and a more hydrophilic copolymer as a model matrix for sustained protein delivery [4].

Prior to injection, the microspheres were tested for cytotoxicity, which has been used as in-vitro biocompatibility test for several biodegradable polymers [8,14,15]. The in-vivo biocompatibility of the PEGT/PBT microspheres was evaluated for 24 weeks in rabbits. Both subcutaneous and intramuscular injection sites were investigated. The in-vivo degradation of the microspheres was studied microscopically and compared to the in-vitro degradation. In addition, the effects of sterilization of the PEGT/PBT microspheres by gamma irradiation on the molecular weight and cytotoxicity were evaluated.

Materials and methods

Materials

The poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis (Bilthoven, The Netherlands) and were indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt% PEG-terephthalate and **c** (=100-b) the wt% PBT. The hydrophobic 300PEGT53PBT47 copolymer and the hydrophilic 1000PEGT71PBT29 copolymer were selected for this study (composition determined by NMR). The average molecular weight (M_w) determined by GPC (relative to PMMA standards) was 78.4 kg/mole for 300PEGT53PBT47 and 85.8 kg/mole for 1000PEGT71PBT29. The PEGT/PBT copolymers contained 0.2 wt% α-tocopherol as antioxidant (determined by HPLC). Phosphate buffered saline (PBS), (pH 7.4) and minimum essential medium (MEM) were purchased from Life Technologies Ltd (Paisley, Scotland). Polyvinylalcohol (PVA, 13.000-23.000 g/mole, 87-89% hydrolyzed) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). Chloroform purchased from Fluka Chemie GmbH (Buchs, Switzerland) was of analytical grade. NPBI (Emmer-Compascuum, The Netherlands) was the supplier of water for injection and saline buffer (0.9% sodium chloride). Dimethylthiazol dephenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co (St Louis, USA). Para rubber (Hilversum Rubber Factory, Hilversum, The Netherlands) and ultra high molecular weight (UHMW) polyethylene (Goodfellow, Cambridge, England) were used as controls in cytotoxicity tests.

Preparation of PEGT/PBT microspheres

Microspheres of the PEGT/PBT copolymers were prepared by an oil-in-water (o/w) emulsion method. The oil phase consisted of PEGT/PBT copolymer (25 g) dissolved in 175 ml chloroform. The polymer solution was poured into 400 ml PBS containing 4% (w/v) of PVA. After 5 minutes stirring at 1100 rpm, 1500 ml water for injection was added. The oil-in-water emulsion was stirred at constant speed for 16 hours at room temperature. The microspheres were collected by using sieves with mesh widths of 25 μm and 75 μm. The fraction between 25 μm and 75 μm was washed with water for injection and freeze-dried for 48 hours.

Sterilization

Microspheres, packed under vacuum in two aluminum pouches, were sterilized by gamma irradiation. A minimum irradiation dose of 25 kGy was applied in a JS6500 Tote Box Irratiator at Gammaster B.V. (Ede, The Netherlands).

Microspheres characterization

The size (number and volume weight mean diameter) and the size distribution of microspheres suspended in water were determined with an optical particle sizer (Accusizer, model 770, Santa Barbara, California, USA). Water was used as eluent. Mean particle diameters (number weight mean (Dn) and volume weight mean (Dv) according to Edmunson [16]) were calculated with Nicomp Particle Sizing Systems, Accusizer C770 software version 2.54.

A Philips XL 30 Environmental Scanning Electron Microscope (ESEM) was used to evaluate the surface characteristics of the microspheres. Samples were sputter-coated with a thin gold layer.

To study the effect of the sterilization process, the molecular weight of the microspheres before and after gamma irradiation was determined using Gel Permeation Chromatography (GPC). Samples were eluted in 0.02M sodiumtrifluoroacetate (NaF_3Ac) in hexafluoroisopropanol (HFIP) through a Polymer Labs HFIP gel guard column (50 x 7.5 mm) and two PL HFIP gel analytical columns (300 x 7.5 mm). Flow rate was 1 ml/min and a Refraction Index (RI) detector was used. Column temperature was 40ºC and sample concentration was 20 mg/ml. The molecular weights (M_n and M_w) were determined relative to polymethylmethacrylate (PMMA) standards.

Cytotoxicity

Microspheres, before and after sterilization, were tested for cytotoxicity towards the growth, morphology and metabolism of fibroblasts. The cytotoxicity test (MEM-extract test, MTT test system, 72 hours incubation) was conducted according to the ISO 10993/EN 30993 standard. In this study, 100 mg of the dry microspheres (equals at least 120 cm^2) were extracted at 37^oC for 24 hours in 20 ml medium. The medium consisted of minimum essential medium (MEM) supplemented with 10% fetal calf serum. 60 cm^2 of Para rubber was extracted identically for a positive control. As a negative control

material, UHMW polyethylene (60 cm^2) was used. The maximum negative control was cells cultured in standard medium.

A monolayer of cells (mouse lung fibroblasts, L929) were grown to 70-80% confluency, which was examined and scored microscopically. Then the cells were challenged with an extract of the microspheres (n=6), the negative control material (n=6), the positive control material (n=6), and with medium only (n=6). After exposure to the extract for 72 hours at 37°C, the medium was removed, leaving a film of medium in each well, and the cells were examined and scored microscopically for cytotoxic effects: confluency of the monolayer and change of cellular morphology. These qualitative scores are corrected for the negative control. The grades of cytotoxicity are given in Table 1.

Table 1: Criteria for scores in cytotoxicity test

Grade	Confluency of monolayer[1]	Change of cell morphology[*,1]	Inhibition of cell metabolism[*,2]
0	100 %	no differences from negative control	<10%
1	90-100%	Slight changes, few cell different	10-30%
2	60-90%	Mild changes, some cells different/rounded	30-50%
3	30-60%	Moderate changes, many cells rounded	50-70%
4	<30%	Severe changes, about all cells show morphologic change	70-100%

[*] compared to negative control
[1] qualitatively determined
[2] quantitatively determined

Quantitative scores were obtained by addition of dimethylthiazol dephenyltetrazolium bromide (MTT), a water soluble tetrazolium salt yielding a yellowish solution, to each culture being assayed. MTT is converted into an insoluble purple formazan dye by mitochondrial dehydrogenase enzymes. Only active mitochondrial dehydrogenases of living cells will convert MTT into the insoluble purple formazan dye. The cultures were incubated for 3 hours at 37°C. After introduction of absolute isopropanol, cells are lysed and the precipitated formazan is dissolved. Formazan concentrations are quantitatively determined by measuring the Optical Density (OD) at 570 nm with background correction of the OD at 690 nm. The mean OD570 value obtained for the negative control is standardized as 0% inhibition. The mean OD570 value of the positive control is standardized as 100% inhibition. The mean OD570 value of a test sample is expressed as % inhibition, resulting in a cytotoxicity grade (Table 1). Finally, the cytotoxic response is

graded by addition of the mean score for microscopic change and the mean score for growth inhibition.

Implantation

The rabbit model was chosen to evaluate the biocompatibility of the microspheres. New Zealand White rabbits (3 – 3.5 kg) were anaesthetized and injected subcutaneously and intramuscularly under sterile conditions. Prior to subcutaneous injection, per dose 150 mg microspheres were suspended in 1.0 ml of a sterile 0.9% saline solution. Per rabbit, 3 doses of 300PEGT53PBT47 microspheres were injected on the right flank and 3 doses of 1000PEGT71PBT29 microspheres were injected on the left flank. For the intramuscular injection, per dose 50 mg of microspheres were suspended in 0.5 ml of sterile saline. Three doses of 300PEGT53PBT47 microspheres (right musculus paravertebralis) and 3 doses of 1000PEGT71PBT29 microspheres (left musculus paravertebralis) were injected per rabbit. At 1, 4, 12 and 24 weeks after injection, one rabbit was sacrificed by using an overdose euthasate. The microspheres were removed with excess surrounding tissue for evaluation. The animal experiment in this study was performed according to the legal guidelines concerning animal welfare ISO 10993 part 2, 1997, European Directive 86/609/EEC and to the Dutch Laboratory Animal Act.

Characterization in-vivo samples

After explantation, the samples remained for 1 to 2 days in the fixative (4% formaldehyde, 2% glutaraldehyde) after which they were washed in PBS and dehydrated in graded series of ethanol. For histological analyses, pieces of the samples were embedded in glycol methacrylate. Subsequently, the samples were sawed by using a microtome and stained with methylene blue and basic fuchsin. The slides were examined under a light microscope (Nikon Eclipse E400) and scored according to the following system: 0 = no infiltration up to 5 = severe infiltration of leukocytes, lymphocytes, macrophages and foreign body giant cells. The slides were also evaluated for the presence of fibroblasts, neovascularization and the formation of a fibrous capsule around the microspheres.

Scanning Electron Microscopy (SEM) was used to evaluate the morphology of the explanted microspheres and surrounding tissue. Following dehydration by series of

ethanol, pieces of the samples were critical point dried from carbon dioxide in a Balzers model CPD 030 Critical Point Dryer. The dried samples were sputter-coated with a thin gold layer and studied on a Philips XL 30 Environmental Scanning Electron Microscope (ESEM).

In vitro degradation

To determine the in-vitro degradation, microspheres (approximately 0.25 gram) were immersed in 10 ml PBS (pH 7.4) at 37°C in a shaking bath for 1, 4, 8, 15 and 24 weeks. Each week, the buffer was refreshed. After 1, 4, 8, 15 and 24 weeks, respectively, the microspheres were collected by centrifugation and freeze-dried. Subsequently, the molecular weight (M_w) was determined by GPC analyses as described above.

Results and discussion

Microspheres characteristics

To prepare PEGT/PBT microspheres, a polymer solution was dispersed in an aqueous poly(vinyl alcohol) solution, resulting in an oil-in-water (o/w) emulsion. Hardening of the microspheres was accomplished by evaporation of the organic solvent. For this in-vivo study, two types of poly (ether-ester) copolymers were selected; a hydrophobic copolymer with PEG segments of 300 g/mole and 47 wt% PBT (300PEGT53PBT47) and a more hydrophilic copolymer: 1000PEGT71PBT29 (M_w PEG = 1000 g/mole, 29 wt% PBT). The molecular weights of the microspheres prior to and after gamma irradiation were determined by GPC analyses. The number average molecular weight (M_n) and the weight average molecular weight (M_w) are given in Table 2. The M_w is in the range of 76.5 – 92.7 kg/mole, all with a molecular weight distribution (M_w/M_n) of about 2, which is expected for polymers prepared by a polycondensation reaction.

As shown in Table 2, the weight average molecular weights of both copolymer compositions increased due to gamma irradiation. This observation is in contrast to the effect of gamma irradiation on poly(lactide-co-glycolide) (PLGA) copolymers. Compared to other sterilization methods (radio-frequency glow discharge plasma treatment and ethylene oxide sterilization), gamma irradiation was considered to be the

most damaging towards PLGA [17]. Several studies on PLGA based microspheres showed a decrease in molecular weight up to 40% depending on the irradiation dose and copolymer composition [18-20]. The decrease in molecular weight was the result of chain scission through radical formation due to gamma irradiation [18,19]. Because of the chain scission, gamma-irradiation affected the stability [18] and the degradation [19,20] of these microspheres. The presence of aromatic segments in the PEGT/PBT copolymers might explain the different effect of gamma irradiation on these copolymers, as aromatic segments are less susceptible to radical formation than the aliphatic segments [21]. Besides, crosslinks can be formed in the case of recombination of radicals, which will increase the molecular weight of the copolymer. The possibility of recombination is greater as the mobility of the chain increases [21]. On the other hand, the PEG segments present in the copolymers are known to be susceptible for oxidation due to gamma irradiation [22]. Possibly, the presence of the antioxidant α-tocopherol in the PEGT/PBT copolymers and the absence of oxygen during sterilization prevented cleavage due to gamma irradiation. The 1000PEGT71PBT29 microspheres showed a higher increase in weight average molecular weight than the 300PEGT53PBT47 microspheres (Table 2). Less aromatic segments and higher segment mobility probably resulted in more radical formation and subsequent recombination (cross-linking) in the 1000PEGT71PBT29 microspheres.

Table 2: Microsphere characteristics before and after γ irradiation

Microspheres	M_n (kg/mole)	M_w (kg/mol)	M_w/M_n	Dn[1] (μm)	Dv[2] (μm)	Dv/Dn
300PEGT53PBT47 before γ	38.8	76.5	2.0	24.7	35.0	1.4
300PEGT53PBT47 after γ	38.6	78.4	2.0	24.6	35.2	1.4
1000PEGT71PBT29 before γ	47.3	84.6	1.8	22.4	35.5	1.5
100PEGT71PBT29 after γ	45.9	92.7	2.0	20.0	32.4	1.6

[1] number weight mean diameter
[2] volume weight mean diameter

Scanning Electron Microscopy (SEM) was used to study the morphology of the dry microspheres prior to injection. The microspheres of both copolymer compositions were round shaped with smooth surfaces (Figure 1). No differences were observed between microspheres before and after gamma irradiation.

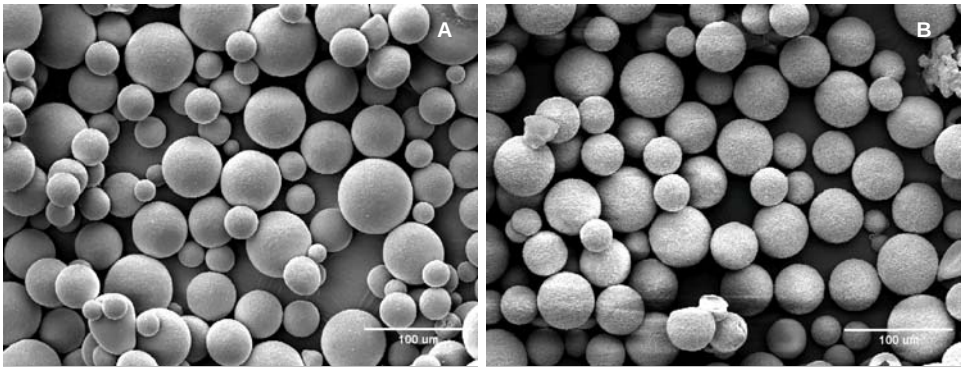


Figure 1: Scanning electron micrographs of sterilized 1000PEGT71PBT29 (A) and 300PEGT53PBT47 (B) microspheres prior to injection.

The number weight mean Dn and volume weight mean Dv of the microspheres measured with the Accusizer are also given in Table 2. The volume weight mean diameters of the 300PEGT53PBT47 and the 1000PEGT71PBT29 microspheres were both around 35 µm. The narrow ratio between the number and the volume weight mean sizes (Dv/Dn) is due to the sieving step, which removed the small particles. The size of the microspheres was not affected by gamma irradiation, in contrast to studies on PLGA microspheres [18,23]. Montari et al. observed an increasing microsphere size with increasing irradiation dose, which was ascribed to aggregates [18]. The 300PEGT53PBT47 and 1000PEGT71PBT29 microspheres were sieved to exclude size effects, although small size differences between batches will not affect the in-vivo behavior. Visscher et al. studied PLGA microspheres with average particle sizes of 30, 79 and 130 µm and noted only minimal differences in the degradation properties for the largest microspheres (130 µm) [24]. Cadée et al. investigated the biocompatibility of both PLGA and crosslinked dextran based microspheres of various sieve fractions [25]. Only, PLGA particles smaller than 10 µm induced an extensive tissue reaction. Macrophages and foreign body giant cells are known to phagocytoze particles of this size [2].

Cytotoxicity

To evaluate the cytotoxicity of the PEGT/PBT microspheres, before and after sterilization, extracts of these materials were tested towards the growth, morphology and metabolism of fibroblasts. In the presence of an extract of UHMW polyethylene, which

served as the negative control, the cell growth was comparable to the medium control cultures (maximum negative control). After exposure to the extract for 72 hours, an almost confluent layer of cells was formed and no morphological changes were observed (grade 0 in Table 1). In contrast, in the presence of Para rubber, which was used as a positive control, no confluent layer could be observed. Lots of cells died or rounded off as a result of the exposure to the Para rubber extract (grade 4 in Table 1). The confluency of the monolayer and the change of cellular morphology after exposure to extracts of PEGT/PBT microspheres, before and after gamma irradiation, were also scored microscopically. These qualitative scores are given in Table 3. For the microspheres of both copolymer compositions, before and after gamma irradiation, no differences with respect to the monolayer and the cell morphology could be observed compared to the negative control. Qualitative scores on the cell metabolism were obtained from the MTT test, which indicates the mitochondrial dehydrogenase activity. The fibroblasts exposed to the extract of the Para rubber (positive control) revealed a strong MTT reduction (Table 3). The extracts of the microspheres, however, showed no inhibition of the cell metabolism compared to the negative control (UHMW polyethylene). From both the qualitative and quantitative scores, it was concluded that the extracts of the microspheres demonstrated no cytotoxic reactivity in this test. The sterilization process by gamma irradiation had no effect on the cytotoxicity of the microspheres.

Table 3: Scores (grades) in cytotoxicity test for microspheres before and after gamma irradiation

Sample	Confluency of monolayer	Change of cell morphology	Inhibition of cell metabolism	Mean score
UHMW polyethylene (negative control)	0	0	0	0
300PEGT53PBT47 before γ irradiation	0	0	0	0
300PEGT53PBT47 after γ irradiation	0	0	0	0
1000PEGT71PBT29 before γ irradiation	0	0	0	0
100PEGT71PBT29 after γ irradiation	0	0	0	0
Para rubber (positive control)	4	4	4	4

This in-vitro biocompatibility experiment focused on the initial cytotoxicity, while the effect of degradation products on the cytotoxicity will be more pronounced in long-term evaluations. Van Loon et al. investigated the cytotoxicity of PEGT/PBT copolymers by real time testing [8]. Samples of these copolymers were degraded in

pseudo-extracellular fluid [26] at 37°C up to 52 weeks. None of the extracts of the tested copolymers showed any cytotoxicity in cell proliferation tests and MTT tests. If these results can be extrapolated for long-term implantation has been tested in the in-vivo biocompatibility study.

In-vivo biocompatibility

The in-vivo biocompatibility of the PEGT/PBT microspheres was evaluated for 24 weeks in rabbits. PEGT/PBT copolymers have been the subject of in-vivo studies before, but all of these studies dealt with larger implants [9,10,12].

Figure 2 shows 300PEGT53PBT47 microspheres after 1 week of subcutaneous implantation in which the tissue response has been started at the surface, while more to the center of the microspheres cluster no tissue response was observed. Later time intervals showed a complete tissue response throughout the cluster of microspheres. The cluster of microspheres injected in a tissue may be considered as an open porous implant. As a result, a period of days or weeks is needed for the progress of the tissue responses from the outer surface of the cluster of microspheres to its center [2,27].

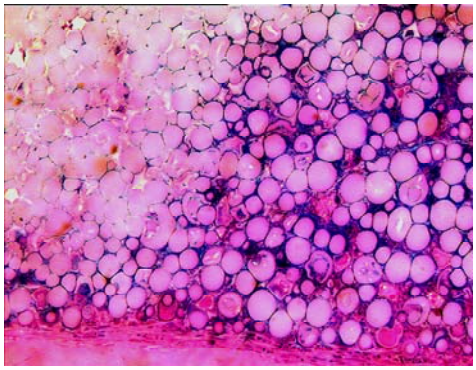


Figure 2: Histology of 300PEGT53PBT47 microspheres injected subcutaneously at 1 week. On the right at the surface of the total microsphere volume the tissue response has been started, while at the left, more in the center, a cluster of microspheres is still present. On the bottom of the picture, the onset of the fibrous capsule formation is seen. Original magnification: 100x

The nature and extent of the tissue reaction can be characterized by the presence of specific cell types, as described by Anderson et al. [2]. Figure 3 gives an overview of the intensities of cell types as a function of time after subcutaneous injection of 300PEGT53PBT47 and 1000PEGT71PBT29 microspheres. After the injury due to the

injection, an acute inflammatory response was started as can be seen from the presence of polymorphonuclear leukocytes. This tissue reaction was of short duration; after 1 week the intensity of polymorphonuclear leukocytes decreased. The low intensity of lymphocytes during the implantation time indicates the occurrence of a mild chronic inflammatory response. The foreign body reaction is expressed by the amount of macrophages and foreign body giant cells at the microspheres/tissue interface [2,28].

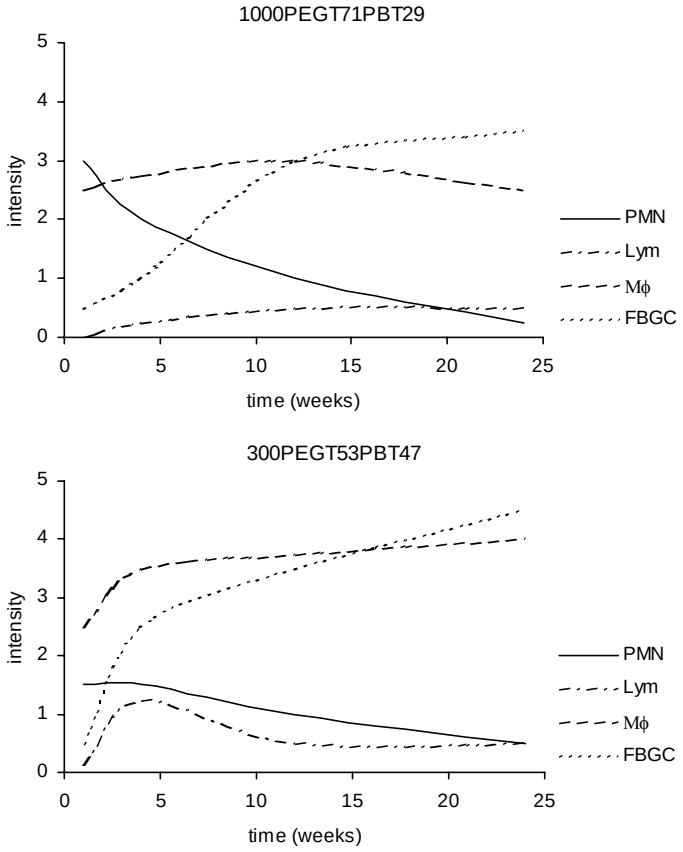


Figure 3: Temporal variation of cell types indicating acute inflammatory response, chronic inflammatory response and foreign body reaction to 1000PEGT71PBT29 and 300PEGT53PBT47 microspheres after subcutaneous injection. Polymorphonuclear leukocytes (PMN), lymphocytes (Lym), macrophages (Mϕ) and foreign body giant cells (FBGC) are indicated.

Macrophages play an important role in the phagocytosis of injured cells and tissue and degradation products from the microspheres [12,29]. Particles that are less than 10 μm in diameter are susceptible to phagocytosis by microphages and foreign body giant cells.

Foreign body giant cells are formed by the fusion of macrophages [30]. Figure 5 shows multinucleated foreign body giant cells present at the surface of 1000PEGT71PBT29 microspheres after 12 weeks of implantation. The intensity of foreign body giant cells increased in time, whereas the number of macrophages remained more or less constant (Figure 3). Macrophages and foreign body giant cells were observed for both the 1000PEGT71PBT29 and 300PEGT53PBT47 microspheres, but the intensities were higher for the 300PEGT53PBT47 microspheres (Figure 3). This observation might indicate that more degradation products were released from the 300PEGT53PBT47 microspheres. Due to the more hydrophilic nature, however, faster degradation is expected for the 1000PEGT71PBT29 microspheres (next paragraph). More likely, the difference in foreign body giant cell intensity is caused by surface properties [31]. The surface of the 1000PEGT71PBT29 microspheres contains hydrophilic PEG chains, which prevents protein adsorption [32]. This can also affect the formation of foreign body giant cell, as this process is surface dependent [2].

Besides phagocytosis, macrophages initiate the formation of granulation tissue, which is the scaffold for tissue repair. The granulation tissue response is characterized by fibroblast infiltration and the development of blood capillaries [30]. If an implant is not phagocytosed, the body tends to completely isolate the foreign implant by forming a fibrous capsule around the implant [29,32,33]. Figure 4 shows the granulation tissue response as observed after subcutaneous injection of 1000PEGT71PBT29 and 300PEGT53PBT47 microspheres. The fibroblast infiltration and the neovasularization increased in time for both types of microspheres, but the intensities for the 1000PEGT71PBT29 microspheres were higher. Fibrous tissue ingrowth and newly formed blood vessels around 1000PEGT71PBT29 microspheres after 12 weeks are shown in Figure 5. Figure 2 shows the onset of fibrous capsule formation for the 300PEGT53PBT47 microspheres after 1 week of subcutaneous implantation. An onset of a fibrous capsule was observed for both 1000PEGT71PBT29 and 300PEGT53PBT47 microspheres. The maximum capsule thickness was 5 – 10 cell layers after 12 weeks, but, surprisingly, decreased to almost no fibrous capsule after 24 weeks. Especially for the application of the microspheres as matrix for drug delivery, the amount of fibrous capsule might be of importance. Studies of Anderson showed that the fibrous capsule influenced the release behavior of the drug [34]. The in-vivo drug release could be delayed, or

prevented by the fibrous capsule. Wood et al isolated fibrous capsules from subcutaneously implanted silastic discs for drug permeability experiments [35]. Three model compounds with various molecular weights were tested in an in-vitro diffusion cell model. These experiments showed that the fibrous capsule is even permeable for dextran with a molecular weight of 70000 g/mole (permeability: 5.6 x 10^{-6} cm s^{-1}). Thus, the fibrous capsule around the microspheres will not necessarily prevent the release of proteins and peptides, but a delayed release might be observed.

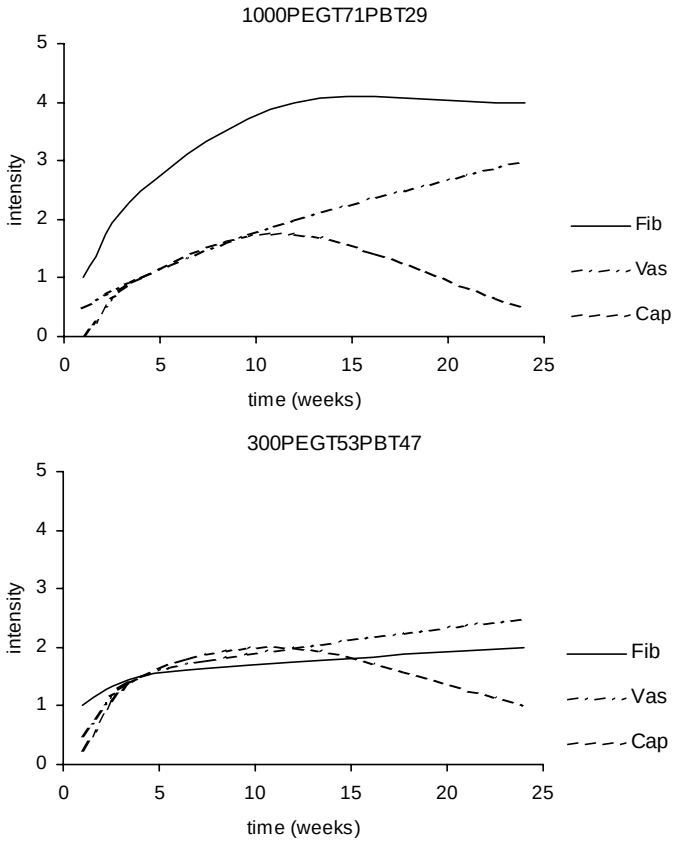


Figure 4: Granulation tissue response to 1000PEGT71PBT29 and 300PEGT53PBT47 microspheres after subcutaneous injection as function of time. The presence of fibroblasts (Fib), newly formed blood capillaries (Vas) and the intensity of fibrous capsule (Cap) are indicated.

Besides the inflammatory response, the foreign body reaction and the granulation tissue response, the slides for histological analyses were checked for tissue necrosis and changes in tissue morphology (like fatty degeneration). These signs of

bioincompatibility were not observed with either the 1000PEGT71PBT29 or the 300PEGT53PBT47 microspheres. From these results, it was concluded that the PEGT/PBT microspheres were well tolerated by the surrounding subcutaneous tissue.

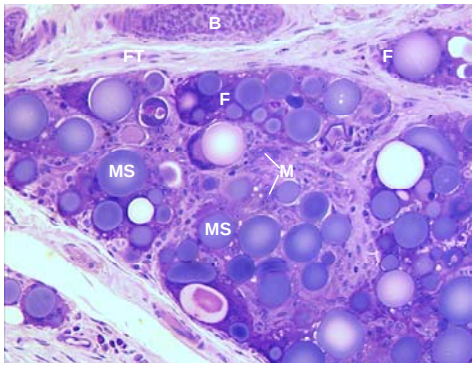


Figure 5: Histology of 1000PEGT71PBT29 microspheres (MS) injected subcutaneously at 12 weeks. Macrophages (M, dark spots) and foreign body giant (F, dark areas) cells surround the microspheres. Fibroblasts produced fibrous connective tissue around clusters of microspheres Blood capillaries (B) within the fibrous tissue (FT) are seen at the top of the figure. Original magnification: 200x.

The results reported above were obtained from the subcutaneously injected microspheres. To study the effect of the injection site on the biocompatibility, the microspheres were also injected intramuscularly. However, evaluation of the intramuscular injection sites was difficult as it was hard to retrieve the microspheres from the muscular tissue. This might be related to the lower amount injected intramuscularly compared to the subcutaneously injected amount. Besides, maybe more degradation or transportation of the microspheres had occurred in the muscular tissue [9]. The few samples of intramuscularly injected microspheres that could be retrieved showed no clear differences in inflammatory and foreign body response compared to the subcutaneously injected microspheres. The granulation tissue response was less obvious for the intramuscularly injected microspheres, as fibrous tissue ingrowth and fibrous capsule were observed to a lesser extent (Figure 6). Previous studies with PEGT/PBT films showed a similar tissue response for intramuscular and subcutaneous implantation in terms of foreign body reaction, ingrowth of fibrous tissue and neovascularization [9]. Usually, a more intense tissue reaction is expected for microspheres, as the surface area is much larger compared to an implant with an identical volume [2]. In some cases, however, the opposite effect was observed. For example, Cadée et al. studied the in-vivo

biocompatibility of both degradable and nondegradable dextran-based microspheres and discs [25,33]. For the discs more exudate and fibrin was observed in the surrounding tissue compared to the microspheres.

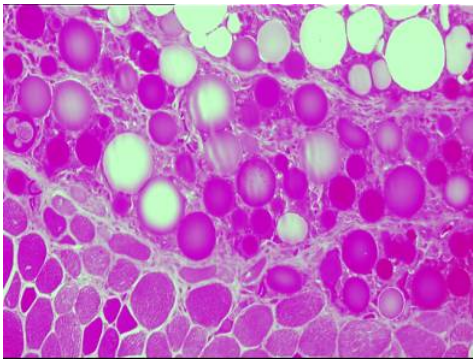


Figure 6: Histology of 1000PEGT71PBT29 microspheres injected intramuscularly at 12 weeks. Macrophages and foreign body giant cells surround the microspheres. Muscle cells are seen at the bottom of the picture. A tiny fibrous capsule is present shown between the muscle cells and the microspheres. Less fibrous tissue was observed for the intramuscularly injected microspheres compared to the subcutaneously injected microspheres (Figure 5). Original magnification: 200x.

Concerning the tissue reaction observed for the microspheres in this study, PEGT/PBT copolymers have good potential as matrices for controlled drug delivery. Repeated injections, however, may enhance the foreign body reaction as was shown for several biomaterials by van Luyn [36]. This phenomenon has to be evaluated for PEGT/PBT matrices. Although the two PEGT/PBT copolymer compositions used in this study varied in hydrophilicity and degradation rate, the in-vivo biocompatibility study on the two types of microspheres showed only marginal differences in the tissue responses.

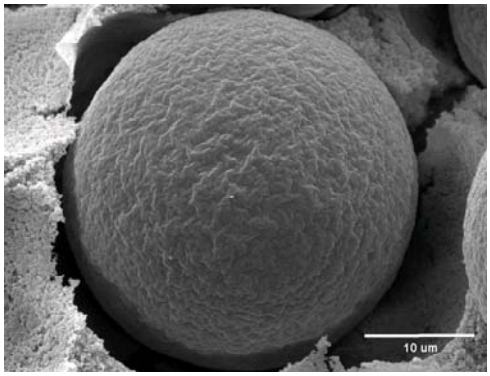


Fig 7: Scanning electron micrograph of 1000PEGT71PBT29 microspheres injected subcutaneously at 24 weeks.

In-vitro and in-vivo degradation

Histology showed intact microspheres up to 12 weeks of implantation (Figures 2, 5 and 6). Even after 24 weeks, no fragmented particles have been observed (Figure 7), although the number of microspheres seemed to be reduced and the amount of fibrous tissue had increased. Histology, however, is hard to use as quantitative method for the determination of mass loss. No signs of degradation were observed at the surfaces of the 300PEGT53PBT47 microspheres. Closer examination, however, of the 1000PEGT71PBT29 microspheres, surrounded by macrophages and foreign body giant cells, showed that biomaterial-tissue interfaces became less defined, which may be a sign of resorption (Figure 8).

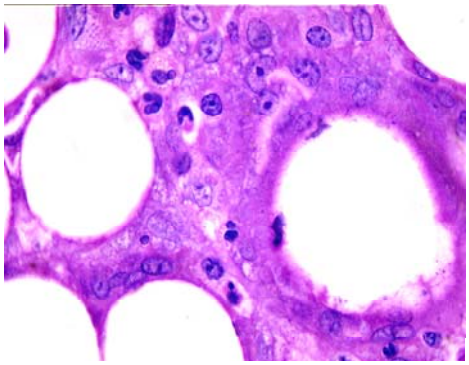


Fig 8: Histology of 1000PEGT71PBT29 microspheres injected subcutaneously at 12 weeks. The biomaterial-tissue interface of the microsphere on the right, surrounded by macrophages and foreign body giant cells, became less defined, which may be a sign of resorption. Original magnification: 1000x.

In previous studies with implants of a similar PEGT/PBT copolymer composition, Transmission Electron Microscopy (TEM) showed phagocytes with intracellular polymeric particles [8-10]. In addition, extensive fragmentation of the implants was visible within 4 weeks, enlarging the total implant surface [9,10]. With increasing PEGT content, the fragmentation of the copolymer was more pronounced [10]. Higher fragmentation rate and smaller average particle size were observed at the intramuscular implantation site as compared to the subcutaneous site [9]. This was attributed to the higher stresses and higher extent of tissue vascularization in muscular tissue. The polymeric fragments, however, varied from 300 up to 1200 μm, which is at least 10 times

larger than the microspheres injected in this study. It is, therefore, unlikely that in our study the microspheres will show fragmentation due to mechanical stresses.

As described for PLGA based microspheres, degradation may have started without being visible as fragmentation or mass loss [2,24,28,37]. As the polymer degrades, no loss of integrity of the microspheres may be observed and the foreign body reaction remains visually the same. In a later stage, the decrease in molecular weight may progress to the point where the integrity of the microspheres can no longer be maintained and the microspheres break down into particles (< 10 μm), which undergo macrophage phagocytosis and complete degradation [2]. To check this hypothesis for the PEGT/PBT microspheres, the in-vitro degradation in PBS was investigated. Figure 9 shows the weight average molecular weight of the microspheres determined by GPC analyses, as function of time. The molecular weight of the 1000PEGT71PBT29 microspheres decreased from 92.7 kg/mole to 37.4 kg/mole in 24 weeks. However, similar to the in-vivo situation, Scanning Electron Microscopy showed no signs of fragmentation or mass loss of the microspheres. Apparently, the point where the integrity of the microspheres can no longer be maintained had not been reached after 24 weeks of degradation. In addition, the mild tissue reaction already indicated that a low amount of degradation products had been released. For the 300PEGT53PBT47 microspheres, no significant change in molecular weight could be observed.

The degradation mechanisms of poly(ether ester)s have been subject of many studies [11,38-40]. The degradation pathways of PEGT/PBT that are supposed to occur in vivo are hydrolysis and oxidation [38]. The ester bonds can be hydrolyzed and the ether bonds can be oxidized due to the presence of ions or radicals produced by cells [38]. In our in-vitro degradation study on PEGT/PBT microspheres, only the hydrolysis was taken into account. Deschamps et al studied the effect of radicals on the degradation of PEGT/PBT films in-vitro [39]. Incubation in a $H_2O_2/CoCl_2$ solution had a dramatic effect on the molecular weight and the mechanical properties of the samples. Based on these results, the in-vivo degradation of the microspheres was expected to be faster than the in-vitro degradation in PBS.

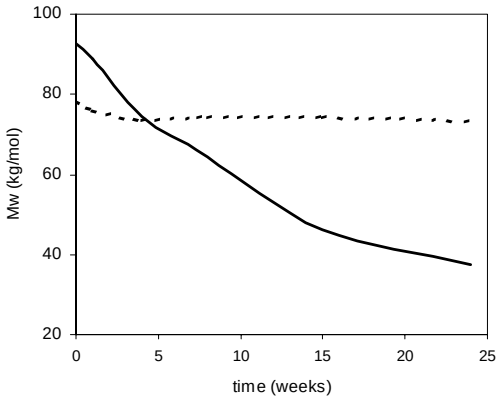


Fig 9: The weight average molecular weights as determined by GPC of 1000PEGT71PBT29 (solid line) and 300PEGT53PBT47 (dotted line) microspheres degraded in PBS versus time.

The in-vivo degradation of the microspheres was slower than reported in previous studies with macroscopic PEGT/PBT implants [9,10]. This might limit the suitability for repeated injections, in particular for the release of peptides from hydrophobic matrices like the 300PEGT53PBT47 matrices. For the sustained release of proteins, however, more hydrophilic PEGT/PBT copolymer compositions are available, which show a faster degradation [10]. The in-vivo behavior of these faster degrading copolymers are the subject of further experiments. In addition, unloaded microspheres were evaluated in this study, while for controlled release applications the microspheres will be loaded with a drug. The biological activity of the incorporated drug may alter the tissue response [2]. In addition, a different internal structure due to the presence of the drug, may affect the degradation behavior as well.

Conclusion

Microspheres of a hydrophobic and a hydrophilic PEGT/PBT copolymer were shown to be biocompatible by in-vitro and in-vivo studies. Extracts of these microspheres showed no cytotoxic reactivity in the in-vitro cytotoxicity test. The microspheres could be sterilized by gamma irradiation without increasing the cytotoxicity and decreasing the copolymer molecular weight. PEGT/PBT microspheres injected subcutaneously and intramuscularly in rabbits showed a mild tissue response in vivo, in terms of the

inflammatory response, the foreign body reaction and the granulation tissue response. The in-vivo degradation was slower than expected from previous studies with large implants, although an in-vitro degradation experiment showed a decrease in molecular weight due to hydrolysis. For the delivery of proteins, more hydrophilic faster degrading PEGT/PBT polymers (with higher PEGT wt% and longer PEG segments) are currently evaluated. In addition, future work will focus on the synthesis of poly(ether-ester) copolymers with enhanced degradation behavior.

References

1. R. Langer, Polymeric delivery systems for controlled drug release, Chem. Eng. Commun. 1980;6:1-48.
2. Anderson JA, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Del Rev 1997;28:5-24.
3. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J Control Rel 2000;64(1-3):179-192.
4. Van Dijkhuizen-Radersma R, Péters FLAMA, Stienstra NA, Grijpma DW, Feijen J, de Groot K, Bezemer JM. Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers. Biomaterials 23 (2002) 1527-1536.
5. Radder AM, Leenders H, Van Blitterswijk CA. Application of PEO/PBT copolymers for bone replacement. J Biomed Mat Res 1996;30:341-351.
6. Beumer GJ, Van Blitterswijk CA, Ponec M. Biocompatibility of degradable matrix induced as a skin substitute: an in vivo evaluation. J Biomed Mat Res 1994;28:545-552.
7. Bakker D, Van Blitterswijk CA, Hesseling SC, Koerten HK, Grote JJ. Biocompatibility of a polyether urethane, polypropylene oxide and a polyether polyester copolymer. A qualitative study of three alloplastic tympanic membrane materials in rat middle ear. J Biomed Mater Res 1990;24:489-515.
8. Van Loon, Thesis University of Leiden, The Netherlands, 1995
9. Beumer GJ, Van Blitterswijk CA, Ponec M. Degradation behaviour of polymeric matrices in (sub)dermal and muscle tissue of the rat: a quantitative study. Biomaterials 1994;15(7):551-559.
10. Van Blitterswijk CA, Van de Brink J, Leenders H, Bakker D. The effect of PEO ratio on degradation, calcification and bone bonding of PEO/PBT copolymer (PolyActive). Cell and Materials 1993;3(1):23-36.
11. Reed AM, Gilding DK. Biodegradable polymers for use in surgery - poly(ethylene oxide) poly(ethylene terephthalate) (PEO/PET) copolymers: 2. In vitro degradation. Polymer 1981;22:499-504.
12. Bakker D, Van Blitterswijk CA, Hesseling SC, Grote JJ, Deams WT. Effect of implantation site on phagocyte/polymer interaction and fibrous capsule formation. Biomaterials 1988;9:14-21.

13. Radder AM, Leenders H, Van Blitterswijk CA. Interface reactions to PEO/PBT copolymers (Polyactive®) after implantation in cortical bone. J Biomed Mat Res 1994;28:141-151.
14. De Groot CJ, Van Luyn MJA, Van Dijk-Wolthuis WNE, Cadée JA, Plantinga JA, Den Otter W, Hennink WE. In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. Biomaterials 2001;22:1197-1203.
15. Cordewener FW, Van Geffen MF, Joziasse CAP, Schmitz JP, Bos RRM, Rozema FR, Pennings AJ. Cytotoxicity of poly(96L/4D-lactide): the influence of degradation and sterilization. Biomaterials 2000;21:2433-2442.
16. Edmunson IC. Particle-size analysis. In: Bean HS, Beckett AH, Couless JF, editors. Advances in Pharmaceutical Sciences. London: Academic Press;1967:95-179
17. Holy CE, Cheng C, Davies JE, Shoichet MS. Optimization the sterilization of PLGA scaffolds for use in tissue engineering. Biomaterials 2001;22:25-31.
18. Montanari L, Costantini M, Ciranni Signoretti E, Valvo L, Santucci M, Bartolomei M, Fattibene P, Onori S, Faucitano A, Conti B, Genta I. Gamma irradiation effects on poly(DL-lactide-co-glycolide) microspheres. J Control Rel 1998;56:219-229.
19. Bittner B, Mäder K, Kroll C, Borchert H-H, Kissel T. Tetracycline-HCl-loaded poly(DL-lactide-co-glycolide) microspheres prepared by spray drying technique: influence of γ-irradiation on radical formation and polymer degradation. J Control Rel 1999;59:23-32.
20. Spenlehauer G, Vert M, Benoit JP, Boddaert A. In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials 1989;10:557-563.
21. Nair PD, Sreenivasan K, Jayabalan M. Multiple gamma radiation sterilization of polyester fibers. Biomaterials 1988;9:335-338.
22. Decker C. Radiation-induced oxidation of solid poly(ethylene oxide) II: Mechanism. J Polym Sci 1977;15:799-813.
23. Montanari L, Cilurzo F, Valvo L, Faucitano A, Buttafave A, Groppo A, Genta I, Conti B. Gamma irradiation effects on stability of poly(lactide-co-glycolide) microspheres containing clonazepam. J Control Rel 2001;75:317-330
24. Visscher GE, Pearson JE, Fong JW, Argentieri GJ, Robinson RL, Maulding HV. Effect of particle size on the *in vitro* and *in vivo* degradation rates of poly(DL-lactide-co-glycolide) microcapsules. J Biomed Mat Res 1988;22:733-746.
25. Cadée JA, Brouwer LA, Den Otter W, Hennink WE, Van Luyn MJA. A comparative biocompatibility study of microspheres based on crosslinked dextran or poly(lactic-co-glycolic)acid after subcutaneous injection in rats. J Biomed Mater Res 2001;56(4):600-609.
26. Homsy CA. Biocompatibility in selection of materials for implantation. J Biomed Mat Res1970;4:341-356.
27. Holder WD, Gruber HE, Moore AL, Culberson CR, Anderson W, Burg KJL, Mooney DJ. Cellular ingrowth and thickness changes in poly-l-lactide and polyglycolide matrices implanted subcutaneously in the rat. J Biomed Mat Res 1998;41:412-421.
28. Visscher GE, Robinson RL, Maulding HV, Fong FW, Pearson JE, Argentieri GJ. Note: Biodegradation of and tissue reaction to poly(DL-lactide) microspheres. J Biomed Mat Res 1986;20:667-676.

29. Anderson JM. In vivo biocompatibility of implantable delivery systems and biomaterials. Eur J Pharm Biopharm 1994;40:1-8.
30. Anderson JM, Langone JJ. Issues and perspectives on the biocompatibility and immunotoxicity evaluation of implanted controlled release systems. J Control Rel 1999;57;107-113.
31. Tabata Y, Ikada Y. Phagocytosis of polymer microspheres by macrophages. Adv Polym Sci 1990;94:107-141.
32. Park H, Park K. Biocompatibility issues of implantable drug delivery systems. Pharm Res 1996;13(12):1770-1776.
33. Cadée JA, Van Luyn MJA, Brouwer LA, Plantinga JA, Van Wachem PB, De Groot CJ, Den Otter W, Hennink WE. In vivo biocompatibility of dextran-based hydrogels. J Biomed Mater Res 200;50:397-404.
34. Anderson JM, Niven H, Pelagalli J, Olanoff LS, Jones RD. The role of the fibrous capsule in the function of implanted drug-polymer sustained release systems. J Biomed Mat Res 1981;15:889-902.
35. Wood RC, LeCluyse EL, Fix JA. Assessment of a model for measuring drug diffusion through implant-generated fibrous capsule membranes. Biomaterials 1995;16(12):957-959.
36. Van Luyn MJA, Plantinga JA, Brouwer LA, Khouw IMSL, De Leij LFMH, Van Wachem PB. Repetitive subcutaneous implantation of different types of (biodegradable) biomaterials alter the foreign body reaction. Biomaterials 2001;22:1385-1391.
37. Visscher GE, Robison RL, Maulding HV, Fong JW, Pearson JE, Argentieri GJ. Biodegradation of and tissue reaction to 50:50 poly(DL-lactide-co-glycolide) microcapsules J Biomed Mat Res 1985;19:349-365.
38. Goedemoed JH, Hennink WH, Bezemer JM, Feijen J, Van Blitterswijk CA, De Bruijn JD. Polyetherester copolymers as drug delivery matrices. European Patent Application 97202533.2, 1998.
39. Deschamps AA, Claase MB, Sleijster MWJ, De Bruijn JD, Grijpma DW, Feijen J. Design of segmented poly(ether ester) materials and structures for tissue engineering of bone. J Control Rel, 2002;78(1-3):175-86.
40. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. A controlled release system for proteins based on poly(ether-ester) block-copolymers: polymer network characterization. J Control Rel 1999;62(3):393-405.

Chapter 4

Control of vitamin B_{12} release from Poly(ethylene glycol)/ Poly(butylene terephthalate) multiblock copolymers

R. van Dijkhuizen-Radersma, F.L.A.M.A. Péters, N.A. Stienstra,
D.W. Grijpma, J. Feijen, K. de Groot, J.M. Bezemer

Abstract

The release of vitamin B_{12} (1355 D) from matrices based on multiblock copolymers was studied. The copolymers were composed of hydrophilic poly(ethylene glycol)-terephthalate (PEGT) blocks and hydrophobic poly(butylene terephthalate) (PBT) blocks. Vitamin B_{12} loaded films were prepared by using a water-in-oil emulsion method. The copolymer properties, like permeability, could be varied by increasing the PEG-segment length from 300 up to 4000 g/mole and by changing the wt% of PEGT. From permeation and release experiments, the diffusion coefficient of vitamin B_{12} through PEGT/PBT films of different compositions was determined. The diffusion coefficient of vitamin B_{12} was strongly dependent on the composition of the copolymers. Although an increased wt% of PEGT (at a constant PEG-segment length) resulted in a higher diffusion coefficient, a major effect was observed at increasing PEG-segment length. By varying the copolymer composition, a complete release of vitamin B_{12} in 1 day up to a constant release for over 12 weeks was obtained. The release rate could be effectively tailored by blending copolymers with different PEG-segment lengths. The swelling and the crystallinity of the matrix could explain the effect of the matrix composition on the release behavior.

Introduction

Extensive investigations have been carried out on polymers for controlled release systems for peptides and proteins [1,2]. Most of this work has focused on poly(lactide-co-glycolide) (PLGA) [3]. Recently, a series of poly(ethylene glycol) terephthalate / poly(butylene terephthalate) (PEGT/PBT) multiblock copolymers was studied as matrix for controlled drug delivery [4]. This polymer system is currently under (pre)clinical investigation for a wide range of biomedical applications, including bone replacement [5], and cartilage [6,7], muscle [7] and skin repair [8]. Many in-vivo and in-vitro studies have shown that PEGT/PBT multiblock copolymers are biocompatible and can be made biodegradable [8-15]. In 2000, a degradable cement restrictor composed of PEGT/PBT obtained market clearance from the FDA.

In earlier publications, PEGT/PBT copolymers were shown to be a successful matrix for (zero order) controlled release of large molecules, like proteins (lysozyme (14.5 kD) and albumin (67 kD)) [4]. A long time constant release of proteins was obtained by a combination of diffusion and degradation. The protein release rate could be tailored by varying the composition of the copolymer, with preservation of the protein activity. To our best knowledge, no experiments dealing with the release of small molecules from PEGT/PBT matrices have been reported. A lot of pharmaceuticals, however, are small molecules like peptides. Currently, leuprolide (1270 D) loaded PLGA microspheres are on the market for the treatment of prostate cancer [16]. Also, research has been carried out on the development of salmon calcitonin (3500 D) release systems based on, for example, poly(glycolic acid) [17]. In this study, the application of PEGT/PBT copolymer as matrix for controlled release of small water-soluble molecules, like peptides, was investigated. As a model, vitamin B_{12} (1355 D) was used, because it has the same size as leuprolide and it is easily detectable by its red color. The vitamin B_{12} release was studied from solvent cast PEGT/PBT films. We investigated the effect of the matrix composition on the vitamin B_{12} release behavior by using a series of PEGT/PBT copolymers and blends of these copolymers.

Materials and methods

Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis (Bilthoven, The Netherlands). The poly (ether-ester) copolymers vary in PEGT/PBT weight ratio (80/20-30/70) and PEG segment length (300, 600, 1000 and 4000) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt% PEG-terephthalate and **c** (=100-b) the wt% PBT. Phosphate buffered saline (PBS), (pH 7.4) was purchased from Life Technologies Ltd (Paisley, Scotland). Chloroform purchased from Fluka Chemie GmbH (Buchs, Switzerland) was of analytical grade. Vitamin B_{12} (cyanocobalamin, approximately 99%) and hexafluoroisopropanol (HFIP) were obtained from Sigma Chemical Co (St Louis, USA).

PEGT/PBT copolymer characterization

Proton NMR spectra were recorded on a Bruker ARX-400 operating at ≥ 200 MHZ. $C_2D_2Cl_4$ was used as solvent without internal standard. The PEGT/PBT copolymer composition was calculated as described elsewhere [18]. The intrinsic viscosity [η] was determined of a solution of 0.5 g/dl of polymer in chloroform using a Schott Gerâte Ubbelohde viscosimeter (DIN) type 0c at 25°C. Thermal analysis of the polymers was carried out with a Perkin-Elmer Pyris 1 differential scanning calorimeter. Calibration was performed with pure indium and zinc. Samples (10-15 mg) were heated from 40°C to 250°C at a heating rate of 10°C/min. We calculated the % of crystallized polymer from the area under the curve (ΔH) as described in detail by Fakirov et al. [19,20].

Preparation of (vitamin B_{12}-loaded) PEGT/PBT films

PEGT/PBT copolymer (1 g) was dissolved in 7 ml chloroform and cast on a glass plate using a casting knife. Copolymers having a PBT content above 50 wt% were dissolved in a mixture of 6 ml chloroform and 1 ml hexafluoroisopropanol. The solvent was slowly evaporated at room temperature and, subsequently, the films were dried under vacuum overnight. The resulting films had a thickness of 50-100 μm.

For the preparation of vitamin B_{12} loaded films, a vitamin B_{12} solution in PBS was emulsified in a polymer solution using an ultra turrax (IKA Labortechnik T25) for 30 s at 19 krpm. The resulting water-in-oil emulsion was cast on a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and freeze dried for at least 16 hrs.

To study the influence of the matrix composition on the vitamin B_{12} release behavior, a series of copolymers was used, containing 20-70 wt% PBT and PEG-segments with molecular weights varying from 300 to 4000 g/mole. Besides changing the composition of the copolymer, blends of copolymers containing approximately 50 wt% PBT and PEG-segment lengths of 300 and 600 g/mole were also prepared to vary the polymer matrix properties. For these experiments, 0.6 ml of vitamin B_{12} in PBS (10 mg/ml) was incorporated per g copolymer.

Swelling in PBS

Dry films (± 1.77 cm^2) were weighed and immersed in PBS at 37°C in a shaking bath. After at least 3 days, the weight of the samples was determined after surface water was removed by blotting the surface with a tissue. The water-uptake (in ml water per g polymer) was calculated from the weight increase. The equilibrium volume swelling ratio Q was determined according to the following equation (1.2 equals the density of all PEGT/PBT copolymers):

$$Q = 1 + \frac{1.2 \cdot (M_{swollen} - M_{dry})}{M_{dry}} \qquad \text{(I)}$$

From the water-up take, also the number of water molecules per ethylene glycol (EG) unit in the copolymer could be calculated. The number of water molecules per gram of polymer was determined by using equation (II), in which 18 is the molar mass of water.

$$N_{H_2O} = \frac{(M_{swollen} - M_{dry})}{18 \cdot M_{dry}} \qquad \text{(II)}$$

The number of EG units per gram of polymer was calculated from the weight fraction of PEGT and the PEG segment length according to the following equation:

$$N_{EG} = \frac{\phi_{PEGT} \cdot PEG}{(PEG + 130) \cdot 44} \qquad \text{(III)}$$

in which ϕ_{PEGT} is the weight fraction of poly(ethylene glycol) terephthalate, *PEG* is the PEG segment length, 130 is the molecular weight of a terephthalate unit and 44 equals the molecular weight of one EG unit. The total number of water molecules per ethylene glycol unit in a PEGT/PBT copolymer is the quotient of equation (II) and equation (III).

Degradation in PBS

To determine the degradation of polymer matrices, dry films (approximately 0.25 gram, 50-100 µm thickness) were immersed in 50 ml PBS at 37°C in a shaking bath. Each week, the buffer was refreshed. After seven weeks, the films were freeze dried and subsequently, the intrinsic viscosity [η] was determined as described above.

Permeability of equilibrium swollen membranes for vitamin B_{12}

The permeability of PEGT/PBT films for vitamin B_{12} (Mw = 1355 D) was measured using a 2 x 7.5 ml two chamber diffusion apparatus with an effective membrane area of 2.27 cm^2. Membranes were swollen to equilibrium in deionized water at 37°C before they were placed between the two chambers. The donor compartment was filled with a 2 mg/ml vitamin B_{12} solution and deionized water was added to the receptor side. The compartments were stirred at 500 rpm. in a thermostatic incubator at 37°C. Samples of the donor and receptor chambers were taken at various time points. The vitamin B_{12} concentration of the samples was determined using a SLT 340 ATTC microplate reader (λ = 340 nm). The permeability coefficients (*P*) were calculated from the UV absorbance data by the following equation (IV):

$$\ln\left(1 - 2\frac{C_t}{C_0}\right) = -\frac{2A}{Vl}Pt \qquad \text{(IV)}$$

in which C_t is the vitamin B_{12} concentration in the receptor cell at time *t*, C_0 is the initial vitamin B_{12} concentration in the donor compartment, *A* is the membrane surface area, *V* is the volume of each cell, *l* is the membrane thickness, and *P* is the permeability coefficient

[21]. The diffusion coefficient (D) of vitamin B_{12} in the various PEGT/PBT films was evaluated by:

$$D = \frac{P}{K_d} \qquad \text{(V)}$$

in which K_d is the partition coefficient. The partition coefficient, defined as the equilibrium ratio of the solute concentration in the film to that in the surrounding solution, was determined by solute uptake experiments. For each PEGT/PBT composition, three disks (2.85 cm^2), previously equilibrated in deionized water, were incubated in vitamin B_{12} solutions (2 mg/ml, 10 ml). After 14 days, the films were taken out of the solution, blotted with a tissue and immersed in 2 ml deionized water. The concentration of vitamin B_{12} released in the solutions was determined photospectroscopically as described above. The concentration of the solute in the films after immersion in the vitamin B_{12} solution was calculated from the total amount of released vitamin B_{12} and the volume of the equilibrium swollen films.

Due to the low diffusion rate of vitamin B_{12} through PEGT/PBT films made from copolymers with PEG-segments of 300 g/mole, it was not possible to measure a significant quantity of permeated vitamin B_{12} within a reasonable time interval using the diffusion apparatus. Therefore, permeability of vitamin B_{12} through PEGT/PBT membranes with 300 g/mole-segments was evaluated from release experiments.

Vitamin B_{12} release from PEGT/PBT films

The vitamin B_{12} release from the vitamin B_{12} loaded PEGT/PBT films was investigated by incubating pieces of the films (± 1.77 cm^2) in 1.5 ml PBS. Vials were continuously shaken at 37°C and samples of the release medium were taken at various time points. The vitamin B_{12} concentration of the buffer was determined using an EL 312e microplate bio-kinetics reader (λ = 380 nm). The buffer was refreshed after sampling. The thickness of the swollen films was measured using a micrometer.

Results and discussion

Polymer matrix characteristics

To study the possibilities of applying PEGT/PBT copolymers as a matrix for the controlled release of vitamin B_{12}, a series of PEGT/PBT copolymers was investigated. The copolymers varied in PEG-segment length and in PEGT/PBT ratio. By varying the composition, the copolymer properties could be changed. The properties of PEGT/PBT multiblock copolymers are mainly determined by their phase separated morphology. The soft hydrophilic PEGT blocks are flexible and responsible for the water-uptake, whereas the hard hydrophobic PBT blocks are rigid and give the matrix its stiffness. The structure and morphology of segmented poly(ether ester)s has been the subject of many studies [22 for a review].

Fakirov et al. found that domains of four different types may exist in PEGT/PBT copolymers: crystalline PBT, amorphous PBT, amorphous PEG and a mixed amorphous phase [23]. The ratio of the different phases is dependent on the polymerization conditions, copolymer composition and the thermal and mechanical history of the sample [22]. Concerning the effect of the copolymer composition, for polymers with a low PBT content and consequently short PBT sequences, no crystalline PBT phase was found. Increasing the molecular weight of the PEG-segments at a constant PEGT/PBT ratio, or increasing the PBT content at a constant PEG segment length will increase the average block length of the PBT segments and thus facilitate crystallization. The copolymer is physically crosslinked by these crystalline domains. The more the copolymer is crosslinked, the smaller the mesh widths in the matrix and consequently, the less permeable the matrix will be [24].

The PEGT/PBT copolymers used in this study as a matrix for the controlled release of vitamin B_{12} varied in PEG-segment length from 300 – 4000 g/mol. The weight percentages of PBT were determined by NMR and are given in Table 1. The intrinsic viscosity of the copolymers was in the range of 0.64 – 1.24 dl/g, depending on the copolymer composition. For controlled release systems, the structure and properties of the water-swollen matrices are of primary interest. Therefore, the swelling behavior of solvent cast, dense PEGT/PBT films (50-100 μm thickness) was investigated as a function of the copolymer composition. The equilibrium swelling ratio Q was reached

within three days (Table 1). For the copolymers used in this study, Q varied from 1.1 for copolymers with 300 g/mole PEG-segments up to 1.3 - 1.7 for 600 g/mole, 1.4 - 1.9 for 1000 g/mole and 1.7 – 3.7 for 4000 g/mole PEG-segments. Thus, as expected, the longer the PEG-segment length, the higher the swelling ratio Q. At increasing PEG-segment length, the effect of the PEG content on the swelling increased. This is in good agreement with earlier observations [18].

Table 1: Equilibrium swelling of PEGT/PBT films and diffusion and partition coefficients of vitamin B_{12} as function of the copolymer composition.

Polymer composition (NMR)	Swelling ratio Q	Diffusion coefficient D (cm^2/s) from diffusion cells	Diffusion coefficient D (cm^2/s) from release	Partition coefficient K_d (-)
300PEGT32PBT68	1.11 ± 0.03	n.d.	$(1.7 \pm 0.2) \times 10^{-14}$	n.d.
300PEGT53PBT47	1.08 ± 0.01	n.d.	$(6.0 \pm 2.7) \times 10^{-13}$	n.d.
300PEGT61PBT39	1.09 ± 0.00	n.d.	$(1.2 \pm 0.4) \times 10^{-12}$	n.d.
600PEGT40PBT60	1.34 ± 0.02	$(2.4 \pm 0.4) \times 10^{-9}$	$(1.6 \pm 0.6) \times 10^{-9}$	0.25 ± 0.05
600PEGT51PBT49	1.29 ± 0.03	$(3.9 \pm 0.9) \times 10^{-9}$	$(1.7 \pm 0.0) \times 10^{-9}$	0.35 ± 0.08
600PEGT57PBT43	1.38 ± 0.02	n.d.	$(1.4 \pm 0.6) \times 10^{-9}$	n.d.
600PEGT77PBT23	1.66 ± 0.01	$(1.7 \pm 0.6) \times 10^{-8}$	n.d.	0.50 ± 0.20
1000PEGT41PBT59	1.41 ± 0.03	$(2.5 \pm 0.1) \times 10^{-8}$	n.d.	0.26 ± 0.01
1000PEGT60PBT40	1.73 ± 0.01	$(4.4 \pm 0.3) \times 10^{-8}$	n.d.	0.33 ± 0.01
1000PEGT71PBT29	1.92 ± 0.13	$(3.2 \pm 0.3) \times 10^{-8}$	n.d.	0.73 ± 0.07
4000PEGT32PBT68	1.66 ± 0.03	$(7.5 \pm 0.1) \times 10^{-8}$	n.d.	0.41 ± 0.05
4000PEGT55PBT45	2.62 ± 0.01	$(1.9 \pm 0.1) \times 10^{-7}$	n.d.	0.60 ± 0.02
4000PEGT82PBT18	3.66 ± 0.05	$(1.5 \pm 0.2) \times 10^{-7}$	n.d.	1.40 ± 0.20

n.d.: not determined

Generally, it is accepted that three molecules of water can be bound to each ethylene glycol unit [25]. This has been investigated using various techniques, including NMR spectroscopy, analysis of the water-PEG phase diagram and differential thermal analysis [26 and references herein]. However, in studies on segmented polyether polyurethanes with 2000 g/mole PEG-segments, although the number of bound water molecules increased with an increase in PEG content, it did not reach the generally accepted value of three [27]. This was caused by the interaction of the PEG segments with the other, more hydrophobic, segments [27,28]. This effect will be even larger for smaller PEG-segment lengths [26]. We calculated the number of water molecules for

different of PEGT/PBT copolymers from the water uptake according to equations (II) and (III). The number of water molecules increased with increasing PEG segment length from 0.5 for copolymers with 300 g/mole segment lengths up to almost 3 water molecules per ethylene glycol unit for 1000PEGT70PBT30. The copolymers with PEG-segment lengths of 4000 g/mole exceeded the value of 3 water molecules per ethylene glycol unit. This is probably caused by the presence of both bound and free water molecules [29], although it cannot be excluded that the other copolymer compositions also contained free water in the matrix. It has been described that the state of the water molecules influences the permeability characteristics of the matrix [30]. The presence of free water enhances the solute diffusion rate compared to matrices containing only bound water.

In earlier publications, we reported a correlation between the equilibrium swelling ratio, the mesh size and the permeability of PEGT/PBT copolymers [18]. The mesh size is the space between neighboring chains in the polymer network, and can be considered as an indication for the available space in the matrix for solute diffusion [31]. An increase in the equilibrium swelling ratio of the PEGT/PBT matrices resulted in an increasing mesh size. Therefore, at increasing PEG-segment length, an increase in diffusion coefficient of the vitamin B_{12} through the PEGT/PBT matrix is expected.

Permeability of PEGT/PBT films for vitamin B_{12}

This study investigated the diffusion rate of vitamin B_{12} through PEGT/PBT matrices for different copolymer compositions. By using diffusion cells, we could determine the permeability of copolymers with PEG-segments of 600, 1000 and 4000 g/mole for vitamin B_{12}. Figures 1A and 1B present typical results of the amount of vitamin B_{12} permeated as a function of time. In order to compare the different membranes with each other, a correction was made for the membrane thickness by dividing time by the thickness of the swollen films (usually between 50 and 100 μm). Figure 1A clearly shows that the permeability of PEG/PBT films for vitamin B_{12} is strongly dependent on the PEG-segment length. Copolymers with relatively long PEG segments, such as 4000PEGT55PBT45 display a high permeability compared to copolymers with less shorter PEG segments, such as 600PEGT51PBT49. At a constant PEG-segment length, the permeability increased with increasing wt% PEGT (Figure 1B). For copolymers with

PEG-segments of 300 g/mole, the permeability was too low to measure within a reasonable time interval.

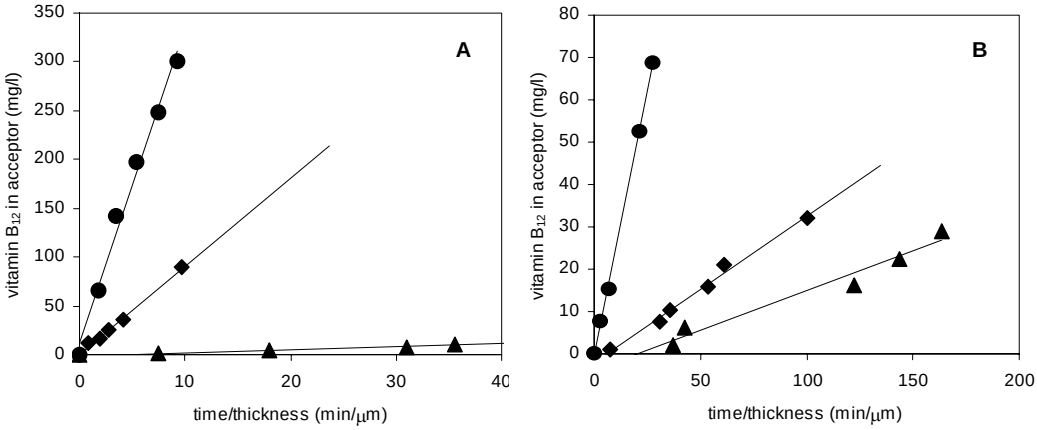


Figure 1: Permeation of vitamin B_{12} through PEGT/PBT films with (A) various PEG segment lengths (4000PEGT55PBT45 (●), 1000PEGT60PBT40 (◆) and 600PEG51PBT49 (▲)) and (B) various wt% PEGT at constant PEG-segment length (600PEG77PBT23 (●), 600PEG51PBT49 (◆) and 600PEG40PBT60 (▲)). To correct for differences in thickness of the films, time is divided by the thickness.

From the permeation experiments, the permeability coefficients P of the PEGT/PBT membranes for vitamin B_{12} were determined using equation (IV). P ranged from 1.2 x 10^{-9} cm^2/s for the 600PEGT40PBT60 composition to 2.1 x 10^{-7} cm^2/s for 4000PEGT80PBT20. To obtain the vitamin B_{12} diffusion coefficient for the various PEGT/PBT copolymers, K_d had to be determined. In Table 1, the partition coefficients are presented as a function of the copolymer composition. The K_d values observed are close to one, indicating that no interaction between solute and matrix occurred. As can be expected for a hydrophilic drug in the absence of interaction with the matrix, the partitioning of vitamin B_{12} in PEGT/PBT films increased with increasing degree of swelling of the copolymers. The vitamin B_{12} diffusion coefficients, calculated from the results of the permeability and partition experiments, are given in Table 1.

Due to the low diffusion rate of vitamin B_{12} through copolymers with 300 g/mole PEG-segment lengths, it was not possible to detect the vitamin B_{12} concentrations in the diffusion cell. Therefore, the diffusion coefficients of vitamin B_{12} through the copolymers with 300 g/mole PEG-segments were calculated from the release experiments. To compare the two methods, the diffusion coefficients of vitamin B_{12}

through some matrices of PEGT/PBT copolymers with 600 g/mole PEG-segments lengths were determined using both methods. For this monolithic system, in which the vitamin B_{12} solution is expected to be homogeneously mixed with the matrix, the total release can be described according to the following equations [32]:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{Dt}{\pi l^2}} \qquad \text{(VI)}$$

for $M_t/M_\infty < 0.6$;

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\exp\left(-\frac{\pi^2 Dt}{l^2}\right) \qquad \text{(VII)}$$

for $M_t/M_\infty > 0.4$; in which, M_∞ is the total amount of vitamin B_{12} in the matrix, M_t is the amount of vitamin B_{12} released at time t and l is the thickness of the film. The diffusion coefficients of vitamin B_{12} through the PEGT/PBT series with 300 g/mole PEG-segment lengths were obtained by plotting the fractional vitamin B_{12} released M_t/M_∞ versus the square root of time. Representative plots are given in Figure 2. From the first part of the curves ($M_t/M_\infty < 0.6$), the diffusion coefficient D was calculated by using equation (VI). The vitamin B_{12} release from copolymers with 600 g/mole PEG-segment lengths was too fast to be able to use equation (VI). Therefore, the diffusion coefficient D of vitamin B_{12} through these copolymers was calculated from the vitamin B_{12} release by using equation (VII).

The diffusion coefficients calculated from the diffusion cell experiments and from the initial release are both given in Table 1. The diffusion coefficient of vitamin B_{12} was approximately 10^{-7} cm^2/s and 10^{-8} cm^2/s for PEGT/PBT matrices with 4000 g/mole PEG-segment lengths and 1000 g/mole PEG-segments, respectively. The diffusion coefficients for vitamin B_{12} through copolymers with 600 g/mole PEG-segments calculated by both methods were in the same range (10^{-9} cm^2/s), indicating agreement between the methods. In matrices with PEG-segment lengths of 300 g/mole, the diffusion coefficient was in the range of 10^{-12}-10^{-14} cm^2/s. In comparison, the diffusion coefficient of vitamin B_{12} in water is 3.79×10^{-6} cm^2/s [21]. From these results, it can be concluded that the PEG-segment length is the important determining factor of the diffusion coefficient. At a constant PEG-segment length, the diffusion coefficient can be

modulated to a certain extent by varying the PEGT content. A similar trend was observed in the equilibrium volume swelling ratio Q of the polymers with different PEG-segments lengths (Table 1).

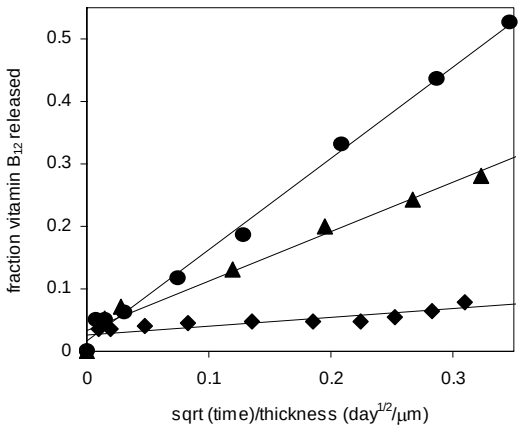


Figure 2: Initial release of vitamin B_{12} as function of the square root of time from 300PEGT32PBT68 (◆), 300PEGT53PBT47 (▲) and 300PEGT61PBT39 (●) films for determination of the diffusion coefficients. To correct for differences in thickness of the films, the square root of time is divided by the thickness (n=3).

However, the swelling can only partly explain the trend in diffusion coefficient. For example, within the 300 g/mole PEG-segment length series a difference in diffusion coefficient is observed, while no significant difference in swelling can be noticed. Probably, the diffusion rate is also affected by the matrix morphology. At a constant PEG-segment length of 300 g/mole, with increasing wt% of PBT, a higher percentage of crystallinity in the film was expected as described above. DSC measurements confirmed this hypothesis (Table 2). The crystallinity in the film increased from 1.3 % at 40 wt% of PBT up to 11 % of crystallinity at 70 wt% of PBT. Because the crystallites can be considered as impermeable [33], most of the vitamin B_{12} transport takes place through the amorphous domains. Thus, the crystallites reduce the available space for solute diffusion and increase the characteristic diffusion length [33]. Moreover, due to the existence of a crystalline polymer fraction, the solute diffusion coefficient through the amorphous phase is modified [34,35]. This is caused by a decrease in swelling of the amorphous phase at increasing crystallinity. Expansion of the network is hampered, because the crystallites act as crosslinks and impede the swelling process. Thus, the decreasing diffusion rate of

vitamin B_{12} through the 300 g/mole matrices could be the result of a higher crystallinity at higher wt% of PBT.

Table 2: Crystallinity and intrinsic viscosity prior to and after 12 weeks of in-vitro degradation in PBS as function of polymer composition.

Polymer composition (NMR)	Crystallinity (%) (DSC)	Intrinsic viscosity (t=0) (dl/g)	Intrinsic viscosity (t=7 weeks) (dl/g)
300PEGT32PBT68	11.0	n.d.	n.d.
300PEGT53PBT47	2.3	0.64	0.66
300PEGT61PBT39	1.3	0.68	0.65
600PEGT57PBT43	n.d.	0.92	0.69
1000PEGT71PBT29	n.d.	0.90	0.54

n.d.: not determined

Sustained release of vitamin B_{12}

For biomedical applications as controlled release devices, the long-term release of drugs is of particular interest. Therefore, only copolymers with 300 g/mole PEG-segment lengths and copolymers with 600 and 1000 g/mole segments and low wt% PBT were selected for the release study. As could be expected from the diffusion coefficients, the release of vitamin B_{12} from films was strongly dependent on the composition of the copolymers. Although an increased wt% of PEGT (at a constant PEG-segment length) resulted in an increasing vitamin B_{12} release rate, a major effect was observed at increasing PEG-segment length. The release of vitamin B_{12} was complete in 1 day for copolymers with PEG-segments of 600 g/mole and 1000 g/mole, while the release from copolymers with 300 g/mole PEG-segments continued for over 12 weeks (Figure 3).

The release profiles of both copolymers with 300 g/mole PEG-segment lengths, shown in Figure 3, indicate an almost constant release of vitamin B_{12} in time. If the release were only determined by diffusion, a first order release would be expected. Zero order release was also obtained for proteins, like lysozyme, from PEGT/PBT matrices with 600 and 1000 g/mole PEG-segments [4]. This was explained by an increase in diffusion coefficient in time due to polymer degradation. However, considering the time period of the release, degradation of copolymers with 300 g/mole PEG-segment lengths will not play an important part. Viscosity measurements proved this hypothesis. Unloaded PEGT/PBT films were degraded in PBS at 37°C for 7 weeks. The intrinsic viscosity of the polymer was measured prior to and after degradation of the films in PBS.

From Table 2, it can be seen that the intrinsic viscosity decreased for the PEGT/PBT copolymers with 600 g/mole and 1000 g/mole PEG-segments lengths. However, the intrinsic viscosity remained unchanged for films of copolymers with 300 g/mole PEG-segments after 7 weeks in PBS, indicating that no degradation has taken place. Instead, the zero order release of vitamin B_{12} from 300 g/mole PEGT/PBT matrices might be caused by the low water-uptake of these copolymers and the solubility of vitamin B_{12} in water. The relatively short PEG-segments resulted in a low water-uptake (Table 1). The solubility of vitamin B_{12} in water is 12 mg/ml [36]. Due to the low amount of water present in the PEGT/PBT matrix, only a fraction of the vitamin B_{12} will be dissolved. The vitamin B_{12} loaded matrices prepared from copolymers with 300 g/mole PEG-segments should be considered as a dispersed system [32]. As long as solid vitamin B_{12} is present, the concentration gradient within and outside the matrix is constant, resulting in a zero order release. In this mechanism, the dissolving of the vitamin B_{12} is assumed to be the rate-limiting step and the concentration gradient inside the matrix is neglected. Although this mechanism can describe the zero order release, it does not explain the difference in release rate within the 300 g/mole series. As discussed before, also the morphology of the matrix plays a role. It has to be noted that the equations (VI) and (VII) describe a dissolved system. Since our system may contain undissolved drug, the obtained values can only be considered as a first approximation.

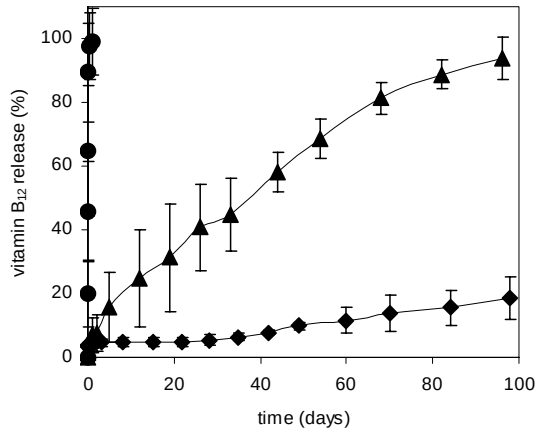


Figure 3: Cumulative release of vitamin B_{12} from PEGT/PBT films of different compositions (◆: 300PEGT32PBT68, ▲: 300PEGT61PBT39, ●: 600PEGT57PBT43) (n=3; ± s.d.).

Effect of copolymer blending on vitamin B_{12} permeability

To overcome the gap between the release rates of vitamin B_{12} of the copolymers with different PEG-segments lengths, blends of 300PEGT53PBT47 and 600PEGT51PBT49 were prepared. Because of the large difference in the diffusion coefficients of these two copolymers, adding of only 5 wt% 600PEGT51PBT49 is sufficient to increase the release rate of vitamin B_{12} to a large extent (Figure 4). By blending of copolymers with different PEG-segment lengths, the release rate can be effectively tailored from 1 day up to 12 weeks.

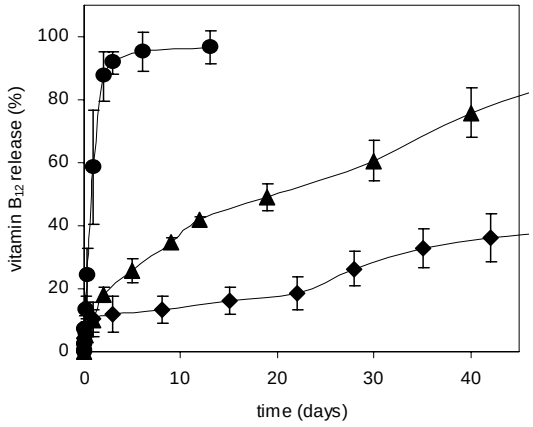


Figure 4: Cumulative release of vitamin B_{12} from 300PEGT53PBT47 / 600PEGT51PBT49 blends (◆: 100% 300PEGT53PBT47, ▲: 95% 300PEGT53PBT47 / 5% 600PEGT51PBT49, ●: 75% 300PEGT53PBT47 / 25% 600PEGT51PBT49) (n=3; ± s.d.)

The effect of blending on the release rate was further evaluated by calculating the diffusion coefficients from the release curves using equations (VI) and (VII). The experimental diffusion coefficients are given in Figure 5. In this figure, also the average diffusion coefficients are shown as function of the blend composition, calculated from equation (VIII):

$$D_{av} = \phi_{300} D_{300} + \phi_{600} D_{600} \qquad \text{(VIII)}$$

in which D_{300} is the diffusion coefficient of vitamin B_{12} through 300PEGT53PBT47, D_{600} is the diffusion coefficient through 600PEGT51PBT49 and ϕ_{300} and ϕ_{600} are the weight fractions of the copolymers. Equation (VIII) is based on the assumption that the blends

can be considered as molecularly mixed matrices, as the two closely related copolymers were blended in solution.

By comparison of the experimental diffusion coefficient calculated from the release curves and the average diffusion coefficient calculated by equation (VIII), the following can be observed. The experimental and the average diffusion coefficients of the blends containing more than 25 wt% of 600PEGT51PBT49 were in the same range. However, a large difference could be seen for low percentages (2 wt%-15 wt%) of 600PEGT53PBT47 in the matrix. The experimental diffusion coefficients of these blends were significantly lower than the calculated average diffusion coefficient. So, the effect of blending on the release rate cannot be simply explained by calculating the average diffusion coefficient from the diffusion coefficients of the copolymers (300PEGT53PBT47 and 600PEGT51PBT49). To determine the mechanism behind the effect of blending on the diffusion coefficient, another parameter that could be affected by blending, the swelling, was determined.

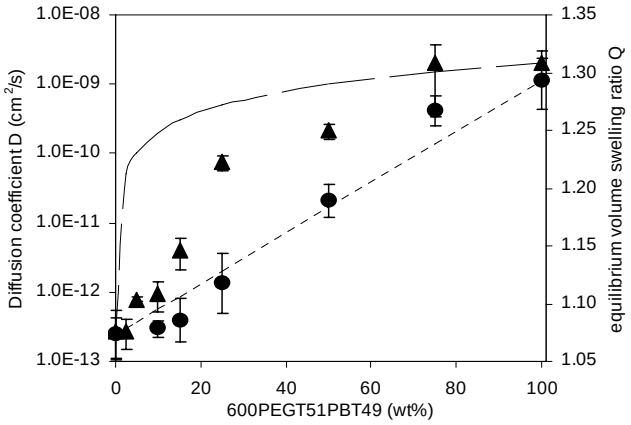


Figure 5: Experimental diffusion coefficients calculated from the release experiments of vitamin B_{12} through films of 300PEGT53PBT47 / 600PEGT51PBT49 blends (▲) and the volume swelling ratio Q of these films (●) as a function of the wt% 600PEGT51PBT49. The calculated average diffusion coefficients (- - -) and swelling ratios (····) are indicated by dotted lines.

The equilibrium volume swelling ratio Q of films cast from solutions of 300PEGT53PBT47 and 600PEGT51PBT49 mixtures in chloroform is also given in Figure 5. Identical to the diffusion coefficient, the average volume swelling ratios of the blends are calculated and indicated in the figure. It can be seen, that the swelling ratio Q

is not directly proportional with the amount of 600PEGT51PBT49. Although the swelling increased with increasing amount of 600PEGT51PBT49, the swelling was only affected significantly after adding of 25 wt%. At low wt% of 600PEGT51PBT49 the experimentally determined swelling is lower than expected from the calculated average swelling. Apparently, the hydrophilic 600 g/mole segments have difficulties expanding in the stiff hydrophobic matrix based on the 300PEGT53PBT47.

However, the release rate was already affected after adding of 5 wt% of 600PEGT51PBT49 (Figure 4). Therefore, as seen for the different copolymer compositions the increase in release rate cannot fully be explained by an increase in swelling. To investigate whether the increase in release rate is caused by a difference in matrix structure, DSC analyses were carried out. The crystallinity of the matrix, calculated from the DSC measurements, is given in Table 3 as a function of the blend compositions.

Table 3: Swelling ratio Q and crystallinity for blends of 300PEGT53PBT47 and 600PEGT51PBT49.

wt% 300PEGT53PBT47	wt% 600PEGT51PBT49	Swelling ratio Q	Crystallinity (%) (DSC)
100	0	1.07 ± 0.02	2.3
90	10	1.08 ± 0.01	5.3
85	15	1.09 ± 0.02	4.2
75	25	1.12 ± 0.01	1.8
50	50	1.19 ± 0.01	2.3
25	75	1.27 ± 0.01	n.d.
0	100	1.29 ± 0.03	2.0

n.d.: not determined

The crystallinity depends on the PBT block length and the average distance between the PBT blocks. The latter is strongly dependent on the PEG-segment length, whereas the PEGT/PBT ratio does not alter the spacing significantly [19,20]. Although the PBT blocks in 600PEGT51PBT49 are larger than in 300PEGT53PBT47, which could increase crystallinity, the distance between these blocks is larger in 600PEGT51PBT49, which could decrease crystallinity. This might explain why no difference in crystallinity was observed between these two polymers (Table 2). However, the crystallinity increased significantly by adding 10 wt% and 15 wt% of 600PEGT51PBT49. Possibly, the longer

PBT blocks of the 600PEGT51PBT49 copolymer facilitate crystallization with the smaller, but more available, PBT blocks of the 300PEGT53PBT47. Thus, the fact that the diffusion coefficient of vitamin B_{12} through the blends (2 wt% - 15 wt% of 600PEGT53PBT47) is lower than expected, may be caused by a combination of unexpected swelling and crystallinity behavior.

Conclusion

The results of this study show that PEGT/PBT multiblock copolymers can be successfully used as matrix for controlled release of small water-soluble molecules. By varying the copolymer composition, a complete release of vitamin B_{12} in 1 day up to a constant release for over 12 weeks can be obtained. The release rate can be effectively tailored by blending copolymers with different PEG-segment lengths. The swelling and the crystallinity of the matrix determine the diffusion coefficient of vitamin B_{12} through PEGT/PBT copolymers. In general, the swelling can mainly be varied by changing the PEG-segment length. At a constant PEG-segment length, the crystallinity increases at increasing wt% PBT. For a number of PEGT/PBT copolymers used in this study, no in-vitro degradation was observed after 7 weeks. Further studies will focus on in-vivo degradation of a series of PEGT/PBT copolymers. Furthermore, the results of this study will be used to develop controlled release systems for pharmaceutically relevant peptides.

References

1. Langer R. Polymeric delivery systems for controlled drug release. Chem Eng Commun 1980;6:1-48.
2. Schwendeman SP, Costantino HR, Gupta RK, Langer R. Peptide, protein, and vaccine delivery from implantable polymeric systems: progress and challenges. In: Park K, editor. Controlled drug delivery, challenges and strategies. Washington, D.C. Am Chem Soc, 1996. p. 229-267.
3. Couvreur P, Puisieux F. Nano- and microparticles for the delivery of polypeptides and proteins. Adv Drug Delivery Rev 1993;10:141-162.
4. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J Control Rel 2000;64(1-3):179-192.

5. Radder AM, Leenders H, Van Blitterswijk CA. Application of PEO/PBT copolymers for bone replacement. J Biomed Mat Res 1996;30:341-351.
6. Kuijer R, Drees M, Douw C, Bouwmeester P, Bulstra S. Histological analysis of repair of fresh and old cartilage defects in the rabbit knee using Polyactive. Sixth Biomaterials Congress, Kamuela, Hawaii, 2000.
7. Papadaki M, Mahmood T, Gupta P, Claase MB, Grijpma DW, Riesle J, Van Blitterswijk CA, Langer R. The different behaviors of skeletal muscle cells and chondrocytes on PEGT/PBT block copolymers are related to the surface properties of the substrate. J Biomed Mater Res 2001;54(1):47-58.
8. Beumer GJ, Van Blitterswijk CA, Ponec M. Biocompatibility of degradable matrix induced as a skin substitute: an in vivo evaluation. J Biomed Mat Res 1994;28:545-552.
9. Reed AM, Gilding DK. Biodegradable polymers for use in surgery - poly(ethylene oxide) poly(ethylene terephthalate) (PEO/PET) copolymers: 2. In vitro degradation. Polymer 1981;22:499-504.
10. Van Loon JA. Thesis University of Leiden, Leiden, The Netherlands, 1995.
11. Beumer GJ, Van Blitterswijk CA, Bakker D, Ponec M. Cell-seeding and in vitro biocompatibility evaluation of polymeric matrices of PEO/PBT copolymers and PLLA. Biomaterials 1993;14:598-604.
12. Beumer GJ, Van Blitterswijk CA, Ponec M. Degradative behaviour of polymeric matrices in (sub)dermal and muscle tissue of the rat: a quantitative study. Biomaterials 1994;15:551-559.
13. Van Blitterswijk CA, Van den Brink J, Leenders H, Bakker D. The effect of PEO ratio on degradation, calcification and bone-bonding of PEO/PBT copolymer (Polyactive). Cells and Materials 1993;3:23-36.
14. Radder AM, Van Loon JA, Puppels GJ, Van Blitterswijk CA. Degradation and calcification of a PEO/PBT copolymer series. J Mater Sci: Mater in Med 1995;6:510-517.
15. Bakker D, Van Blitterswijk CA, Hesseling SC, Koerten HK, Kuijpers W, Grote JJ. Biocompatibility of a polyether urethane, polypropylene oxide, and a polyether polyester copolymer. A qualitative and quantitative study of three alloplastic tympanic membrane materials in the rat middle ear. J Biomed Mater Res 1990;24:489-515.
16. Okada H. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. Adv Drug Delivery Rev 1997;28:43-70.
17. Lee KC, Soltis EE, Newman PS, Burton KW, Metha RC, DeLuca PP. In vivo assessment of salmon calcitonin sustained release from biodegradable microspheres. J Control Rel 1991;17:199-206.
18. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. A controlled release system for proteins based on poly(ether ester) block-copolymers: polymer network characterization. J Control Rel 1999;62(3):393-405.
19. Fakirov S, Gogeva T. Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 1. Poly(ether/ester)s with various polyester : polyether ratios. Makromol Chem 1990;191:603-614.
20. Fakirov S, Gogeva T. Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 2. Effect of polyether length. Makromol Chem 1990;191:615-624.
21. Colton CK, Smith KA, Merrill EW, Farrell PC. Permeability studies with cellulosic membranes. J. Biomed Mater Res 1971;5:459-488.

22. Adams RK, Hoeschele GK, Witsiepe WK. Thermoplastic polyether ester elastomers. In: Holden G, Legge NR, Quirk R and Schroeder HE, editors. Thermoplastic elastomers, 2nd edition. Munich: Hansen Publishers, 1996. p. 191-225.
23. Fakirov S, Apostolov AA, Boeseke P, Zachmann HG. Structure of segmented poly(ether ester)s as revealed by synchrotron radiation. J Macromol Sci 1990;Phys B29:379-395.
24. Hickey AS, Peppas NA. Mesh size and diffusive characteristics of semicrystalline poly(vinyl alcohol) membranes prepared by freezing/thawing technique. J Membrane Sci 1995;107:229-237.
25. Liu KJ, Parsons JL. Solvent effects on the preferred conformation of poly(ethylene glycols). Macromolecules 1969;2:529-533.
26. Antonsen KP, Hoffman AS. Water structure of PEG solutions by differential scanning calorimetry measurements. In: Milton Harris J, editor. Poly(ethylene glycol) chemistry: Biotechnical and biomedical applications. New York: Plenum Press, 1992. p. 15-28.
27. Yui N, Katanaoke K, Yamada A, Sakurai Y. Novel design of microreservoir-dispersed matrices for drug delivery formulations: Regulative drug release from poly(ethylene oxide)- and poly(tetramethylene oxide)-based segmented polyurethanes. J Control Rel 1987;6:329-342.
28. Schneider NS, Illinger JL, Karasz FE. The interaction of water with polyurethanes containing block copolymer soft segments. J App Polymer Sci 1993;47:1419-1425.
29. Wisniewski S, Kim SW. Permeation of water-soluble solutes through poly(2-hydroxyethyl methacrylate) and poly(2-hydroxyethyl methacrylate) crosslinked with ethylene glycol dimethacrylate. J Membrane Sci 1980;6:299-308.
30. McNeill ME, Graham NB. Properties controlling the diffusion and the release of water-soluble solutes from poly(ethylene oxide) hydrogels 1. Polymer composition. J Biomater Sci Polymer Edn 1993;4(3):305-322.
31. Canal T, Peppas NA. Correlation between mesh size and equilibrium degree of swelling of polymeric networks. J Biomed Mater Res 1989;23:1183-1193.
32. Baker RW, Lonsdale HK. Controlled release: Mechanisms and rates. In; Tanquarry AC, Lacey RE, editors. Controlled release of biologically active agents. New York, Plenem Press, 1974. p. 15-71.
33. Peterlin A. Dependence of diffusive transport on morphology of crystalline polymers. J Macromol Sci 1975;Phys B11(1):57-87.
34. Harland RS, Peppas NA. Solute diffusion in swollen membranes VI: A model for diffusion in heterogeneous media. Polymer Bulletin 1987;18:553-556.
35. Harland RS, Peppas NA. Solute diffusion in swollen membranes VII. Diffusion in semicrystalline networks. Colloid Polym Sci 1989;267:218-225.
36. Budavari S, O'Neil MJ, Smith A, Heckelman PE, Kinneary JF, editors. The Merck Index, twelfth edition. Whitehouse Station, NJ, Merck & Co, 1996. 10152. Vitamin B_{12}.

Chapter 5

Stability aspects of salmon calcitonin entrapped in poly(ether-ester) sustained release systems

R. van Dijkhuizen-Radersma, H.M. Nicolas, M. van de Weert,
M. Blom, K. de Groot, J.M. Bezemer

Abstract

Poly(ether-ester)s composed of hydrophilic poly(ethylene glycol)-terephthalate (PEGT) blocks and hydrophobic poly(butylene terephthalate) (PBT) blocks were studied as matrix for the controlled release of calcitonin. Salmon calcitonin loaded PEGT/PBT films were prepared from water-in-oil emulsions. The initial calcitonin release rate could be tailored by the copolymer composition, but incomplete release of calcitonin was observed. FTIR measurements indicated aggregation of calcitonin in the matrix, which was not due to the preparation method of the matrices, but due to the instability of calcitonin in an aqueous environment. Release experiments showed the susceptibility of calcitonin towards the composition of the release medium, in particular to the presence of metal ions. With increasing amount of sodium ions, a decrease in the total amount of released calcitonin was observed due to enhanced aggregation. The calcitonin had to be stabilized in the matrix to prevent aggregation. Incorporation of sodium dodecyl sulphate (SDS) as a stabilizer in PEGT/PBT matrices increased the percentage of calcitonin released, but could not avoid aggregation on a longer term.

Introduction

Calcitonin (CT), a polypeptide of 32 amino acids (3500 Da) has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of osteoclastic bone resorption [1-5]. The treatment of bone-related diseases, including Paget's disease, hypercalcemia and osteoporosis, requires frequent administration of relatively high calcitonin dosages. Different routes have been studied to deliver calcitonin more effectively, including oral [4,5], nasal [6,7] and vaginal [8] delivery.

To reduce the number of doses that have to be administered, controlled release systems using biodegradable polymers are under investigation. Incorporation of the calcitonin in a polymeric matrix can protect the peptide from rapid clearance and provide a sustained release. Calcitonin release systems based on poly(lactic acid) [9,10], poly(lactic-glycolic acid) (PLGA) [11], poly(glycolic acid) (PGA) [12] and poly(caprolactone) (PCL) [13] have been studied so far. In this study, poly(ether ester) multiblock copolymers were investigated as potential release matrix for calcitonin. The polymers were composed of repeating blocks of hydrophilic poly(ethylene glycol) terepththalate (PEGT) and hydrophobic poly(butylene terephthalate) (PBT). These biocompatible and degradable PEGT/PBT copolymers have successfully been applied as matrix for the controlled release of proteins and peptides [14,15]. Quantitative release of fully active lysozyme has been reported from these multiblock copolymers [14]. A potential additional advantage of PEGT/PBT is the presence of hydrophilic polymer chains, which may improve the stability of calcitonin [16,17].

Like many proteins and peptides [18,19], the therapeutic use of calcitonin is hampered by its physical instability [20]. In aqueous solutions calcitonin has a pronounced tendency to aggregate into long, thin fibrillar aggregates, yielding a viscous and turbid dispersion [21]. Extensive research has been performed on stabilization of calcitonin in aqueous solutions containing organic acids [16,22-27], surfactants [25,26], sugars [16,19] and polymers like polyvinylpyrolidone [16,23] and polyvinylalcohol [16]. Also other solvents, like ethanol [16,28], methanol [28], propylene glycol [25,28] and dimethyl sulfoxide [28], have been evaluated. Stability problems may also occur in sustained release systems, due to the high concentration of calcitonin in the polymer matrix. Therefore, this study focussed on the stabilization of calcitonin in the matrix.

Salmon calcitonin (sCT) was used in this study because it is the most potent of the calcitonins available, well tolerated and clinically effective.

Materials and methods

Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis NV (Bilthoven, The Netherlands). The poly (ether-ester) copolymers varied in PEGT/PBT weight ratio (80/20-40/60) at a constant PEG segment length (600 g/mole) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt% PEG-terephthalate and **c** (=100-b) the wt% PBT. Phosphate buffered saline (PBS), (pH 7.4) and sodium dodecyl sulphate (SDS) were purchased from Life Technologies Ltd (Paisley, Scotland). Merck (Darmstadt, Germany) supplied acetic acid (100%), sodium chloride (NaCl) and sodium dihydrogen phosphate (NaH_2PO_4). Chloroform (analytical grade), carboxymethyl cellulose sodium salt (CMC, medium viscosity) and D(-)-mannitol (extra pure) were purchased from Fluka Chemie GmbH (Buchs, Switserland). Sigma Chemical Co (St Louis, USA) supplied Tween 80 and dextran (average mol wt 41,272). Polyvinylalcohol (PVA, 13,000-23,000 g/mole, 87-89% hydrolyzed) and D(+)-trehalose (dihydrate 99%) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). Bachum AG (Bubendorf, Switzerland) was the supplier of salmon calcitonin (sCT), which was a gift of PowderJect Pharmaceuticals PLC (Oxford, United Kingdom).

Preparation of salmon calcitonin-loaded PEGT/PBT films

For the preparation of salmon calcitonin loaded films, an aqueous salmon calcitonin solution (0.6 ml, 20 mg/ml) was emulsified with a polymer solution (1 g PEGT/PBT in 7 ml $CHCl_3$) using an ultra turrax (IKA Labortechnik T25) for 30 s at 19 krpm. The resulting water-in-oil emulsion was cast on a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and freeze dried for at least 16 hrs. The resulting films had a thickness of 50-100 μm. To study the

influence of the matrix composition on the salmon calcitonin release behaviour, the wt% PEGT was varied between 40 and 80 wt%.

The aqueous calcitonin solution was varied to evaluate the effect of the solution composition on the calcitonin release. PBS (pH 7.4) was used as the standard aqueous phase, while also experiments with acetic acid (0.0002M, pH=4.3) and a phosphate buffer (0.1M NaH_2PO_4, pH=4.3) as water phase were carried out. SDS was added to a acetic acid solution (0.0002M, pH=4.3) to study the effect of this additive on the calcitonin stability.

Salmon calcitonin release from PEGT/PBT films

The salmon calcitonin release from the salmon calcitonin loaded PEGT/PBT films was investigated by incubating pieces of the films (± 1.77 cm^2) in 1 ml release medium. Vials were continuously shaken at 37°C and samples of the release medium were taken at various time points. The salmon calcitonin concentration of the buffer was determined using a micro bichinchoninic acid (BCA) protein assay on an EL 312e microplate bio-kinetics reader (λ = 570 nm). The buffer was refreshed after sampling.

The standard in-vitro release buffer used in this study was PBS (0.16M Na^+, pH 7.4). The release of calcitonin was also measured in acetic acid (0.0002M, pH=4.3) and a phosphate buffer (0.1M NaH_2PO_4, pH=4.3). The influence of the presence of sodium ions in the release medium was evaluated by varying the amount of sodium chloride in water.

Infrared spectroscopy and calcitonin structure analysis

Infrared spectroscopy of calcitonin loaded and unloaded PEGT/PBT films was performed in transmission mode on a Bio-Rad FTS6000 FTIR spectrometer (Cambridge, USA), equipped with a dTGS detector. 2048 scans were averaged at 2 cm^{-1} resolution. The calcitonin loaded film was measured in dry state directly after preparation and after incubation in PBS for two days at 37°C. Calcitonin, dissolved in PBS, was measured in a transmission cell with CaF_2 windows and a 6 micron mylar® spacer (Graseby SpecAc, Orpington, United Kingdom). Peptide conformation was analyzed by the 2nd derivative in the Amide I region [29], after a 13-point Savitzky-Golay smoothing to remove white noise. For the calcitonin in PBS solution, the solvent background was first interactively subtracted to obtain a flat baseline in the region 2300-1800 cm^{-1}. Any polymer

contribution to the solid state spectra was interactively subtracted to obtain a flat baseline between 1710 and 1730 cm^{-1} [30-32].

Screening of stabilizers to reduce calcitonin aggregation

The stability of salmon calcitonin in solution was evaluated by using UV spectroscopy [24,25,32]. The optical dispersion of calcitonin solutions (20 mg/ml) in PBS or acetic acid (0.0002M, pH=4.3) was recorded on a Lambda 40 UV-VIS spectrometer (Perkin Elmer, Norwalk, USA). Aggregation of the calcitonin was monitored by measuring the increase in turbidity at 340 nm as a function of time. The calcitonin solutions were stored at 37^{o}C between the measurements. Various stabilizers were evaluated for their ability to prevent calcitonin aggregation. Surface-active compounds (Tween 80, SDS), sugars (trehalose, mannitol) and large organic molecules (CMC, PVA, dextran) were added at different concentrations to freshly prepared calcitonin (20 mg/ml) solutions in PBS or acetic acid (0.0002M, pH=4.3).

Results and discussion

Calcitonin release in PBS from PEGT/PBT films

Calcitonin release kinetics from PEGT/PBT films were first evaluated as a function of copolymer composition. Previous studies with vitamin B_{12} (1355 D), showed a constant release over 12 weeks from PEGT/PBT copolymers with 300 g/mole PEG-segments [15]. These copolymer compositions had a very low equilibrium swelling ratio. Being three times the size of vitamin B_{12}, salmon calcitonin release would require more swollen matrices to allow diffusion. Therefore, copolymers with PEG-segment lengths of 600 g/mole were selected for this study. The PEGT/PBT copolymers varied between 40 and 80 wt% PEGT. A higher wt% PEGT resulted in a higher swelling of the matrix [14,15].

The copolymer composition determined the calcitonin release in PBS to a large extent (Figure 1). 60 % of the encapsulated calcitonin was released within two days for the 600PEGT80PBT20 film, whereas a slower initial release was observed for the copolymers with lower wt% PEGT. For all compositions, the calcitonin release was incomplete. The slower release of calcitonin from 600PEGT60PBT40 and the

600PEGT40PBT60 copolymers can be explained by the lower degree of swelling of the polymer matrix.

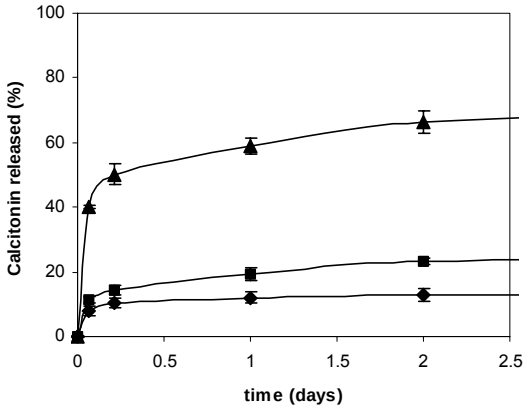


Figure 1: Salmon calcitonin release from 600PEGT40PBT60 (●), 600PEGT60PBT40 (◆) and 600PEG80PBT20 (▲) films in PBS (n=3; ± s.d.).

The slow diffusion through the PEGT/PBT matrix does not explain the observed incomplete calcitonin release (Figure 1). Possibly, calcitonin aggregation has occurred in the matrices, either during the release experiments or during the preparation process. FTIR measurements have been preformed on calcitonin loaded PEGT/PBT films to investigate this further. Bauer et al. already showed that FTIR spectroscopy provides a sensitive analytical tool to monitor aggregation phenomena of calcitonin in aqueous solutions [34].

Integrity of calcitonin in PEGT/PBT matrix

Calcitonin loaded films and aqueous calcitonin solutions were analyzed by FTIR to detect conformation changes of calcitonin. FTIR analysis of the calcitonin-loaded films was somewhat hampered by the low amount of calcitonin in the film. Within that limitation, the FTIR spectrum did not indicate any obvious change in secondary structure of calcitonin directly after preparation of the films (Figure 2). However, when the film was incubated for 2 days in PBS, the secondary structure of the calcitonin remaining in the film was markedly different. A clear band at 1630 cm^{-1} is observed, which is typical of intermolecular β-sheet formation (non-covalent aggregation). A calcitonin solution stored for 20 hours at room temperature also indicated the early onset of aggregation (Figure 2).

It is well-known that calcitonin is unstable upon incubation in aqueous solution [3,6,7,20]. Bauer et al. reported an increase in time for both α and β-contents in human calcitonin solutions determined by ATR-FTIR [34]. Arvinte et al. related these α helical and β-sheet components to the fibrillated state of aggregated calcitonin [33]. Thus, the aggregation of calcitonin in PEGT/PBT films is most likely caused by incubation in solution, rather than by the preparation method.

Cholewinsky et al. have described several degradation pathways of calcitonin in aqueous solutions [20]. Besides pH and temperature, the presence of metal ions is known to facilitate aggregation of calcitonin. The PEGT/PBT films described above contained a significant amount of sodium ions, as the calcitonin was incorporated in these films as a solution in PBS. In addition, the release experiments have been carried out in PBS as release medium as well. To study the effect of the presence of sodium ions on the release of calcitonin from PEGT/PBT films, the composition of both the incorporated aqueous calcitonin solution as well as the release medium have been varied.

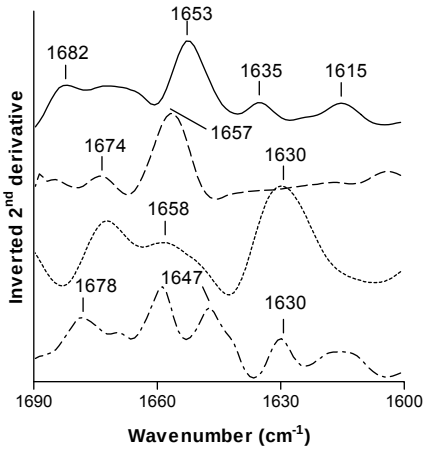


Figure 2: Inverted 2nd derivative spectra in the Amide I region of calcitonin in solution (solid line), in a 600PEGT60PBT40 film (dashed line) directly after preparation, in a 600PEGT60PBT40 film (dotted line) after 1 day incubation in PBS at 37°C, and in solution after 1 day incubation at room temperature (dash-dotted line).

Effect of sodium ions on calcitonin release

For the release experiments described below the 600PEGT60PBT40 copolymer was selected. The release of calcitonin was measured as a function of the inner phase and release buffer compositions. Either a 0.1 M phosphate or a 0.0002 M acetic acid buffer were used, both formulated at pH 4.3 to exclude pH effects. It is known that calcitonin is

less prone to aggregation at acidic pH [24] in acetic acid solutions [16,25]. As shown in Figure 3, variation of the composition of the release buffer composition has a major influence on the calcitonin release. Using phosphate buffer as release medium, the total calcitonin release is only 20-25%, whereas acetic acid buffer results in a release of 80-100%. Variation of the inner phase composition has much less influence on the total amount of calcitonin released, with acetic acid buffer as inner phase yielding a slightly higher total release than phosphate buffer. This suggests that the calcitonin release profile is mainly determined by the release medium composition. The complete release in the acetic acid release medium indicated that no aggregation of the calcitonin had occurred, possibly due to the absence of metal ions.

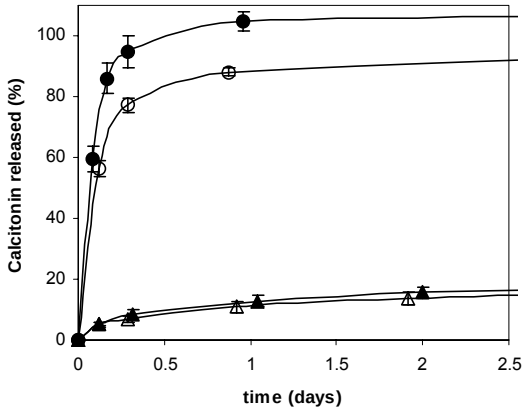


Figure 3: Salmon calcitonin release from 600PEGT60PBT40 films with phosphate buffer (▲) and acetic acid (●) as inner phase. Release medium: 0.1 M NaH_2PO_4 (open symbols) and 0.0002 M acetic acid (filled symbols) (n=3; ± s.d.).

The effect of the sodium ions in the release medium on the calcitonin release was evaluated in more detail. Samples of the calcitonin loaded films were incubated in aqueous solutions containing various sodium chloride concentrations. In water without sodium chloride, the release was complete within 2 days (Figure 4). With increasing sodium concentration, however, the calcitonin release became incomplete, indicating calcitonin aggregation in the matrix (Figure 4). In a release medium containing 150 mM sodium chloride, only 15% of the calcitonin was released from 600PEGT60PBT40 matrices. Apparently, the sodium ions had diffused from the release buffer into the

polymer matrix, inducing calcitonin aggregation. As the sodium ion concentration in blood is 142 mM, the calcitonin should be stabilized in the matrix to avoid aggregation.

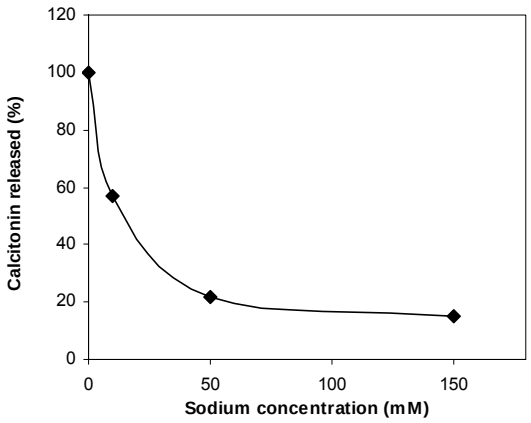


Figure 4: Effect of sodium ions in release medium on the total amount of salmon calcitonin released from 600PEGT60PBT40 films.

Effect of additives on calcitonin stability and long term release

To be able to select the proper stabilizer for calcitonin in the matrix, several additives have been investigated in solutions of calcitonin in PBS and acetic acid. The samples were checked periodically for the presence of a precipitate, a gel or turbidity, which are indicative of fibrillation [33]. Table 1 shows the effect on the stability of calcitonin solutions with several additives.

Table 1: Stability of salmon calcitonin (20 mg/ml) in PBS with several additives.

Additive	Concentration (% w/v)	Stabilization period[1]
-	-	2 hours
Trehalose	1	4 hours
Mannitol	1	< 1 hour
SDS	1	5 days
Tween 80	1	6 hours
PVA	1	< 1 hour
Dextran	1	6 hours
CMC	0.5	< 1 hour

[1] time period in which no turbidity change was observed

Sodium dodecyl sulphate (SDS) was the only additive that showed a long-term stabilizing effect. Therefore, the effect of SDS was studied in more detail in both PBS and acetic acid (Table 2). By increasing the concentration of the SDS from 0 to 2 % (w/v), the stability of the calcitonin solutions varied. Surprisingly, the stability of the solutions decreased with increasing SDS concentration up to 0.5% (w/v), while the stability increased at higher SDS concentrations. Baudyš et al. attributed the stabilizing effect of SDS to the formation of a very stable complex between calcitonin and SDS in SDS micelles [25,26]. Although the critical micelle concentration value for SDS is 0.24 %, we only observed a long-term stabilizing effect at SDS concentrations of 1 % and higher. This might be due to the relatively high calcitonin concentration used in our study. Possibly, at lower SDS concentrations, the number of potential calcitonin binding sites in SDS micelles was exhausted due to the high calcitonin concentration [26]. Partial stabilized calcitonin molecules apparently aggregate even faster than unstabilized calcitonin (no SDS).

Table 2: Stability of calcitonin solution (20 mg/ml) in PBS or 0.0002 M acetic acid at various SDS concentrations.

SDS (% w/v)	Buffer	Stabilization period[1]
0	PBS	2 hours
0.24	PBS	1 hour
0.5	PBS	< 1 hour
1.0	PBS	5 days
2.0	PBS	> 2 months
0	acetic acid	> 2 months
0.24	acetic acid	2 hours
0.5	acetic acid	< 1 hour
1.0	acetic acid	> 2 months
2.0	acetic acid	> 2 months

[1] time period in which no turbidity change was observed

Compared to the PBS solutions, an increased calcitonin stability was already observed for acetic acid containing solutions in the absence of SDS. Several other groups, therefore, have added acetic acid to stabilize calcitonin in solution [16,25-27]. In our release experiments described above, we also observed a (small) positive effect of acetic acid in the inner phase on the total amount of calcitonin released (Figure 3). Therefore,

acetic acid was chosen as inner phase to evaluate the effect of SDS on the sustained release of calcitonin from PEGT/PBT films. The release from 600PEGT60PBT40 films, containing 0, 1 and 2 % SDS in the inner phase, has been measured in PBS. Figure 5 shows an increase in total amount of calcitonin released with increasing SDS concentration. However, similar to the PEGT/PBT matrices without SDS (Figure 1), the release stops after two days, indicating that the calcitonin has aggregated during the release period. Apparently, the SDS concentration was not high enough to fully stabilize the calcitonin in the matrix. This could be either due to the diffusion of SDS out of the matrix during the release experiment, or due to the high concentration of calcitonin in the matrix compared tot the solutions used in the turbidity experiment. The increasing amount of calcitonin released with increasing SDS concentration may be the result of the stabilizing effect of the SDS on the calcitonin, but it could also be due to a more open matrix structure. The presence of SDS in the water-in-oil emulsion may destabilize the emulsion, resulting in more open matrix, which in turn will increase the calcitonin diffusion rate. Consequently, a larger amount of calcitonin will be released before aggregation has started.

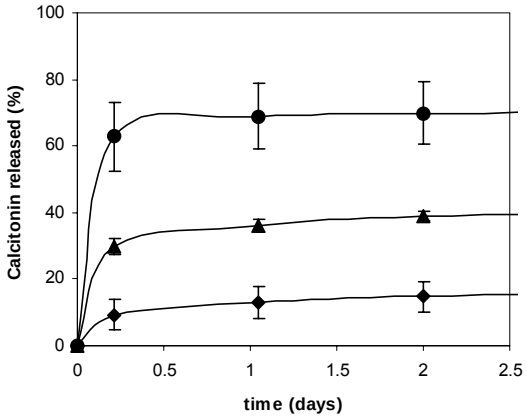


Figure 5: Salmon calcitonin release from 600PEGT60PBT40 films with acetic acid as inner phase containing 0 % (◆), 1 % (▲) and 2 % (●) of SDS (w/v). Release in PBS (n=3; ± s.d.).

Conclusion

In order to obtain a sustained release system for salmon calcitonin, calcitonin loaded PEGT/PBT films were prepared from water-in-oil emulsions. Although the initial calcitonin release rate could be tailored by the copolymer composition, no complete release of calcitonin was observed. FTIR measurements indicated aggregation of calcitonin in the matrix, which was not due to the preparation method of the matrices, but due to the instability of calcitonin in an aqueous environment. Release experiments showed the susceptibility of calcitonin towards the release medium, in particular to the presence of metal ions in the release medium. With increasing amount of sodium ions, a decrease in the total amount of released calcitonin was observed due to aggregation. The calcitonin had to be stabilized in the matrix to prevent aggregation. Comparison of several additives in absorption experiments showed that sodium dodecyl sulphate (SDS) had a long-term stabilizing effect on calcitonin in solution. Incorporation of SDS in PEGT/PBT matrices indeed increased the percentage of calcitonin released, but still no sustained release of calcitonin was obtained.

Further experiments will focus on the stabilizing calcitonin in the matrix by incorporation of other additives. In addition, the activity of calcitonin after release from PEGT/PBT matrices will be investigated by in-vivo experiments.

References

1. A.M. Parfitt, Bone and plasma calcium homeostasis, Bone 8 (1987) S1-S8.
2. S.D. Boden, F.S. Kaplan, Calcium homeostasis, Orthopedic Clin. North Am. 21 (1990) 31-42.
3. S. Wallach, G. Rousseau, L. Martin, M. Azria, Effects of calcitonin on animal and in vitro models of skeletal metabolism, Bone 25 (1999) 509-516.
4. Y-H. Lee, P.J. Sinko, Oral delivery of salmon calcitonin, Adv. Drug. Del. Rev. 42 (2000), 225-238.
5. M. Torres-Lugo, N.A. Peppas, Transmucosal delivery systems for calcitonin: a review, Biomaterials 21 (2000) 1191-1196.
6. K. Morimoto, H. Katsumata, T. Yabuta, K. Iwanaga, M. Kakemi, Y. Tabata, Y. Ikada, Evaluation of gelatin microspheres for nasal and intramuscular administrations of salmon calcitonin, Eur. J. Pharm. Sci. 13 (2001) 179-185.

7. S.L. Law, C.L. Shih, Characterization of calcitonin-containing liposome formulations for intranasal delivery, J. Microencap. 18 (2001) 211-221.
8. J.L. Richardson, M. Miglietta, P.A. Ramires, M. Rochira, A.N. Fisher, N.F. Farraj, L. Illum, L.M. Benedetti. Vaginal administration of calcitonin in rats ans sheep using microspheres of hyaluronane esters, Proceed. Intern. Symp. Control. Rel. Bioact. Mater. 20 (1993).
9. M.Asano, M. Yoshida, H. Omichi, T. Mashimo, K. Okabe, H. Yuasa, H. Yamanaka, S. Morimoto, H. Sakakibara, Biodegradable poly(DL-lactic acid) formulations in a calcitonin delivery system, Biomaterials 14 (1993) 797-799.
10. A.J. Millest, J.R. Evans, J.J. Young, D. Johnstone, Sustained release of salmon calcitonin in vivo from lactide:glycolide copolymer depots. Calcif. Tissue Int. 52 (1993) 361-364.
11. R.V. Diaz, M. Llabrés, C. Évora, One-month sustained release microspheres of ^{125}I-bovine calcitonin, in vitro-in vivo studies, J. Control. Release 59 (1999) 55-62.
12. K.C. Lee, E.E. Soltis, P.S. Newman, K.W. Buron, R.C. Mehta, P.P. DeLuca, In vivo assessment of salmon calcitonin sustained release from biodegradable microspheres, J. Control. Release 17 (1991) 199-206.
13. Th.F. Vandamme, J.L. Gillard, Poly-ε-caprolactone microspheres containing calcitonin : preparation, morphology and release properties, Proceed. Intern. Symp. Control. Rel. Bioact. Mater. 20 (1993).
14. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices, J. Control. Rel. 64 (2000) 179-192.
15. R. van Dijkhuizen-Radersma, F.L.A.M.A. Péters, N.A. Stienstra, D.W. Grijpma, J. Feijen, K. de Groot, J.M. Bezemer, Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers, Biomaterials 23 (2002) 1527-1536.
16. T. Arvinte, A. Cudd, J. Philips, Fibrillated calcitonin pharmaceutical compositions, US patent 5, 593, 962, Jan 14, 1997.
17. S. Sakuma, Y. Ishida, R. Sudo, N. Suzuki, H. Kikuchi, K. Hiwatari, A. Kishida, M. Akashi, M. Hayashi, Stabilization of salmon calcitonin by polystyrene nanoparticles having surface hydrophilic polymeric chains, against enzymatic degradation, Int. J. Pharm. 159 (1997) 181-189.
18. J.L. Cleland, A.J.S. Jones, Stable formulations of recombinant human growth hormone and interferon-γ for microencapsulation in biodegradable microspheres, Pharm. Res. 13 (1996) 1464-1475.
19. G. Zhu, S.R. Mallery, S. P. Schwendeman, Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide), Nature Biotech. 18 (2000) 52-56.
20. M. Cholewinsky, B. Lückel, H. Horn, Degradation pathways, analytical characterization and formulation strategies of a peptide and a protein Calcitonin and human growth hormone in comparison, Pharm. Acta Helvetiae 71 (1996) 405-419.
21. H.H. Bauer, U. Aebi, M. Häner, R. Hermann, M. Müller, H.P. Merkle, Architecture and polymorphism of fibrillar supramolecular assemblies produced by in-vitro aggregation of human calcitonin, J. Struc. Bio. 115 (1995) 1-15.
22. W. Stern, J.P. Gilligan, Oral salmon calcitonin pharmaceutical products, US patent 5, 912, 014, Jun 15, 1999.

23. P.A. Veronesi, E. Peschechera, A.M. Veronesi, Pharmaceutical non inorganic saline solutions for endonasal administration of a calcitonin, US patent 6, 087, 338, Jul 11, 2000.
24. K.C. Lee, Y.J. Lee, H.M. Song, C.J. Chun, P.P. DeLuca, Degradation of synthetic salmon calcitonin in aqueous solution, Pharm. Res. 9 (1992) 1521-1523.
25. M. Baudyš, D. Mix, S.W. Kim, Stabilization and intestinal absorption of human calcitonin, J. Control. Release 39 (1996) 145-151.
26. M. Baudyš, S.W. Kim, stabilisation and oral delivery of calcitonin, US patent 5, 726,154 Mar 10, 1998.
27. A. Cudd, T. Arvinte, R.E. Gaines Das, C. Chinni, I. MacIntyre, Enhanced potency of human calcitonin when fibrillation is avoided, J. Pharm. Sci. 84 (1995) 717-719.
28. C.L. Stevenson, M.M. Tan, Solution stability of salmon calcitonin at high concentration for delivery in an implantable system, J. Peptide Res. 55 (2000) 129-139.
29. H. Susi, D.M. Byler, Protein structure by fourier transform infrared spectroscopy: second derivative spectra. Biochem. Biophys. Res. Commun. 115 (1983) 391-397.
30. T.-H. Yang, A. Dong, J. Meyer, O.L. Johnson, J.L. Cleland, J.F. Carpenter, Use of infrared spectroscopy to assess secondary structure of human growth hormone within biodegradable microspheres. J. Pharm. Sci. 88 (1999) 161-165.
31. M. van de Weert, R. van Dijkhuizen-Radersma, J.M. Bezemer, W.E. Hennink, D.J.A Crommelin. Reversible Aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer. Eur. J. Pharm. Biopharm. 54 (2002) 89-93.
32. M. van de Weert, R. van 't Hof, J. van der Weerd, R.M.A. Heeren, G. Posthuma, W.E. Hennink, D.J.A. Crommelin, Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques, J. Control. Rel. 68 (2000) 31-40.
33. T. Arvinte, A. Cudd, A.F. Drake, The structure and mechanism of formation of human calcitonin fibrils, J. Biol. Chem. 268 (1993) 6415-6422.
34. H.H. Bauer, M. Müller, J. Goette, H.P. Merkle, U.P. Fringeli, Interfacial adsorption and aggregation assosiated chages in secondary structure of human calcitonin monitored by ATR-FTIR spectroscopy, Biochem. 33 (1994) 12276-12282.

Chapter 6

In-vitro and in-vivo evaluation of salmon calcitonin loaded poly(ether-ester) microspheres

R. van Dijkhuizen-Radersma, H.M. Nicolas,
K. de Groot, J.M. Bezemer

Abstract

Salmon calcitonin (sCT) was successfully encapsulated in poly(ether-ester) microspheres using a water-in-oil-in-water emulsion. The presence of sodium chloride in the outer water phase increased the encapsulation efficiency, but decreased the total amount of sCT released from the microspheres. The in-vitro release could be tailored effectively by varying the copolymer composition. The in-vivo study in rats showed the release of active calcitonin by a decreased calcium concentration and an increase in parathyroid hormone plasma level. Pathology showed no treatment-related findings and no indication for systemic toxicity.

Introduction

Calcitonin (CT), a polypeptide of 32 amino acids (3500 Da) has a physiological role in the regulation of calcium homeostasis and is a potent inhibitor of osteoclastic bone resorption [1]. It decreases the calcium plasma concentration, resulting in a secretion of parathyroid hormone (PTH), which promotes reactions in the body opposite to CT [2]. The treatment of bone-related diseases including Paget's disease, hypercalcemia and osteoporosis, requires frequent administration of relatively high CT dosages. Incorporation of the CT in a polymeric matrix may protect the peptide from rapid clearance and provide a sustained release [3]. Various biocompatible and degradable polymers have been applied as matrix for the controlled release of proteins and peptides, including poly(ether-ester)s based on poly(ethylene glycol) terephthalate/ poly(butylene terephthalate) (PEGT/PBT) [4,5]. Previously, the in-vitro release of CT from PEGT/PBT films has been described [6]. In this paper, we report the results of in-vitro and in-vivo studies on CT loaded PEGT/PBT microspheres. The in-vitro study focuses on the encapsulation and release rate of sCT. In the in-vivo study, the pharmacodynamic effect of the sCT microspheres was investigated as well as the pathology. Salmon calcitonin (sCT) was used in this study because it is the most potent of the calcitonins available, well tolerated and clinically effective [7].

Materials and methods

Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis NV (Bilthoven, The Netherlands). The poly (ether-ester) copolymers varied in PEGT/PBT weight ratio (80/20-40/60) and in PEG segment length (300-600 g/mole) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG molecular weight, **b** the wt% PEG-terephthalate and **c** (=100-b) the wt% PBT. Merck (Darmstadt, Germany) supplied sodium dihydrogen phosphate (NaH_2PO_4). Chloroform (analytical grade) was purchased from Fluka Chemie GmbH (Buchs, Switserland). Polyvinylalcohol (PVA, 13,000-23,000 g/mole, 87-89% hydrolyzed) was

obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). Bachum AG (Bubendorf, Switzerland) was the supplier of salmon calcitonin (sCT).

Preparation of PEGT/PBT microspheres

Salmon calcitonin loaded microspheres were prepared by a water-in-oil-in-water emulsion method. An aqueous sCT solution (0.6 ml, 20 mg/ml) was emulsified in a solution of 1 g PEGT/PBT in 7 ml of chloroform. The w/o emulsion was added to a secondary water phase consisting of 50 ml 0.1 M NaH_2PO_4 solution containing 4% PVA under rapid stirring. After 5 minutes, 100 ml 0.1 M NaH_2PO_4 solution was added. The double emulsion was stirred for 2 hrs in order to evaporate the solvent. The hardened microspheres were collected, washed and freeze dried overnight. The secondary water phase composition was varied to evaluate the effect on the encapsulation of sCT in the microspheres. 0.1 M NaH_2PO_4 solution was used as standard, while also experiments with 0.1 M NaH_2PO_4 solution containing 150 mM NaCl were carried out. To study the influence of the matrix composition on the sCT release behavior, 600PEGT60PBT40 and 300PEGT80PBT20 copolymers were used. Also microspheres of blends of these two copolymer compositions were prepared.

Characterization of microspheres

A Philips XL 30 Environmental Scanning Electron Microscope (ESEM) was used to evaluate the surface characteristics and the size of the microspheres. Samples were sputter-coated with a thin gold layer.

Encapsulation efficiency

The encapsulation efficiency of sCT in the PEGT/PBT microspheres was determined indirectly. After preparation of the microspheres, the secondary water phase was analyzed for the sCT concentration, indicating the amount of sCT that was not encapsulated. The sCT concentration was determined by using bichinchonic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

In-vitro calcitonin release

The in-vitro release of sCT was determined by incubating the microspheres (approx. 50 mg) in 1.0 ml of a 0.1 M NaH_2PO_4 (pH=4.5) solution at 37^{o}C. The sCT concentration in the release medium was determined at various time points by using micro bichinchonic acid (BCA) protein assay. The release medium was refreshed after sampling.

In-vivo study

The in-vivo effect of the sCT loaded microspheres was evaluated subcutaneously in Wistar rats (n=6). Prior to injection, 100 mg microspheres of a blend of 600PEGT60PBT40 and 300PEGT80PBT20 (50/50) were resuspended in 1 ml water for injection. Blood samples were taken at various time points during four weeks and analyzed for calcium (by Atomic Absorption Spectrometry (AAS)) and PTH (by a ^{125}I radioimmuno assay (RIA)) plasma concentrations. After 6 weeks the animals were sacrificed and complete necropsies were performed. Macroscopic and microscopic observations were carried out on all relevant tissues and organs.

Results & Discussion

Microspheres characteristics

The sCT loaded PEGT/PBT microspheres obtained using the double emulsion method were smooth and spherical (Figure 1). The size distribution was narrow and the average diameter was around 70 μm.

Encapsulation efficiency

The encapsulation efficiency of sCT in the PEGT/PBT microspheres was determined from the sCT concentration in the secondary water phase. Using additives in the external aqueous phase can increase the encapsulation efficiency. NaCl was found to be the most effective additive by Uchida et al. [8]. Figure 2 shows the encapsulation of sCT in 600PEGT60PBT40 microspheres with and without NaCl in the secondary water phase. Adding NaCl increased the encapsulation efficiency from 70 to 90% (100% equals 12 mg sCT per gram of polymer).

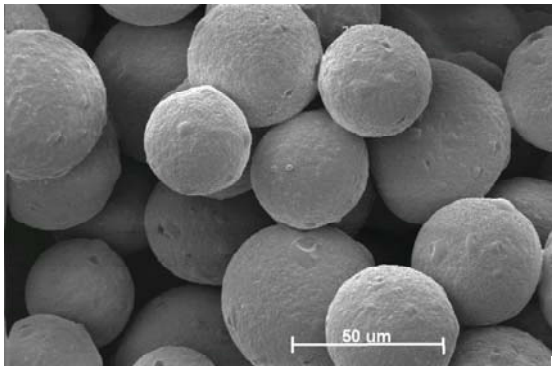


Figure 1: Scanning electron micrograph of calcitonin loaded 600PEGT60PBT40 microspheres.

However, it was previously reported that sodium ions had a negative effect on the stability of calcitonin [6]. The presence of sodium ions in the release medium induced aggregation of the sCT in the matrix, resulting in a decrease of the total amount of released sCT. Figure 2 shows the total amount of sCT released from the microspheres. The presence of NaCl in the secondary water phase clearly reduced the amount of released sCT from the microspheres. Apparently, the sCT has aggregated in the matrix during preparation inhibiting a complete release. The microspheres prepared in the absence of NaCl showed an almost complete release of the encapsulated sCT. Therefore, it was decided to prepare microspheres for future experiments in a secondary water phase without NaCl, despite of the lower encapsulation efficiency.

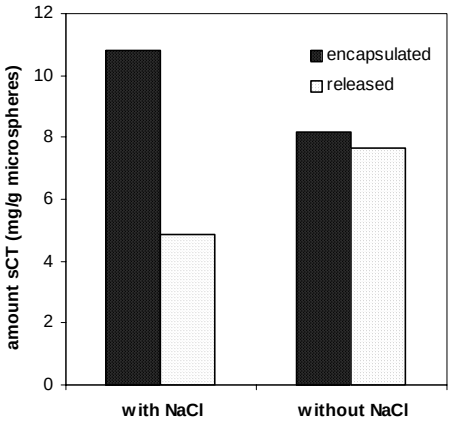


Figure 2: Total amount of sCT encapsulated and released from 600PEGT60PBT40 microspheres prepared with and without NaCl in secondary water phase.

In-vitro release modulation

Calcitonin loaded PEGT/PBT microspheres were prepared to investigate the effect of the copolymer composition on the in-vitro release. The copolymers used in this study varied in PEG segment length (300 and 600 g/mole) and PEGT/PBT ratio. A complete sCT release within 7 days was observed from the 600PEGT60PBT40 copolymer, whereas no sCT was released from the 300PEGT80PBT20 microspheres (Figure 3). Apparently, the mesh size of the 300PEGT80PBT20 copolymer is too small to allow calcitonin diffusion. The longer PEG-segments in the 600PEGT60PBT40 resulted in a higher swollen matrix [4,5], which facilitated sCT diffusion. In agreement with previous experiments [5], the sCT release rate could be tailored by blending of the two copolymers.

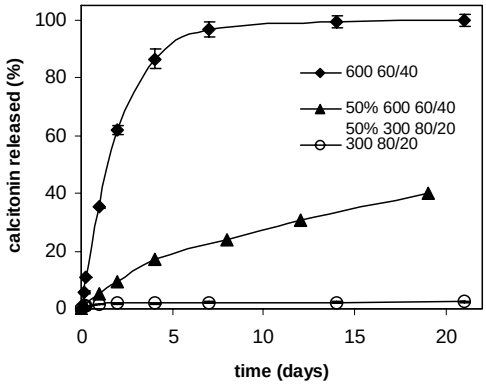


Figure 3: sCT release from PEGT/PBT microspheres in phosphate buffer (pH=4.5) (n=3; ± s.d.).

In-vivo study

The sCT loaded microspheres based on the blend of 600PEGT60PBT40 (50%) and 300PEGT80PBT20 (50%) were selected for the in-vivo study. After subcutaneous injection of the microspheres, the calcium level clearly dropped, indicating the sCT was still active after release (Figure 4). After 5 days the calcium concentration in the plasma was back at the normal level. This could be attributed to the natural counteracting effect of PTH. Due to the low calcium level, caused by the released sCT, PTH was secreted (Fig. 2), which increased the calcium concentration back to the normal level. However, it cannot be excluded that the sCT release from the PEGT/PBT had stopped after 4 days.

A similar calcium plasma concentration profile has been reported for rats that were injected with sCT loaded poly(glycolic acid) based microspheres [3], but the

PEGT/PBT microspheres in our study showed a longer lasting calcium drop. The hypocalcemic effect in the study of Lee et al. was observed for 24 hrs, while the serum sCT levels were sustained for over 5 days [3].

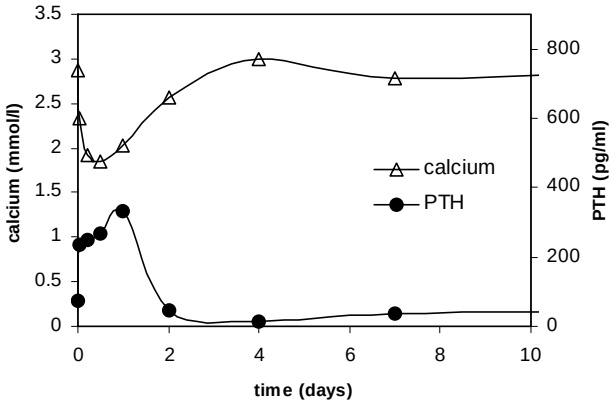


Figure 4: Calcium and PTH plasma levels in rats after injection of sCT loaded PEGT/PBT microspheres.

Macroscopic and microscopic examinations after complete necropsies did not reveal any treatment-related findings concerning the main organs and tissues. In the subcutis of the injection site, a fibrous capsule encapsulating the microspheres was observed seen in several rats (Figure 5). This formulation of granulation tissue is considered to be a normal tissue response after implantation of microspheres [9,10]. There was no indication of systemic toxicity.

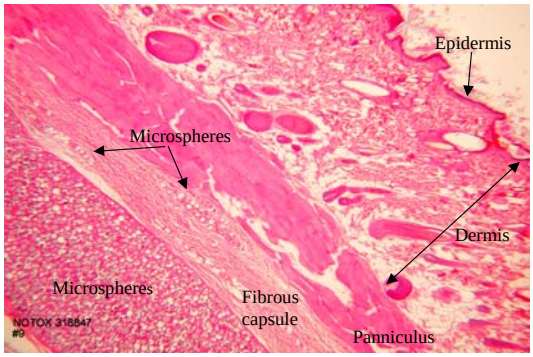


Figure 5: Histology of sCT microspheres (MS) injected subcutaneously in rats after 6 weeks. A fibrous capsule (containing some microspheres) surrounded the field of microspheres . Also epidermis, dermis (including hair follicles and glands) and panniculus (muscle layer) are visible. Original magnification: 2x.

Conclusions

Salmon calcitonin (sCT) was successfully encapsulated in PEGT/PBT microspheres using a water-in-oil-in-water emulsion. The presence of sodium chloride in the outer water phase increased the encapsulation efficiency, but decreased the total amount of sCT released from the microspheres. The release of sCT could be tailored effectively by varying the polymer matrix composition. The in-vivo study showed that the sCT was still active after release from PEGT/PBT microspheres in rats. The calcium level was decreased due to the release of active sCT. The calcium concentration was back to normal levels after 5 days, most likely due to the secretion of PTH. Pathology showed no treatment-related findings and no indication for systemic toxicity.

References

1. Lee, Y.H., Sinko, P.J. 2000. Oral delivery of salmon calcitonin. Adv. Drug Del. Rev., 42, 225-238.
2. Torres-Lugo, M., Peppas, N.A, 2000. Transmucosal delivery systems for calcitonin: a review. Biomaterials, 21, 1191-1196.
3. Lee, K.C., Soltis, E.E., Newman, P.S., Buron, K.W., Mehta, R.C., DeLuca, P.P., 1991. In vivo assessment of salmon calcitonin sustained release from biodegradable microspheres. J. Control. Rel., 17, 199-206.
4. Bezemer, J.M., Radersma, R., Grijpma, D.W., Dijkstra, P.J., Feijen, J., Van Blitterswijk, C.A., 2000. Zero-order release of lysozyme from poly(ethylene glycol)/poly(butylene terephthalate) matrices. J. Control. Rel., 64, 179-192.
5. Van Dijkhuizen-Radersma, R., Péters, F.L.A.M.A., Stienstra, N.A.,. Grijpma, D.W, Feijen, J., De Groot, K., Bezemer, J.M., 2002a. Control of vitamin B_{12} from poly(ethylene glycol)/poly(butylene terephthalate) multiblock copolymers. Biomaterials, 23, 1527-1536.
6. Van Dijkhuizen-Radersma, R., Nicolas, H.M., Van de Weert, M., Blom, M., De Groot, K., Bezemer, J.M., 2002b. Stability aspects of salmon calcitonin entrapped in poly(ether-ester) sustained release systems. Int. J. Pharm., 248, 229-237.
7. Stevenson, J.C., Evans, I.M.A., 1981. Pharmacology and therapeutic use of calcitonin. Drugs 21, 257-273.
8. Uchida, T., Yoshida, K., Goto, S., 1996. Preparation and characterization of polylactic acid microspheres containing water-soluble dyes using a novel w/o/w emulsion solvent evaporation method. J. Microencapsulation, 13, 219-228.

9. Anderson, J.A., Shive, M.S., 1997. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv. Drug Del. Rev. 28, 5-24.
10. Van Dijkhuizen-Radersma, R., Hesseling, S.C., Kaim, P.E., De Groot, K., Bezemer, J.M., 2002c. Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation. Biomaterials, 23, 4719-4729.

Chapter 7

In vitro / in vivo correlation for ^{14}C-methylated lysozyme release from poly(ether-ester) microspheres

R. van Dijkhuizen-Radersma, S.J. Wright, L.M. Taylor,
B.A. John, K. de Groot, J.M. Bezemer

Abstract

The purpose of this study was to obtain an in-vitro / in-vivo correlation for the sustained release of a protein from poly(ethylene glycol) terephthalate (PEGT) / poly(butylene terephthalate) (PBT) microspheres. Radiolabelled lysozyme was encapsulated in PEGT/PBT microspheres via a water-in-oil-in-water emulsion. Three microsphere formulations varying in copolymer composition were administered subcutaneously to rats. The blood plasma was analysed for radioactivity content representing released lysozyme at various time points post-dose. The in-vitro release was studied in phosphate buffered saline (PBS). The encapsulation efficiency, calculated from the radioactivity in the outer water phase of the emulsion, varied from 60% to 87%. Depending on the PEG segment length and wt% PEGT, the lysozyme was released completely in-vitro within 14 to 28 days without initial burst. ^{14}C-methylated lysozyme could be detected in the plasma over the same time courses. The in-vitro/in-vivo correlation coefficients obtained from point-to-point analysis were greater than 0.96 for all microsphere formulations. In addition, less then 10% of administered radioactivity remained at dose site at 28 days for the microsphere formulations, indicating no notable retention of the protein at the injection site. The in-vitro release in PBS and the in-vivo release in rats showed an excellent congruence independent of the release rate of ^{14}C-methylated lysozyme from PEGT/PBT microspheres.

Introduction

With the increasing number of therapeutic proteins on the pharmaceutical market, extensive investigations are carried out on biodegradable polymers for controlled release systems for these proteins [1]. Most of this work has focused on matrices composed of poly(lactide-co-glycolide) (PLG) because of their good biocompatibility, low toxicity and biodegradation characteristics [2]. However, at present there is considerable concern about the effects of PLG on protein stability [3-6]. With respect to the release kinetics, frequently an initial burst has been reported for protein release from PLG matrices followed by a plateau and subsequently result in incomplete release [6-8]. Recently, a series of biodegradable poly(ether ester) multiblock copolymers composed of repeating blocks of hydrophilic poly(ethylene glycol) terephthalate (PEGT) and hydrophobic poly(butylene terephthalate) (PBT) was introduced as a matrix for controlled release systems [9,10]. Many in-vivo and in-vitro studies have proven the biocompatibility of these PEGT/PBT multiblock copolymers [11,12] and in 2000, a degradable cement restrictor composed of PEGT/PBT obtained market clearance from the FDA.

In previous publications, PEGT/PBT copolymers were shown to be a successful matrix for controlled release of proteins [9,10]. An important advantage of this system is that through modulation of the copolymer composition, the required release profile can be obtained. In-vitro experiments showed a complete release of lysozyme varying from minutes to months depending on the amount and length of the PEG segments in the matrix [9,10]. Through the release mechanism, which is based on diffusion of the protein due to swelling and degradation of the matrix, zero order release profiles could be obtained without an initial burst. In addition, the in-vitro released protein retained its integrity [13] and was fully active [9].

However, for many release systems, the in-vivo release behaviour is very often not predicted by the in-vitro release [7,14-16]. In case of subcutaneous injection of protein loaded polymer microspheres, the protein input into the systemic circulation depends not only on the release rate, but also on the diffusion rate of the protein through the subcutaneous tissue [6,17] or the lymphatic transport system [18,19]. A free protein formulation should always be included as a reference in in-vivo evaluations of sustained release formulations, to be able to obtain the elimination rate of the protein in the body.

However, it cannot be excluded that the rate of clearance for the slow release system differs from the bolus injection [6,20]. After taking into account the reference, in most cases the in-vivo release still differs from the in-vitro release concerning profile shape and/or time-course [7,14-16]. This can be caused by instability of the protein in the in-vitro release medium [4,16], difference in degradation of the polymer matrix in-vitro and in-vivo [21,22] and/or the in-vitro release test set-up (e.g. sink conditions, centrifugation, type of tubes) [8,23]. Various release media without resemblance to the in-vivo situation have been used to overcome the discrepancy which resulted in a proper in-vitro / in-vivo correlation [4,7].

In this in-vitro / in-vivo correlation study, radiolabelled lysozyme was used as a model protein as our polymer system has been evaluated extensively for lysozyme [9,10]. We selected ^{14}C-methylated lysozyme as the relatively long half-life of the radiolabel ($T_{1/2}$ is 5730 years) means that the activity is essentially constant over the test period. We did not consider the commonly used ^{125}I label ($T_{1/2}$: 60 days) [4,24] suitable for sustained release experiments, as after 4 weeks only 72% of the initial radioactivity remains and more importantly the radiolabel readily dissociates from the protein in-vivo, making data interpretation difficult. ^{14}C-methylated lysozyme loaded PEGT/PBT microspheres were prepared by a water-in-oil-in-water emulsion. Loaded microspheres of various copolymer compositions were administered subcutaneously to rats. The blood plasma was analysed for radioactivity concentrations at several time points. The in-vitro release was studied in phosphate buffered saline (PBS). Lysozyme release in PBS was in good agreement with the in-vivo release from crosslinked gelatine hydrogels [25], although an incomplete lysozyme release in PBS was observed from PLG microspheres [4].

Materials and methods

Materials

A series of poly(ethylene glycol) terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers were obtained from IsoTis NV (Bilthoven, The Netherlands). The poly(ether-ester) copolymers varied in PEGT/PBT weight ratio (60/40-77/23) and in PEG segment length (600 - 1000 g/mole) and are indicated as **a**PEGT**b**PBT**c**, in which **a** is the PEG

molecular weight, **b** the wt% PEG-terephthalate and **c** (=100-b) the wt% PBT (composition determined by NMR). The average weight molecular weight (M_w) determined by GPC (relative to PMMA standards) was approximately 85 kg/mole. Phosphate buffered saline (PBS), (pH 7.4) was purchased from Life Technologies Ltd (Paisley, Scotland). Fluka Chemie GmbH (Buchs, Switzerland) and Rathburn Chemicals Ltd (Walkerburn, Scotland) supplied the chloroform (analytical grade). Hen egg white lysozyme (HEWL) (3x crystallized, dialyzed and lyophilized) was obtained from Sigma (St. Louis, MO, USA). ^{14}C-Methylated lysozyme in 0.01 M sodium phosphate solution (41 μCi/mg, 5μCi/ml) was supplied by Nycomed Amersham plc (Little Chalfont, England). Polyvinylalcohol (PVA, 13,000-23,000 g/mole, 87-89% hydrolyzed) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, USA). Carboxymethyl cellulose sodium salt (CMC, low viscosity 29-70cP) was purchased from Fisher Scientific (Loughborough, UK).

Concentration of lysozyme solution

PD-10 desalting columns (Amersham Biosciences AB, Uppsala, Sweden) were used to exchange the buffer from ^{14}C-methylated lysozyme from 0.01M sodium phosphate to 0.001M sodium phosphate. The column was equilibrated with sodium phosphate buffer (0.001M, 25 ml) and ^{14}C-methylated lysozyme (2 ml) was loaded onto the column, and washed on with a further 0.5 ml buffer (0.001M). The eluate up to this point was discarded. Further buffer (0.001M, 3.5ml) was used to elute the radiolabelled protein which was collected into pre-weighed containers. The ^{14}C-methylated lysozyme now in 0.001M sodium phosphate, was frozen at -70°C prior to freeze drying overnight to concentrate the protein.

Preparation of microspheres

For the preparation of ^{14}C-methylated lysozyme loaded microspheres, an aqueous radiolabelled lysozyme solution (0.6 ml, 55 mg lysozyme (3 mg ^{14}C-methylated and 52 mg non-radiolabelled) per ml PBS) was emulsified with a polymer solution (1 g PEGT/PBT in 7 ml $CHCl_3$) using an ultra turrax (IKA Labortechnik T25) for 30 s at 19 krpm. This water-in-oil emulsion was poured into 50 ml PBS containing 4% (w/v) of PVA. After 5 minutes stirring at 1000 rpm, 100 ml PBS was added. The resulting water-

in-oil-in-water emulsion was stirred at constant speed for 2 hours at room temperature. The microspheres were collected by centrifugation and washed three times with PBS and freeze-dried for 16 hours. To study the influence of the matrix composition on the lysozyme release behaviour, three microsphere formulations were prepared containing 1000PEGT70PBT30, 1000PEGT60PBT40 and 600PEGT77PBT23 copolymers, respectively. To evaluate the effect of the radioactive label, a batch of 1000PEGT70PBT30 microspheres containing non-radiolabelled lysozyme only was prepared as a reference under similar process conditions.

Morphology and size

A Philips XL 30 Environmental Scanning Electron Microscope (ESEM) was used to evaluate the morphology and the size of the microspheres. Samples were sputter-coated with a thin gold layer.

Lysozyme content

The encapsulation efficiency was determined indirectly by measuring the amount of lysozyme that was not encapsulated during the microsphere preparation. The outer water phase of the water-in-oil-in-water emulsion was analysed for the total protein concentration by micro bichinchoninic acid (BCA) protein assay, and for radioactivity content by liquid scintillation counting (LSC). The loading of the microspheres was also determined directly by extraction of the radiolabelled protein from the microsphere formulations, consisting of microspheres suspended in 1% CMC solution in PBS. Aliquots of the dosing formulations were weighed and diluted with chloroform. Both the chloroform layer and the CMC layer were analysed for radioactivity concentration. Assuming a complete release of the encapsulated lysozyme [9], the loading could also be determined from the total amount of lysozyme released in the in-vitro release experiments as described below.

In-vitro release

The in-vitro lysozyme release from the loaded PEGT/PBT microspheres was investigated by incubating 50 mg of microspheres in 1.5 ml PBS. Vials were continuously agitated at 37°C. At various time points, the suspension was centrifuged and 800 µl of the release

medium was removed. After sampling, the volume of each sample was replenished with 800 μl of PBS and the microspheres were resuspended and mixed continuously as before until the next sampling point. The sampled supernatant was analyzed for the total protein concentration by BCA, and for radioactivity content by LSC.

In-vivo study

The lysozyme release from the PEGT/PBT microspheres was studied in Sprague-Dawley rats (male, 230-250 g). The animals were housed in groups of three in a well ventilated environment under controlled and monitored temperature (21 ± 2^{o}C) and humidity (55 ± 15%). Food and water were available ad libitum. Prior to injection, the microspheres were resuspended in 1% CMC solution in PBS. For each controlled release formulation, three rats were injected subcutaneously with approximately 150 mg microspheres (ca. 15 μCi per kg bodyweight). Another three rats received free ^{14}C-methylated lysozyme (15 μCi per kg bodyweight) dissolved in a 1% CMC in PBS solution. One rat from the group administered 1000PEGT70PBT30 microspheres died after 1 hour, however, this was not attributed to administration of ^{14}C-methylated lysozyme. After 28 days the rats were killed.

In-vivo release

The in-vivo release of lysozyme from the PEGT/PBT microspheres was evaluated from the plasma concentrations. At pre-selected time intervals, serial blood samples were collected from the caudal vein (0.4 ml) of each animal and a final terminal sample (8 ml) was collected by cardiac puncture under isoflurane anasthesia. Each blood sample was delivered into a heparinised container and centrifuged. Subsequently, the plasma was transferred to clean polypropylene tubes. The lysozyme plasma concentrations were determined by radioactivity content measurements using LSC. To ensure that only bound ^{14}C label is detected during the in-vivo release study, the proportion of radioactivity associated with the protein fraction was measured by trichloroacetic acid (TCA) precipitation. The remaining plasma was pooled by time point for each microsphere formulation. An aliquot was dispensed in a 15% aqueous solution of TCA in order to precipitate any of the ^{14}C-radiolabel remaining in association with the protein. After centrifugation, the radioactivity content of the supernatant, assumed to represent ^{14}C

unassociated with protein was determined by LSC. Subsequently, the proportion of the total radioactivity content associated with the protein fraction was calculated by difference. After collection of the final sample, the rats were sacrificed and the dose sites (skin and underlying muscle) were excised from the carcasses to evaluate the remaining lysozyme at the injection sites. The explants were solubilized by digestion with a solution of NaOH and Triton X-405 in aqueous methanol. Subsequently, the radioactivity content of the digests were measured by LSC.

In-vitro / in-vivo correlation

To compare the in-vitro and in-vivo release of ^{14}C-methylated lysozyme from the microsphere formulations the radioactivity concentration-time data were processed using the software WinNonlin Pro version 3.1 (Pharsight Corp., Ca., USA).

Results and discussion

In-vitro characteristics

Morphology and size

The lysozyme loaded PEGT/PBT microspheres prepared via a water-in-oil-in-water emulsion were spherical with relatively smooth surfaces (Fig. 1). Some small pores were visible at the surface (Fig. 1B). The internal structure of the microspheres was dense, but also contained some small pores (Fig. 1C). The small pores may originate from the water droplets in the water-in-oil emulsion. The size of the microspheres ranged approximately from 40 to 120 μm (Fig. 1A), which was comparable to previous studies [10].

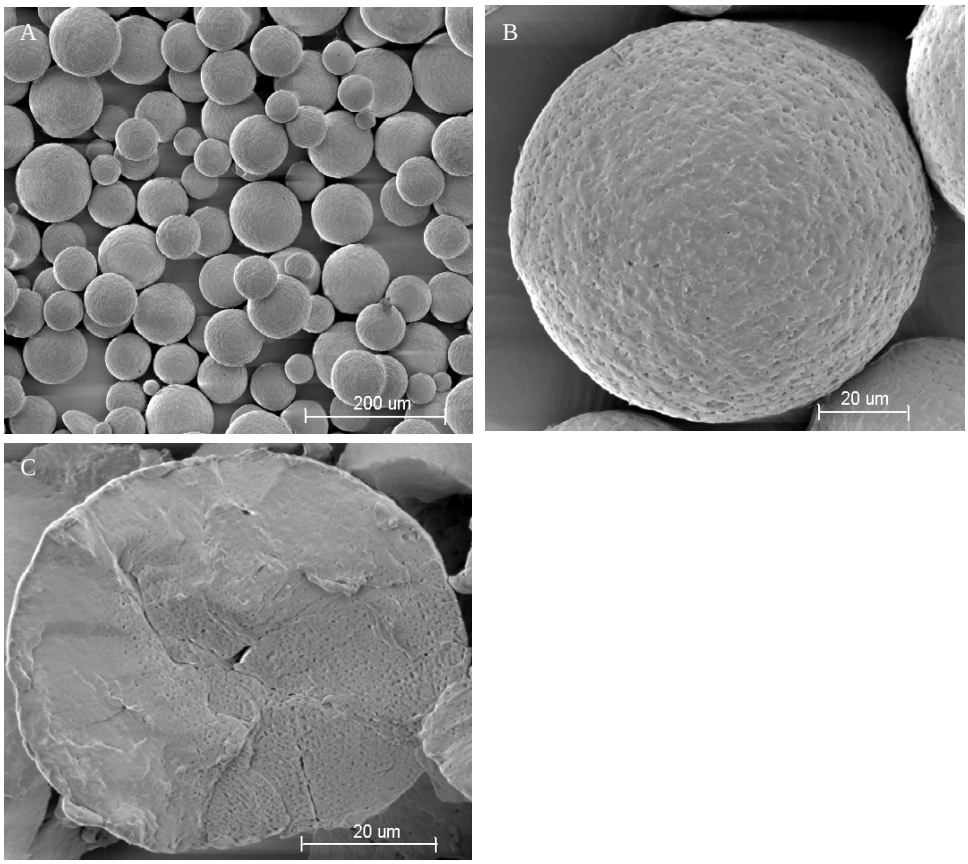


Figure 1: Scanning electron micrographs of lysozyme loaded 1000PEGT70PBT30 microspheres: overview (A), surface (B) and internal structure of fractured microsphere (C).

Lysozyme content

The final content of lysozyme in the microspheres was determined by the efficiency of the encapsulation process. The encapsulation efficiency was calculated in several ways: from the amount of protein or radioactivity in the outer water phase after preparation, from extraction of the radioactivity from the microspheres, and from the total amount of protein or radioactivity released. Figure 2 shows the encapsulation efficiencies calculated by the various methods for the three different copolymer compositions. Although the values obtained depended on the calculation method, the trend in the encapsulation efficiencies determined by the various methods is comparable for all three microsphere formulations. The encapsulation efficiency based on the loading determined by extraction of radioactivity generally showed the lowest values, which is probably due to incomplete

extraction [26]. Although indirect, the encapsulation efficiency based on the amount of protein in the outer water phase is probably the most reliable as no processing steps are required. The difficulty of this method, however, is the small sample size used for analyses compared to the total volume of the outer water phase. Therefore, a small deviation in the protein concentration will result in a large deviation in the encapsulation efficiency. In addition, as only one microsphere batch per copolymer composition was prepared, the encapsulation efficiencies are based on a single measurement. The lower value of the BCA determined encapsulation efficiency from the protein concentration in the outer water phase was possibly caused by the interference of PVA in the BCA analyses.

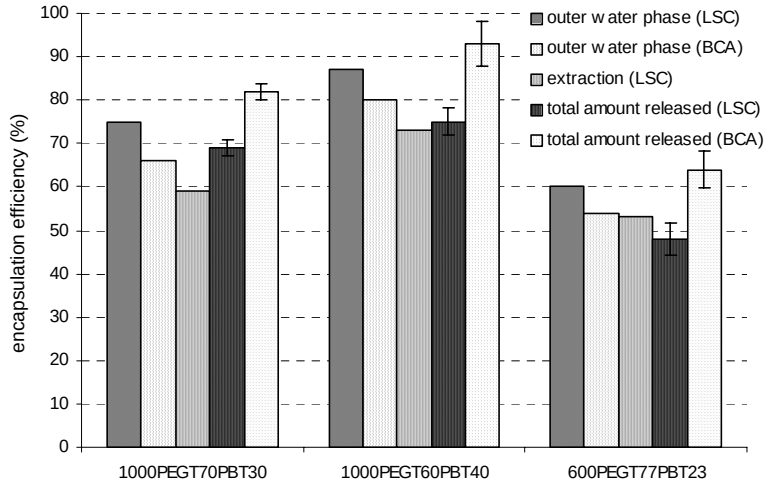


Figure 2: Efficiency of encapsulation of ^{14}C-methylated lysozyme in PEGT/PBT microspheres determined from outer water phase, by extraction and total amount of lysozyme released.

Previous studies showed a complete release of lysozyme from PEGT/PBT matrices [9]. Therefore, the total amount of lysozyme released from microspheres can also be used as indication of the encapsulation efficiency. The higher value for the encapsulation efficiency determined from the total release by protein analyses compared to the encapsulation efficiency based on the radioactivity measured in the outer phase is probably caused by interference of polymer degradation products with the BCA reagent (in this case approximately 10%). The total amount released based on radioactivity is slightly lower than expected from the outer water phase measured for radioactivity. This

might be instability of part of the ^{14}C-methylated lysozyme caused by the processing it has undergone (e.g. labeling and concentration). Subsequently, partly aggregated ^{14}C-methylated lysozyme might prevent a complete release from PEGT/PBT microspheres in contrast to native lysozyme. In conclusion, the various different methods used for determination of the encapsulation efficiency result in calculated values with relatively small variations (± 10%). For the in-vitro release, the encapsulation efficiency based on the radioactivity concentration in the outer water phase was used.

As described before, the encapsulation efficiency depended on the copolymer composition [10]. This phenomenon was attributed to the swelling of the copolymer matrix. A higher swelling of the matrix resulted in a lower encapsulation of the protein in the matrix, due to premature protein release during preparation. The relatively low encapsulation of the 600PEGT77PBT23 microspheres, however, was unexpected and did not correspond with previous results [10].

In vitro release

The in-vitro release of (^{14}C-methylated) lysozyme from PEGT/PBT microspheres was evaluated in PBS. The cumulative release profiles were obtained from radioactivity measurements and corrected for the encapsulation efficiency based on the outer water phase (Figure 3). As shown in previous publications, the protein release rate could be effectively tailored by varying the composition of the copolymer [9,10]. Lysozyme release from 1000PEGT70PBT30 microspheres was complete within 14 days, whereas the 600PEGT77PBT23 microspheres continued releasing for 28 days. Previous studies on PEGT/PBT matrices showed similar release profiles of fully active lysozyme [9,10,13]. Irrespective of the copolymer composition, an initial protein burst did not occur, indicating that the protein was effectively encapsulated in the microspheres. The release profile is attributed to a combination of diffusion of the lysozyme through the swollen matrix and degradation of the matrix [9]. The diffusion coefficient appeared to be highly dependent on the swelling of the polymer matrix, which is mainly determined by the PEG-segment length. At a constant PEG-segment length, the swelling can be modulated to a certain extent by varying the PEGT content. The volume swelling ratio's (Q) of the copolymers used in this study are 1.92, 1.73 and 1.66 for 1000PEGT70PBT30, 1000PEGT60PBT40 and 600PEGT77PBT23, respectively [9]. The order in the swelling

ratios of the copolymers corresponds with the order of the lysozyme release rates from these matrices (Fig. 3).

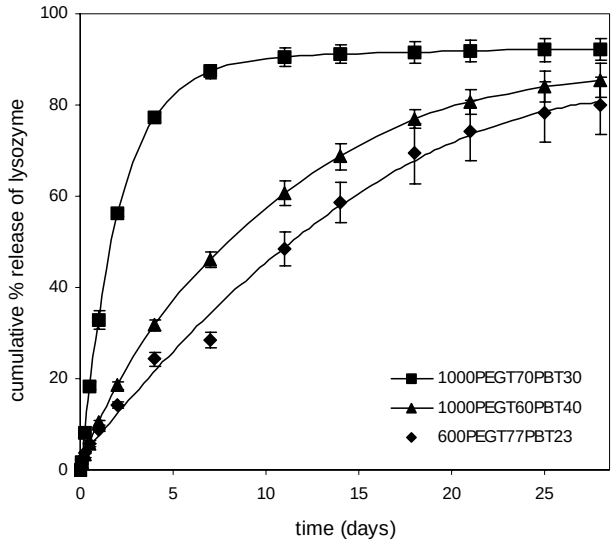


Figure 3: Cumulative in-vitro release of ^{14}C-methylated lysozyme from PEGT/PBT microspheres in PBS (n=3; ± s.d.) based on radioactivity measurements.

Total protein concentration measurements showed similar release profiles to those obtained with radioactivity measurements, although the total estimated release was always lower (by ca. 10%) for the radioactivity measurements. Therefore, it was concluded that the label remained associated with the protein after release from the microspheres. In addition, no significant differences were observed for the batches of 1000PEGT70PBT30 microspheres containing either radiolabelled lysozyme or non-radiolabelled lysozyme (data not shown), indicating that the label had no effect on the lysozyme release.

In vivo study

The in-vivo release of ^{14}C-methylated lysozyme from PEGT/PBT microspheres was evaluated in rats. Plasma radioactivity concentration-time profiles resulting from subcutaneous administration of free ^{14}C-methylated lysozyme and the three microsphere formulations are presented in Figure 4. Following the administration of free ^{14}C-

methylated lysozyme, the plasma radioactivity concentrations declined in a biexponential manner and were below the limit of quantification beyond 14 days post-dose. The mean plasma radioactivity concentration-time profiles following administration of the microsphere formulations indicated that passage of radioactivity into the systemic circulation was fastest for 1000PEGT70PBT30 microspheres and the slowest for 600PEGT77PBT23 microspheres. Furthermore, the mean plasma radioactivity concentrations were below the limit of quantification beyond 14 days post-dose for the 1000PEGT70PBT30 microspheres, but were measurable up to the last sample point (28 days) for the 1000PEGT60PBT40 and 600PEGT77PBT23 microspheres.

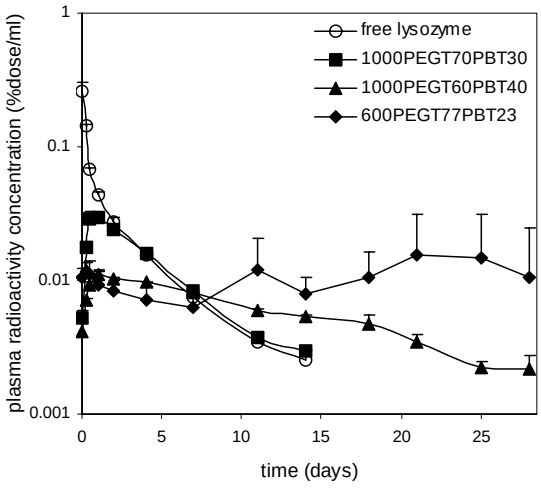


Figure 4: Plasma radioactivity concentrations in rats after administration of single subcutaneous doses of free ^{14}C-methylated lysozyme and microspheres formulations (n=2 1000PEGT70PBT30, n=3; ± s.d. 1000PEGT60PBT40 and 600PEGT77PBT23).

Table 1 shows the pharmacokinetic parameters as derived from the plasma levels. Independent of the microsphere formulation, the maximum plasma concentration was approximately 10 fold lower compared to the C_{max} of the free ^{14}C-methylated lysozyme injection. While C_{max} for the free lysozyme formulation was achieved at the first sampling point (1 hour), the maximum measured concentrations occurred at 24 hours for the 1000PEGT70PBT30 and 1000PEGT60PBT40 formulations. For the 600PEGT77PBT23 microspheres, showing the slowest release in-vitro, C_{max} was not reached until 21 days post dose. However, it should be noted that for this formulation

large variation in plasma radioactivity concentrations was observed for individual animals, particularly at the later time points (coefficient of variation ranged from 17% to 132%). The latter might be explained by either variation in the total amount injected or by variation in the injection depth for this formulation [27].

Table 1. Pharmacokinetic parameters and total amount of radioactivity at dose sites

Formulation	C_{max} (%dose/ml)	T_{max} (hours)	AUC_{all} (% dose.h/ml)	Relative bioavailability (% of free lysozyme)	Radioactivity dose site (total % dose)
Free lysozyme	0.2607	1	6.1 ± 0.2	-	0.3 ± 0.1
1000PEGT70PBT30 microspheres*	0.0294	24	4.0	66	4.5
1000PEGT60PBT40 microspheres	0.0112	24	3.9 ± 0.1	64	9.5 ± 1.4
600PEGT77PBT23 microspheres	0.0156	504	7.1 ± 4.0	117	8.2 ± 3.2

* n=2

The relative bioavailability, based on the area under the curve (AUC_{all}), was calculated relative to the subcutaneous injection of free lysozyme (Table 1). The relative bioavailability for all microsphere formulations was higher than 64%. Relative bioavailabilities of proteins released from microspheres formulations based on poly(lactic-co-glycolic acid) were somewhat lower than we observed. For recombinant human growth hormone (rhGH) bioavailabilities of 33-55% relative to a subcutaneous injection of rhGH have been reported [28]. The low relative bioavailability of 40.7% for bovine derived superoxide dismutase (bSOD) was attributed to instability of bSOD in the PLGA microspheres and the higher susceptibility to enzymatic degradation in subcutaneous tissue of the gradually released bSOD [6]. It should be noted that in our study radioactivity measurements were used, and thus quantifications are not affected by any reduction of protein activity through metabolism. Therefore, the high relative bioavailabilities observed for the PEGT/PBT microsphere formulations cannot be extrapolated to other (biologically active) proteins. In addition, it cannot be excluded that a sampling point within in first hour for the free lysozyme formulation might have increased the C_{max} value, which would result in lower relative bioavailabilities.

The high relative bioavailabilities for the microsphere formulations used in this study indicate that there was no prolonged retention of the protein at the injection site. This observation was confirmed by radioactivity measurements on the dose sites after

sacrificing the animals (Table 1). For all microsphere formulations, less than 10% of the total dose remained at the injection site after 4 weeks. However, the protein release from the 1000PEGT60PBT40 and 600PEGT77PBT23 microspheres was not complete within 4 weeks, which corresponds with the in-vitro release profiles (Fig. 3).

To ensure that only bound ^{14}C label is detected during the in-vivo release study, the plasma was dispensed in trichloroacetic acid (TCA) in order to precipitate the ^{14}C-radiolabel remaining in association with the protein. The proportion of radioactivity associated with precipitable protein was greater than 77% for all time points. Therefore, it was concluded that the radioactivity measurements reflected the release of protein during the in-vivo study. This study did not assess the integrity of the released lysozyme. In-vitro studies, however, have shown a complete release of fully active lysozyme from PEGT/PBT matrices [9,13].

In-vitro / in-vivo correlation

To compare the in-vitro and in-vivo release of ^{14}C-methylated lysozyme from the microsphere formulations, the mean radioactivity concentration-time data of the free ^{14}C-methylated lysozyme injection were modelled using WinNonlin Model 8 (2 compartment IV-Bolus, macro-constants, no lag time, 1st order elimination) in order to provide the reference parameters for deconvolution analysis. The resultant cumulative input of ^{14}C-methylated lysozyme into the plasma following the administration of the microsphere formulations and the in-vitro release of the microsphere formulations are presented in Figure 5. Examination of the plots indicated that, for the 1000PEGT70PBT30 (A) and 1000PEGT60PBT40 (B) microspheres, the in-vitro and in-vivo curves were very similar in shape. Greater variability was observed between in-vitro and in-vivo for the 600PEGT77PBT23 microspheres (C), the slowest release formulation. The latter might be due to variation in injection depth [27] for this formulation as discussed before.

Point to point correlation analysis for each formulation yielded in-vitro/in-vivo correlation coefficients of 0.995, 0.997 and 0.962 for the 1000PEGT70PBT30, 1000PEGT60PBT40 and 600PEGT77PBT23 microspheres, respectively. Independent of the release rate, the in-vitro release and the in-vivo release showed excellent congruence. In contrast to other studies [4,14,15,17], the in-vitro release buffer PBS at 37^{o}C mimicked the in-vivo situation surprisingly well for our release system.

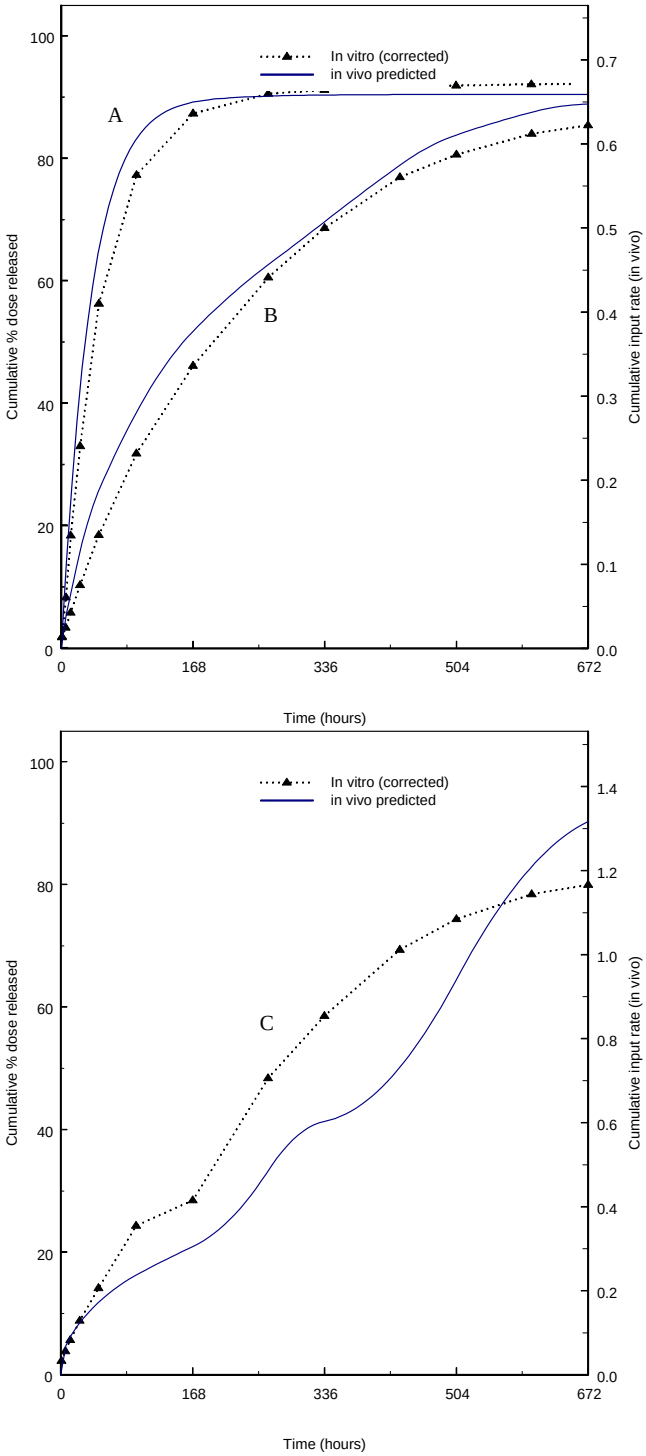


Figure 5: In vitro cumulative release and in vivo cumulative input of ^{14}C-methylated lysozyme from microspheres of 1000PEGT70PBT30 (A), 1000PEGT60PBT40 (B) and 600PEGT77PBT23 (C) based on radioactivity measurements.

The observed in-vivo release very often differs from the in-vitro release concerning shape and/or time-course [4,7,14-16]. In-vivo lysozyme release from PLG microspheres described by Jiang et al. was mimicked the best by in-vitro release in a glycine-HCl buffer (pH 2.5) whereas a slower and more incomplete release was observed in PBS [4]. This was ascribed to the adsorption of lysozyme onto the hydrophobic PLG matrix in PBS. Due to the presence of hydrophilic PEG blocks in our polymer system, incomplete release due to protein adsorption was not observed. The slow in-vitro release compared to the in-vivo release has been reported for many PLG based release systems [7,14,15]. In most of these cases, the release of the active compound is mainly determined by degradation of the polymer matrix. The in-vivo degradation of PLG microspheres was found to be two times faster than the in-vitro degradation, due to the foreign body response [21,22]. Therefore, for degradation controlled release systems, the in-vivo release will differ from the release profile obtained in-vitro. On the contrary, for diffusion controlled sustained release systems, a proper in-vitro / in-vivo correlation has been described [29]. The release mechanism for our PEGT/PBT system is based on diffusion of the protein through the water-swollen matrix. The diffusion of the lysozyme through the matrix is time-dependent due to degradation of the polymer matrix. As the molecular weight decreases, the mesh size in the polymer matrix increases, resulting in an increased diffusion rate [9]. An important degradation mechanism of these PEGT/PBT copolymers is hydrolysis, which occurs both in-vitro (PBS) and in-vivo. Therefore, the perfect in-vitro / in-vivo correlation for the release of lysozyme from PEGT/PBT microspheres found in this study can be explained by the diffusion of lysozyme combined with the hydrolysis of the polymer matrix. Both swelling and degradation of the matrix are determined by the copolymer composition. Of the three compositions used in this study, the 600PEGT77PBT23 copolymer showed the lowest swelling due to the shortest PEG segments. Therefore, the degradation of this copolymer is more important for the lysozyme release, compared to the higher swelling compositions. In case the in-vivo situation affects the degradation rate of the polymer matrix, this effect will be more pronounced for the 600 g/mole PEG segment containing copolymer. This might also be an explanation for the somewhat greater variability observed between the in-vitro and in-vivo release for the 600PEGT77PBT23 microspheres.

The excellent congruence between the in-vitro release in PBS and the in-vivo release of lysozyme from PEGT/PBT microspheres, however, cannot be directly extrapolated to other proteins. In-vivo, the protein-dependent metabolism [17,18,20] and immune response [6,17,28,30] will affect the protein plasma levels. In addition, the size of the protein may determine the route of transport through the tissue into the blood stream [18,19]. On the other hand, the in-vitro release may be affected by the stability and solubility of the protein in the release buffer [3,4,16]. Therefore, for every protein used in a controlled release formulation an in-vitro/ in-vivo correlation should be established.

Conclusion

^{14}C-methylated lysozyme was successfully encapsulated in PEGT/PBT microspheres via a water-in-oil-in-water emulsion. The release rate of radiolabelled lysozyme from PEGT/PBT microspheres could be tailored by varying the copolymer composition. The in-vitro release in PBS and in-vivo release in rats showed an excellent congruence independent of the release rate. The in-vitro/in-vivo correlation coefficients were greater than 0.96. In addition, at sacrifice less then 10% of administered radioactivity remained at the dose site for the microsphere formulations, indicating that there was no notable retention of the protein at the injection site after 28 days.

Referenties

1. S. P. Schwendeman, H. R. Costantino, R. K. Gupta, R. Langer. Peptide, protein, and vaccine delivery from implantable polymeric systems: progress and challenges. In K. Park, editor, Controlled drug delivery, challenges and strategies, Am. Chem. Soc. Washington, D.C., 1996 pp 229-267.
2. P. Couvreur, F. Puisieux. Nano- and microparticles for the delivery of polypeptides and proteins, Adv. Drug Del. Rev. 10:141-162 (1993).
3. W. Lu, T. G. Park. Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems, J. Pharm. Sci. & Technol. 49:13-19 (1995).

4. G. Jiang, B. H. Woo, F. Kang, J. Singh, P. P. DeLuca. Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres, J. Control. Release 79:137-145 (2002).
5. A. Aubert-Pouëssel, D. C. Bibby, M. C. Vernier-Julienne, F. Hindré, J. P. Benoît. A novel in vitro delivery system for assessing the biological integrity of protein upon release from PLGA microspheres, Pharm. Res. 19:1046-1050 (2002).
6. T. Morita, Y. Sakamura, Y. Horikiri, T. Suzuki, H. Yoshino. Evaluation of in vivo release characteristics of protein-loaded biodegradable microspheres in rats and severe combined immunodeficiency disease mice, J. Control. Release 73:213-221 (2001).
7. G. Jiang, W. Qiu, P. P. DeLuca. Preparation and in vitro/in vivo evaluation of insulin-loaded poly(acryloyl-hydroxyethyl starch)-PLGA composite microspheres, Pharm. Res. 20: 452-459 (2003).
8. P. Johansen, G. Corradin, H.P. Merkle, B. Gander. Release of tetanus toxoid from adjuvants and PLGA microspheres: How experimental set-up and surface adsorption fool the pattern, J. Control. Release 56:209-217 (1998).
9. J. M. Bezemer, R. Radersma, D. W. Grijpma, P. J. Dijkstra, J. Feijen, C. A. van Blitterswijk. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices, J. Control. Release 64:179-192 (2000).
10. G. J. Beumer, C. A. van Blitterswijk, M. Ponec. Biocompatibility of degradable matrix induced as a skin substitute: an in vivo evaluation, J. Biomed. Mat. Res. 28:545-552 (1994).
11. R. van Dijkhuizen-Radersma, S. C. Hesseling, P. E. Kaim, K. de Groot K, J. M. Bezemer. Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation, Biomaterials 23:4719-4729 (2002).
12. J. M. Bezemer, R. Radersma, D. W. Grijpma, P. J. Dijkstra, C. A. van Blitterswijk, J. Feijen. Microspheres for protein delivery prepared from amphiphilic multiblock copolymers. 2. Modulation of release rate, J. Control. Release 67:249-60 (2000).
13. M. van de Weert, R. van Dijkhuizen-Radersma, J. M. Bezemer, W. E. Hennink, D. J. A Crommelin. Reversible Aggregation of lysozyme in a biodegradable amphiphilic multiblock copolymer, Eur. J. Pharm. Biopharm. 54:89-93 (2002).
14. Y. Machida, H. Onishi, A. Kurita, H. Hata, A. Morikawa, Y. Machida. Pharmacokinetics of prolonged-release of CPT-11-loaded microspheres in rats, J. Control. Release 66:159-175 (2000).
15. I. Soriano, C. Evora, M. Llabrés. Preparation and evaluation of insulin-loaded poly(DL-lactide) microspheres using an experimental design, Int. J. Pharm. 142:135-142 (1996).
16. J. L. Cleland, O. L. Johnson, S. Putney, A. J. S. Jones, Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development, Adv. Drug Del. Rev. 28:71-84 (1997).
17. T. K. Kim, D. J. Burgess. Pharmacokinetic characterization of ^{14}C-vascular endothelial growth factor controlled release microspheres using a rat model, J. Pharm. Pharmacol. 54:897-905 (2002).
18. C. J. H. Porter, G. A. Edwards, S. A. Charman. Lymphatic transport of proteins after s.c. injection: implications of animal model selection, Adv. Drug. Del. Rev. 50:157-171 (2001).
19. A. Supersaxo, W. R. Hein, H. Steffen, Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration,Pharm. Res. 7:167-169 (1990).

20. C. M. Negrín, A. Delgado, M. Llabrés, C. Évora. In vivo-in vitro study of biodegradable methadone delivery systems, Biomaterials 22:563-570 (2001).
21. M. Tracy, K. L. Ward, L. Firouzabadian, Y. Wang, N. Dong, R. Qian, Y. Zhang. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in-vitro, Biomaterials 20:1057-1062 (1999).
22. G. Spenlehauer, M. Vert, J. P. Benoit, A. Boddaert. In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method, Biomaterials 10:557-563 (1989).
23. T. G. Park, W. Lu, G. Crotts. Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(D,L-lactic acid-co-glycolic acid)microspheres, J. Control Release 33:211-222 (1995).
24. R. V. Diaz, M. Llabrés, C. Évora. One-month sustained release microspheres of ^{125}I-bovine calcitonin: in vitro – in vivo studies, J. Control. Release 59:55-62 (1999).
25. A. J. Kuijpers, P. B. van Wachem, M. J. A. van Luyn, G. H. M. Engbers, J. Krijgsveld, S. A. J. Zaat, J. Dankert, J. Feijen. In vivo and in vitro release of lysozyme from cross-linked gelatin hydrogels: a model system for the delivery of antibacterial proteins from prosthetic heart valves, J. Control. Release 67:323-336 (2000).
26. R. K. Gupta, A. C. Chang, P.Griffin, R. Rivera, Y. Y. Guo. Determination of protein loading in biodegradable polymer microspheres containing tetanus toxoid, Vaccine 15:672-678 (1997).
27. J. Zuidema, F. Kadir, H. A. C. Titulaer, C. Oussoren. Release and absorption rates of intramuscularly and subcutaneously injected pharmaceuticals (II), Int. J. Pharm. 105:189-207 (1994).
28. S. Y. Yen, K. C. Sung, J. J. Wang, O. Y. P Hu. Controlled release of nalbuphine propionate from biodegradable microspheres: in vitro and in vivo studies, Int. J. Pharm. 220:91-99 (2001).
29. J. L. Cleland, A. Daugherty, R. Mrsny. Emerging protein delivery methods, Current opinion in biotechnology 12:212-219 (2001).
30. K. Yamaguchi, J. M. Anderson. Biocompatibility studies of naltrexone sustained release formulations, J. Control. Release 19:299-314 (1992).

Chapter 8

Succinate substituted poly(ethylene glycol)/ poly(butylene terephthalate) copolymers: synthesis and characterization

R. van Dijkhuizen-Radersma, J.R. Roosma, S. Métairie, F.L.A.M.A. Péters, P.E. Kaim, J. de Wijn, P.G. Zijlstra, K. de Groot, J.M. Bezemer

Abstract

Multiblock poly(ether-ester)s based on poly(ethylene glycol), butylene terephthalate and butylene succinate units were synthesized by a two-step melt polycondensation reaction, with the aim of developing a new series of degradable polymers for controlled release applications. The copolymers were characterized with respect to their composition (NMR), thermal properties (DSC) and swelling. The main focus was on the degradation kinetics and release properties of the copolymers. The crystallinity and swelling could be tailored by the PEG segment length and the ratio of the building units. With increasing mol fraction succinate in the hard segment, the swelling increased. The in-vitro degradation was found to occur by molecular weight decrease and mass loss. Substitution of the aromatic terephthalate units by aliphatic succinate units increased the degradation rate of the copolymers. Polymers with PEG segments of 1000 g/mole showed a more pronounced degradation than copolymers containing shorter and longer PEG segments. Model proteins were successfully incorporated and released from the poly(ether-ester) films. Depending on the size of the protein, the release mechanism was based on diffusion of the protein and degradation of the matrix.

Introduction

Amphipilic block copolymers have gained increasing interest for drug delivery applications [1]. Recently, a series of poly (ether-ester) multiblock copolymers composed of poly(ethylene glycol) terephthalate and poly(butylene terephthalate) (PEGT/PBT) was introduced as matrix for controlled release systems [2-4]. Variation of the composition of the copolymers (PEG wt% and PEG molecular weight) allowed easy modulation of the release of several proteins and peptides [3,4]. Extensive in-vitro and in-vivo studies have addressed the degradation behaviour of these type of polymer systems [5,6]. The degradation rate appeared to be strongly dependent on the copolymer composition [3,4]. For some polymer compositions, in particular those rich in PBT, degradation times for more than one year have been reported. For controlled release applications requiring frequently repeated injections, such degradation rates might be too slow [7]. A known method to increase the degradation rate of aromatic polyesters is by (partial) substitution of the aromatic groups by aliphatic groups [8-10]. We evaluated this approach to design poly(ether-ester) copolymers with increased degradation rates as compared to the existing PEGT/PBT multiblock copolymers. Succinate has been selected as the aliphatic group to replace part of the terephthalate, as it is an approved pharmaceutical additive [11].

This paper describes the synthesis of PEGT/PBT copolymers in which part of the butylene terephthalate (BT) sequences are substituted by butylene succinate (BS) units. The resulting PEG(T/S)PB(T/S) have been characterized for their thermal properties, swelling (in water) and in-vitro degradation behaviour. Several model proteins have been used to evaluate the controlled release properties of these novel multiblock copolymers.

Materials and methods

Materials

Dimethyl terephthalate (DMT, purum ≥ 99% (GC)), dimethyl succinate (DMS, purum ≥ 98% (GC)), poly(ethylene glycol) (PEG, purum) 300, 600, 1000, 4000, 1,4-butanediol (BD, purum ~ 99% (GC)), tetrabutyl orthotitanate ($Ti(OBu)_4$), chloroform (analytical

grade) and dichloromethane (analytical grade) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Aldrich Chemical Company, Inc. (Milwaukee, USA) was the supplier of α-tocopherol (Vitamin E, 97 %). Phosphate buffered saline , (pH 7.4) was obtained from Life Technologies Ltd (Paisley, Scotland). Lysozyme (Hen egg white, 3x crystallized, dialyzed and lyophilized), Carbonic Anhydrase (Bovine erythrocytes) and Albumin (Bovine, 98%, initial fraction by heat shock) were purchased from Sigma (St. Louis, MO, USA).

Synthesis of PEG(T/S)PB(T/S) copolymers

The PEG(T/S)PB(T/S) copolymers were synthesized on 1 kg scale in a two-step melt polycondensation reaction in the presence of $Ti(OBu)_4$ as catalyst (0.2wt% of hard segment) and α-tocopherol as antioxidant (0.6 wt%). The first step involved the trans-esterification of dimethyl terephthalate (DMT) and dimethyl succinate (DMS) with PEG and an excess of 1,4-butanediol under a nitrogen atmosphere at approximately 160^{o}C. After the distillation of the methanol, the reactor temperature was increased to 245^{o}C and, subsequently, the pressure was slowly reduced to 0.5 – 1.5 mbar, in order to start the polycondensation. Due to distillation of the 1,4-butanediol and concurrent polymerization, the melt viscosity of the polymer started to increase. At a desired viscosity, the reaction was stopped and the polymer melt was extruded from the reactor and quenched in a cold-water bath. The poly(ether-ester) polymer was dried under vacuum (0.5 mbar) at 50^{o}C for at least 2 days. The resulting multiblock copolymer can be described as being built up of four different segments (Table 1). Various PEG(T/S)PB(T/S) copolymer compositions were prepared by using PEG of different molecular weights and by varying the PEG/DMT/DMS ratios. The obtained copolymer composition is indicated as aPEG(T/S)bPB(T/S)c (T/S=d/e), in which a is the PEG molecular weight, b the combined weight percentage of PEGT and PEGS and c, the combined weight percentage of PBT and PBS. d/e is the molar T/S ratio in the polymer. PEG(T/S) is also referred to as soft segment, PB(T/S) is also referred to as hard segment.

Polymer characterisation

Proton NMR spectra were recorded on a Bruker ARX-400 operating at ≥ 200 MHZ at 343 K. $C_2D_2Cl_4$ was used as solvent without internal standard.

The molecular weights of the copolymers were determined using Gel Permeation Chromatography (GPC). Samples were eluted in 0.02M sodiumtrifluoroacetate (NaTFA) in hexafluoroisopropanol (HFIP) through a Polymer Labs HFIP gel guard column (50 x 7.5 mm) and two PL HFIP gel analytical columns (300 x 7.5 mm). Flow rate was 1 ml/min and both an Ultraviolet (UV, 254 nm) and a Refraction Index (RI) detector were used. Column temperature was 40ºC and sample concentration was 0.3 mg/ml (50 µl injection). The weight average molecular weights (M_w) and the polydispersity (M_w/M_n) were determined relative to polymethylmethacrylate (PMMA) standards. The intrinsic viscosity [η] was determined of a solution of 0.5 g/dl of polymer in chloroform using a Schott Gerâte viscosimeter (DIN) Ubbelohde type 0c at 25°C [12].

Thermal analysis of the polymers was carried out with a Perkin-Elmer Pyris 1 differential scanning calorimeter. Calibration was performed with pure indium and zinc. Samples (10-15 mg) of both dry (stored for over 3 weeks at room temperature) and water-swollen compression moulded sheets were heated from -80°C to 250°C at a heating rate of 10°C/min. We calculated the percentage of crystallized polymer, originating from PBT or PBS sequences in the polymer chains, from the area under the curve (ΔH) of the first scan according to equation (I)

$$X_c(\%) = \frac{\Delta H}{\Delta H_f} \times 100\% \qquad \text{(I)}$$

in which the theoretical heat of fusion for the perfect crystal ΔH_f equals 110.3 J/g for PBS [13] and 144.5 J/g for PBT [14].

The percentage of crystallinity of the hard segment ($\omega_{c\ PBT}$ or $\omega_{c\ PBS}$) was calculated using equation (II)

$$\omega_c = \frac{X_c}{\omega_h} \qquad \text{(II)}$$

in which ω_h is the weight fraction of PBT or PBS, respectively.

The swelling of the copolymers was determined using films. Poly(ether-ester) films were prepared from solutions of 1 g copolymer in 5 ml chloroform. The polymer solutions were cast onto a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and vacuum-dried for at least 16 hrs at 50°C. To measure the degree of swelling of the polymers, dry films (surface ± 1.77 cm^2, ± 100 µm in thickness) were weighed and immersed in phosphate buffered saline at

37°C in a shaking bath. After 3 days, the weight of the samples was determined after surface water was removed by blotting the surface with a tissue. The equilibrium swelling ratio q was determined as the ratio of the equilibrium weight of the swollen samples and the weight of the dry samples.

In-vitro degradation

To evaluate the in-vitro degradation, polymer sheets (1 mm thickness) were compression moulded at 100°C – 220°C using a PHI press ($9 \cdot 10^3$ kg for 2 min). Samples of the sheets (20 mm x 20 mm) were immersed in 50 ml phosphate buffered saline at 37°C in a shaking bath for several time periods. The buffer was refreshed every 2 weeks. After the degradation periods, the residues of the samples were washed with purified water and dried using a vacuum oven at 50°C for at least 16 hours. The in-vitro degradation was monitored by analysing the changes in molecular weight, mass loss and copolymer composition. The molecular weight as function of degradation time was determined using Gel Permeation Chromatography (GPC) as described above. The mass loss was determined by weighing the copolymer sheets prior to and after degradation. The effect of the in-vitro degradation on the composition of the copolymers was studied by NMR measurements as described above.

In-vitro release of proteins

The release properties of the PEG(T/S)PB(T/S) copolymers were evaluated using protein loaded films. A protein solution (0.6 ml, 55 mg/ml) in phosphate buffered saline was emulsified in a polymer solution (1 g polymer in 5 ml dichloromethane) using ultra-turrax mixing (30 s at 19 krpm, IKA Labor technik T25). The resulting water-in-oil emulsion was cast onto a glass plate using a 0.7 mm casting knife. After slow evaporation of the solvent, the film was removed from the glass plate and freeze dried for at least 16 hours. Lysozyme (14.5 kDa), Carbonic Anhydrase (29 kDa) and Bovine Serum Albumine (67 kDa) were used as model proteins.

The protein release from the protein loaded PEG(T/S)PB(T/S) films was investigated by incubating pieces of the films (± 1.77 cm^2) in 1 ml phosphate buffered saline. Vials were continuously shaken at 37°C and samples of the release medium were taken at various time points. The protein concentration of the buffer was determined

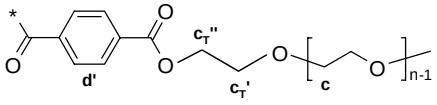
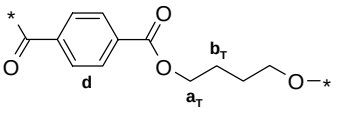
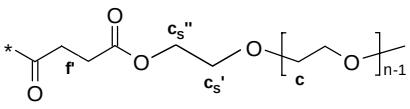
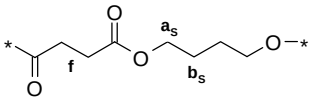
using micro bichinchoninic acid (BCA) protein assay on an EL 312e microplate bio-kinetics reader (λ = 570 nm). The buffer was refreshed after sampling.

Results and discussion

Synthesis

A series of PEG(T/S)PB(T/S) copolymers was synthesized in a two-step melt polycondensation reaction. The copolymers can be described as being built up of four different repeating segments (Table 1). As succinate and terephthalate have a similar reactivity in the polycondensation reaction [15], it is assumed that the four segments are randomly distributed along the polymer chain. The results of the polymerizations are given in Table 2. The composition of the copolymers was calculated from ^{1}H-NMR spectra, a typical spectrum is shown in figure 1. Table 1 indicates the protons from which the integrals originate. The wt% of the soft content (PEG(T/S)) and the molar ratio between terephthalate and succinate (T/S) were calculated from the spectra as described elsewhere[14,16].

Table 1: Segments present in the poly(ether ester) copolymers

Formula	Name	Abbreviation
	Poly(ethylene glycol) terephthalate	PEGT
	Butylene terephthalate	BT
	Poly(ethylene glycol) succinate	PEGS
	Butylene succinate	BS

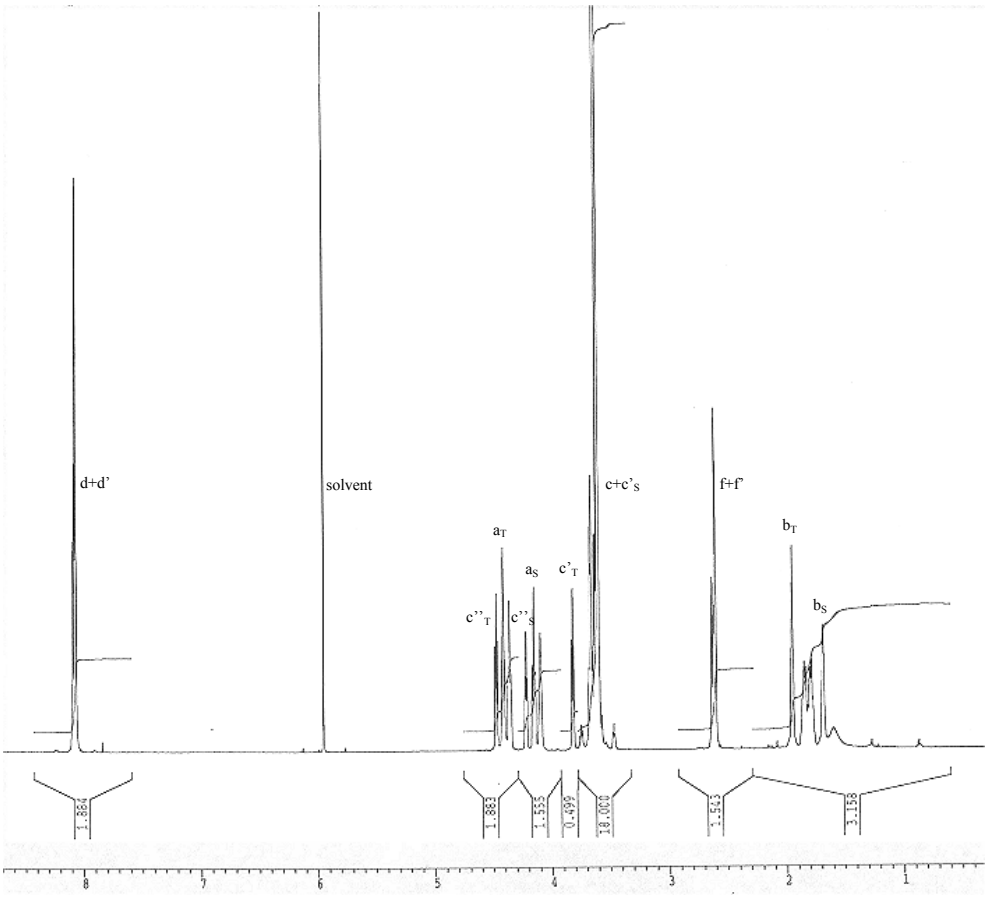


Figure 1: NMR spectrum of 1000PEG(T/S)65PB(T/S)35 containing 45 mol% succinate in hard segment.

The weight average molecular weight (M_w) and the polydispersity (M_w/M_n) of the copolymers are given in Table 2. The M_w is in the range of 93 – 180 kg/mole (relative to PMMA), all with a polydispersity of about 2, which is expected for polymers prepared by a polycondensation reaction. The intrinsic viscosity of the copolymers was in the range of 0.74 – 1.25 dl/g, depending on the copolymer composition (Table 2).

Table 2: Molecular weight, polydispersity, intrinsic viscosity and equilibrium swelling of the poly(ether-ester)s as function of the copolymer composition.

Polymer composition (NMR)	mol% succinate (NMR)	M_w (10^{-3} g/mole) (GPC)	M_w/M_n (GPC)	Intrinsic viscosity (dl/g)	Swelling ratio q
300PEG(T/S)63PB(T/S)37	46.8	109	1.9	0.90	1.12 ± 0.01
300PEGS66PBS34	100	112	1.5	0.95	1.43 ± 0.03
600PEG(T/S)62PB(T/S)38	46.3	98	1.9	0.92	1.78 ± 0.16
600PEGS71PBS29	100	93	1.8	1.04	n.d.
1000PEGT61PBT39	0	n.d.	n.d.	0.74	1.54 ± 0.07
1000PEG(T/S)62PB(T/S)38	11.4	158	2.0	0.97	1.63 ± 0.02
1000PEG(T/S)66PB(T/S)34	45.4	129	2.3	0.99	2.02 ± 0.09
1000PEGS67PBS33	100	126	1.6	0.89	2.27 ± 0.06
1000PEGT71PBT29	0	133	1.9	0.80	1.77 ± 0.11
1000PEG(T/S)73PB(T/S)27	44.8	n.d.	n.d.	1.05	2.45 ± 0.07
1000PEGS72PBS28	100	180	2.1	1.17	2.71 ± 0.06
4000PEG(T/S)66PB(T/S)34	46.2	111	1.9	1.10	2.84 ± 0.06
4000PEGS66PBS34	100	127	1.8	1.25	3.18 ± 0.24

n.d.: not determined

Copolymer properties

The thermal properties of the PEG(T/S)PB(T/S) were determined by differential scanning calorimetry (DSC). In general, three transition temperatures could be observed for the melting and crystallisation of PEG, PBS and PBT. Table 3 shows the melting temperatures, the melting enthalpy and the percentage of crystallized PBS and PBT, respectively, for copolymers with PEG segments of 1000 g/mole and approximately 65 wt% soft segment. The presence of two melting points for the hard segment indicates micro phase separation resulting in PBS rich and PBT rich domains. The melting temperatures of the hard segments in the copolymers were always lower than those of pure PBT and PBS (226^{o}C [17] and 114^{o}C [18], respectively). For the copolymers containing 100 mol% PBT or PBS as hard blocks, this could be attributed to the presence of PEG [19,20]. Fakirov et al. reported for PEGT/PBT copolymers decreasing melting temperatures with increasing amount of PEG [14]. At a fixed PEG content, the melting points were further decreased by introducing mixed (BT and BS) hard segments (Table 3). The lower melting temperatures indicate the formation of smaller or less perfect

crystals [13,21]. This effect is represented by the percentage of crystalline PBT and PBS, which decreased when their relative amounts decreased (Table 3). The random sequence of the comonomeric units in the poly(ether-ester)s hinders or prevents organization of the crystalline lattices of PBS and PBT and less ordered, amorphous, regions are formed. This inhibited crystal growth effect has been observed for several comparable polymer systems [13,22-24]. In addition, crystallization might require a certain minimum length of the PBT or PBS blocks. It has to be noted that with increasing BS content, the average sequence length of the PBT blocks will decrease (and vice versa). The concentration of PBT blocks sufficiently long to be able to crystallize will be low in copolymers with a high PBS content, resulting in a decrease in PBT crystallinity (and visa versa). As observed before for copolymers of PEG and poly(butylene terephthalate) [25] or poly(lactic acid) [26], the PEG segment length also affected the crystallinity of the succinate substituted poly(ether-ester)s. The longer the PEG segment, the higher the percentage of crystalline PBS and PBT (data not shown). The longer soft segments at a constant soft/hard ratio increases the average block length of the hard PB(T/S) segments. The critical block length necessary for crystallization will be more frequently present in longer hard blocks.

Table 3: Thermal properties of the poly(ether-ester)s as function of copolymer composition.

Polymer composition (NMR)	mol% succinate (NMR)	$T_{m\,PBS}$ (^oC)	ΔH_{PBS} (J/g)	$\omega_{c\,PBS}$ (%)	$T_{m\,PBT}$ (^oC)	ΔH_{PBT} (J/g)	$\omega_{c\,PBT}$ (%)
1000PEGT61PBT39	0	-	-	-	170	26.3	46.7
1000PEG(T/S)62PB(T/S)38	11.4	51	<LQ	-	150	13.7	27.0
1000PEG(T/S)66PB(T/S)34	45.4	53	1.8	14.0	108	2.6	8.1
1000PEGS67PBS33	100	65	22.1	60.7	-	-	-

LQ: limit of quantification

The crystallinity of the copolymer may affect the degradation kinetics too a large extent, since hydrolysis takes place preferably at the amorphous parts of polymers [13,15,18,22-24,27,28]. Therefore, the total degree of crystallization derived from PBS and PBT, calculated using equation (I), is given in Figure 2 for copolymers with PEG segments of 1000 g/mole and approximately 65 wt% soft segment. As was expected based on the average sequence length of the PBT and PBS blocks, the lowest crystallinity

was found for the copolymer in which approximately half of the aromatic groups had been replaced by aliphatic groups. This result was also observed visually, as crystalline sheets (containing either 100% T or S) had a whitish appearance and were relatively stiff, while the sheet containing 45 mol% of succinate was transparent and highly flexible. As the poly(ether-ester)s will be applied in aqueous environments, DSC measurements on water-swollen matrices were also performed (data not shown). These analyses showed that the melting peak of PBS completely disappeared in the presence of water.

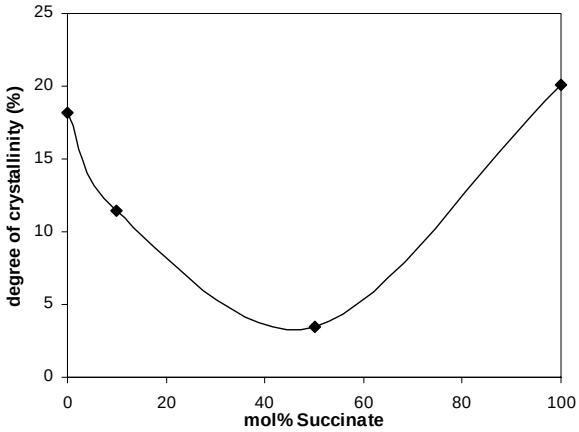


Fig. 2. Percentage crystallinity of 1000PEG(T/S)65PB(T/S)35 sheets derived from PBT and PBS as function of degree of succinate substitution.

The properties of the water-swollen matrices are of primary interest for controlled release applications. For example, the extent of swelling largely determines the rate at which water-soluble drugs, such as proteins, are released from the polymer matrices. For the copolymers used in this study, the equilibrium swelling ratio q varied from 1.1 - 1.4 for copolymers with 300 g/mole PEG-segments up to 2.8 – 3.2 for 4000 g/mole PEG-segments (Table 1). As expected [17,26,29,30], the equilibrium swelling ratio increased with increasing amount of the hydrophilic component, PEG, in the copolymer (compare 1000PEG(T/S)66PB(T/S)34 and 1000PEG(T/S)73PB(T/S)27 both containing 45 mol% S). At a constant amount of soft segment and a fixed mol% succinate, the swelling ratio q increases with increasing length of the hydrophilic PEG-segment (Fig. 3A) [19,29]. However, the increase in swelling levelled off for copolymers containing longer PEG segments. As discussed before, longer PEG blocks will result in

longer hydrophobic blocks that may form stronger physical crosslinks, which restricts the expansion of the copolymer network in water. Figure 3B clearly shows that substitution of the terephthalate groups by succinate groups favours the swelling. The aliphatic succinate containing blocks are less efficient in forming physical crosslinks in the water-swollen hydrogels than the hard segments based on aromatic polyesters, as shown by DSC measurements. In addition, the less hydrophobic nature of the aliphatic segments in comparison with the aromatic polyesters, might also contribute to the efficiency of cross-linking. The higher swelling of copolymers with aliphatic blocks has also been observed for copolymers of PEG and poly(lactic glycolic acid) [31] and poly(ether-ester-amide)s [32]. Besides the effect on the drug diffusion in controlled release applications, the swelling might also affect the degradation rate of the succinate containing copolymers. A higher swelling may result in a faster degradation [19,28].

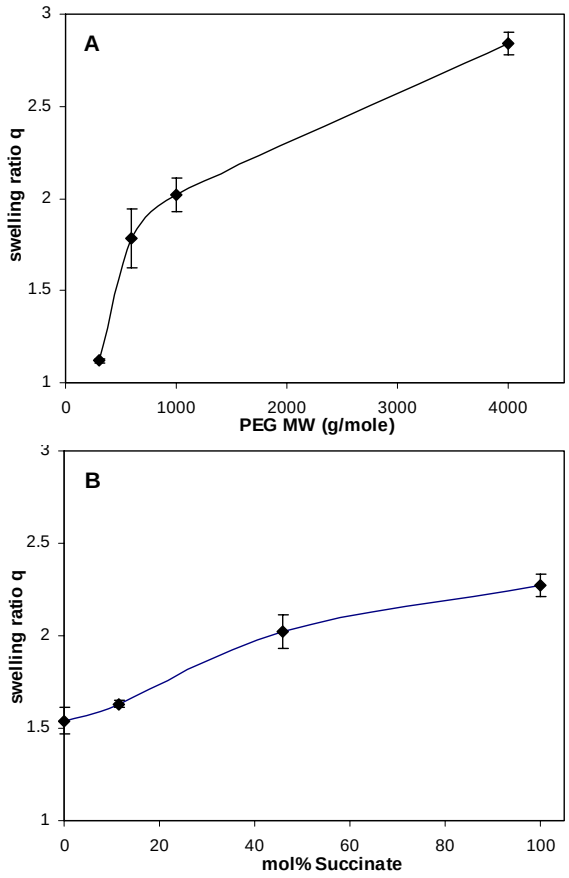


Figure 3: Swelling behaviour of PEG(T/S)PB(T/S) films in phosphate buffered saline at 37ºC: equilibrium swelling ratio q as function of the PEG segment length (65 wt% soft segment, 45 mol% S) (A) and mol% of succinate of the copolymers (PEG MW 1000, 65 wt% soft segment) (B) (n=3, ± s.d.).

In-vitro degradation

To investigate the effect of the substitution of the aromatic BT groups by aliphatic BS groups on the in-vitro degradation, compression moulded sheets were immersed in phosphate buffered saline at 37°C. The in-vitro degradation as function of time was monitored by mass loss (erosion) and changes in molecular weight (hydrolysis) and copolymer composition of the residue. The degree of aliphatic substitution of the aromatic groups by aliphatic groups showed a clear effect on the in-vitro degradation (Fig. 4). At a constant soft/hard ratio, the decrease in molecular weight was faster for poly(ether-ester)s with increasing succinate content (Fig. 4A). After 8 weeks incubation in phosphate buffered saline , a plateau value of 20% of the starting molecular weight was obtained for the residue of the copolymers with high succinate content (45 – 100 mol%). The observed effect of succinate content on the rate of molecular weight decrease is in good agreement with the DSC and swelling measurements. Copolymers with higher succinate substitution show a higher swelling (Fig. 3B), making the ester bonds more accessible for hydrolysis [5,17]. In addition, the aliphatic esters are more susceptible to hydrolysis than aromatic ester, as a result of steric effects [28,33] and flexibility of the chain backbone [8,13]. Unlike what is described for a large number of degradable polymers [13,15,23,34], the observed crystallinity in dry state of the PEGS/PBS copolymer (100 mol% succinate) (Fig 2) did not reduce the degradation rate (Fig. 4). DSC measurements showed the absence of PBS crystals in the presence of water.

The clear effect of the substitution of the terephthalate groups by the succinate groups on the molecular weight of the residue was also observed for the mass loss (Fig. 4B). Sheets of 1000PEGS67PBS33 (100 mol% succinate) completely disappeared within 16 weeks, while no significant erosion could be observed for copolymers with less than 10 mol% of succinate. After 24 weeks, sheets of the 45 mol% succinate containing copolymer showed cracks and hardly any mechanical strength, while no visual changes were observed in time for the sheets of copolymers with 10 mol% or less succinate. The higher the degree of substitution of terephthalate by succinate, the faster the erosion at a constant soft/hard ratio and PEG segment length. When comparing the GPC and the mass loss results, it appears that a similar molecular weight decrease for the copolymers containing, respectively, 45 mol% and 100 mol% of succinate in the hard segment, did not result in a similar mass loss profile. Apparently, the release rates of degradation

products from these two copolymers are different. Göpferich proposed two mechanisms for the release of degradation products: erosion controlled and diffusion controlled release of oligomers [33]. For the erosion controlled release, it is assumed that the different types of degradable bonds in the copolymers are cleaved at different rates. The resulting degradation products are, therefore, released at divergent rates. The diffusion controlled release is based on solubility and diffusivity of the degradation products, resulting in different release rates for different copolymers. In our system, both mechanisms will play a part. As discussed above, the hydrolytic cleavage of the aliphatic ester bonds is faster as compared to that of aromatic ester-bonds. However, in case of a similar molecular weight decrease, the difference in mass loss is diffusion controlled. Succinic acid is soluble in water (77 g/l), whereas terephthalic acid is practically insoluble in water [35]. It is therefore expected that succinate containing oligomers will dissolved at a higher molecular weight than terephthalate containing oligomers. The latter should have a high PEG/aromatic ratio to become soluble [10].

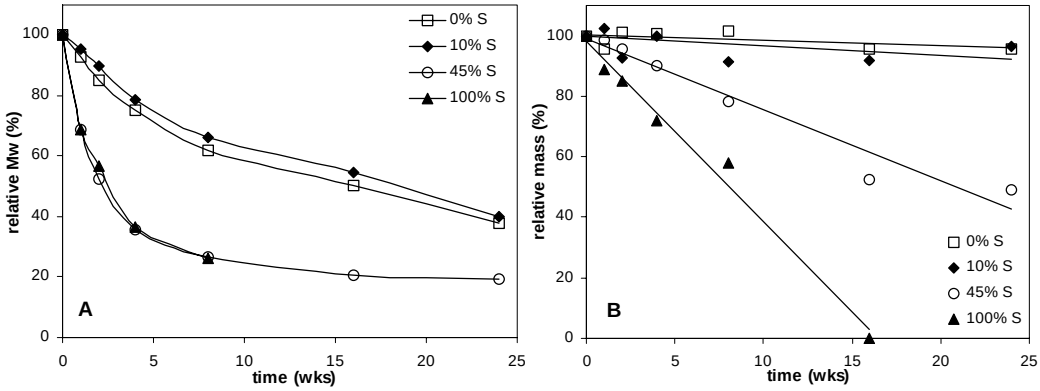


Figure 4: Relative weight average molecular weight (A) and mass (B) of residue as function of degradation time for 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (□), 10 (◆), 45 (○) and 100 (▲) mol% succinate. C: Mass loss rate versus degree of succinate substitution (PEG MW 1000, 65 wt% soft segment).

Due to the preferred hydrolysis of aliphatic ester bonds and the higher solubility of aliphatic oligomers, the copolymer composition might change during degradation [5,17,36]. To investigate this, NMR analyses were performed on the residue (Table 4). The copolymer with 1000 g/mole PEG segments containing 45 mol% succinate still contained 42 mol% succinate after 24 weeks, while half of its initial mass was lost. This

observations indicate that, although hydrolysis of aliphatic ester bonds is preferred and aliphatic oligomers are faster solubilised, the degradation of the poly(ether-ester) will not result in an inert residue consisting of terephthalic groups only. Similar, only minor changes in soft/hard ratio's were observed for the copolymers with significant mass loss, indicating no preferential chain scission of ether-ester bond. Therefore, no residue will remain containing only inert hard segment.

Table 4: Change in copolymer compositions (NMR) and mass loss after 24 weeks incubation in phosphate buffered saline at 37°C.

Polymer composition t = 0	mol% succinate t = 0	Polymer composition t = 24 wk	mol% succinate t = 24wk	Mass loss (%) t = 24 wk
300PEG(T/S)63PB(T/S)37	46.8	300PEG(T/S)62PB(T/S)38	46.5	3
1000PEG(T/S)66PB(T/S)34	45.4	1000PEG(T/S)58PB(T/S)42	41.6	51
4000PEG(T/S)66PB(T/S)34	46.2	4000PEG(T/S)61PB(T/S)39	45.7	7
1000PEGS67PBS33	100	1000PEGS59PBS41[1]	100[1]	42[1]

[1] t=8 wk

Degradation by hydrolysis and subsequent dissolution of the polymer fragments is based on a combination of the swelling [19,28,33] and the average block length of the copolymer's hard segment [8,19]. The higher the PEG segment length, the higher the equilibrium swelling ratio q (Fig. 3A). Increasing the molecular weight of the PEG-segments at a constant soft/hard ratio will increase the average block length of the hard PB(T/S) segments and subsequently reduce the number of easy accessible hydrolysable ether-ester bonds. The effect of the PEG segment length on the in-vitro degradation of the PEG(T/S)PB(T/S) copolymers was evaluated for two series; containing 45 mol% and 100 mol% succinate in the hard segment, respectively (Fig. 5 and 6). For the poly(ether-ester)s in which 45 mol% of the terephthalate had been replaced by succinate, the decrease in molecular weight was slow for the low swelling copolymer containing 300 g/mole PEG segments, while faster decreases were observed for higher swelling compositions (Fig. 5A). However, the fastest molecular weight decrease was observed for the 1000 g/mole PEG segments, while the copolymer containing PEG segments of 4000 g/mole has a higher swelling ratio. This might be due to the longer hard hydrophobic blocks in this polymer, as discussed above. Apparently, copolymers with 1000 g/mole PEG segments have the optimal combination of swelling and hard block length for the

fastest decrease in molecular weight. A similar trend was observed in mass loss experiments, which also showed a maximum degradation rate for the PEG(T/S)PB(T/S) copolymer containing 1000 g/mole PEG segment (Fig. 5B). The limited mass loss of the copolymers containing smaller and longer PEG segments is caused by the slower chain scission (Fig 5A) and the lower solubility of the resulting oligomers.

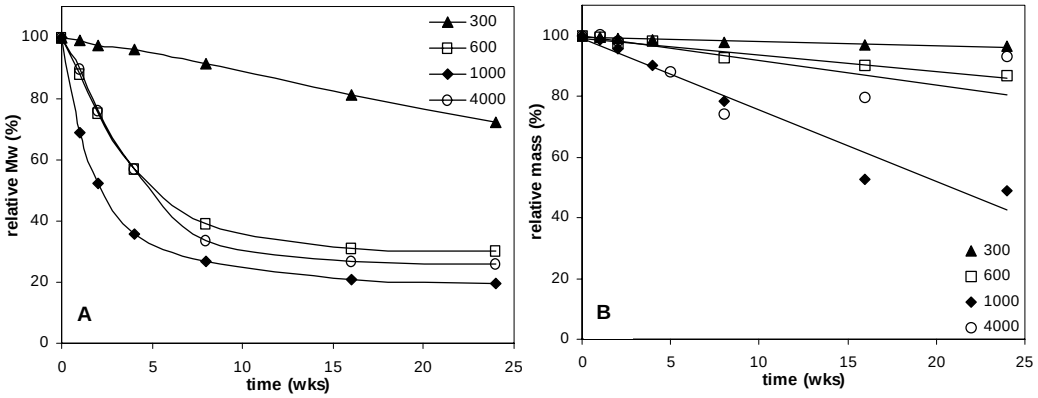


Figure 5: Relative weight average molecular weight (A) and mass (B) of residue as function of degradation time for PEG(T/S)65PB(T/S)35 copolymers containing 45 mol% succinate with PEG segments of 300 (▲), 600 (□), 1000 (◆) and 4000 (○) g/mole.

Figure 6 shows the decrease in molecular weight (A) and mass loss (B) for copolymers based on poly(ethylene glycol) succinate and poly(butylene succinate) as function of PEG-segment length. Due to the higher aliphatic substitution, the degradation of 100% substituted succinate copolymers is faster compared to the 45 mol %. Even for the relatively low swelling copolymer containing 300 g/mole PEG segments, a significant decrease in molecular weight and mass loss could be measured. A major difference is observed for the copolymers with 600 g/mole PEG segments. Hardly any mass loss could be measured for the 45 mol% succinate substituted copolymer, while the 100 mol% substituted copolymer completely disappeared within 16 weeks, similar to the 1000 g/mole PEG segments containing copolymer. Apparently, for the 100% substituted copolymers containing 600 g/mole and 1000 g/mole PEG segments have the optimal combination of swelling and hard block length. This effect is quantified in Figure 6C, showing the mass loss rate as function of the PEG segment length. The mass loss rate was calculated from the slopes of the mass loss curves shown in Fig. 5B and Fig. 6B.

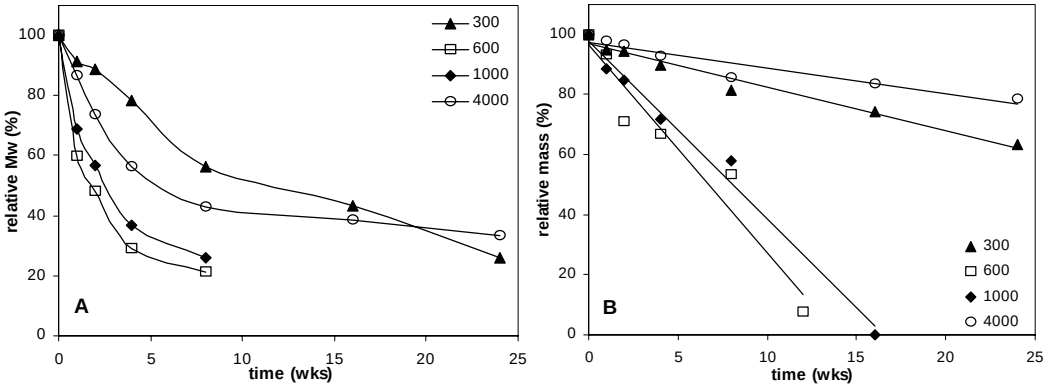


Figure 6: Relative weight average molecular weight (A) and mass (B) of residue as function of degradation time for PEGS65PBS35 copolymers (100% succinate substitution) with PEG segments of 300 (▲), 600 (□), 1000 (◆) and 4000 (○) g/mole.

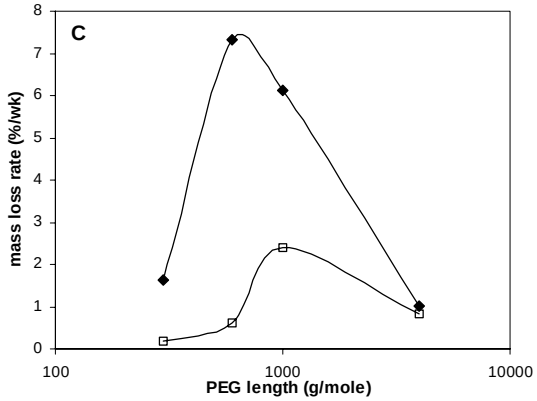


Figure 6C: Mass loss rate versus PEG segment length for copolymer containing 45 mol% (□) and 100 mol% (◆) succinate (65 wt% soft segment).

Surprisingly, different degradation behaviour has been reported for comparable polymer systems based on PEG and PLGA [31] and PEG and PBS [19]. For copolymers based on PEG and PLGA, the mass loss rate increased by increasing PEG segment length from 150 to 8000 g/mole at a constant wt% PEG. Due to the higher hydrophilicity of the longer PEG segments, relatively high molecular weight PLGA segments could be brought in solution by attached PEG segments [31]. Copolymers based on PEG (40 wt%) and PBS showed the opposite behaviour after incubation for 7 days in phosphate buffered saline in the presence of lipase. Increasing the PEG segments from 200 to 2000 g/mole, decreased the mass loss rate greatly, in spite of a remarkable increase in swelling. It was concluded that the hydrolytic degradation of the PEG/PBS copolymers was influenced by

the concentration of degradable ester linkages between PEG and succinic acid, rather than by the swelling [19]. Therefore, the optimum PEG molecular weight for the mass loss rate in our system may be due to the combination of both effects.

The in-vitro study focused on the degradation by hydrolysis of the ester bonds and subsequent dissolution of the polymer fragments. However, in the body, degradation by oxidation of the ether bonds due to the presence of ions or radicals will also play a part [2]. Therefore, in future experiments, degradation will be studied in-vivo. In addition, the biocompatibility of these copolymers should be evaluated.

In-vitro release of proteins

Model proteins of three different sizes were incorporated in PEG(T/S)PB(T/S) matrices. The in-vitro release of lysozyme (14.5 kDa), carbonic anhydrase (29 kDa) and BSA (67 kDa) from films of 1000PEG(T/S)66PB(T/S)34 containing 45 mol% succinate are given in Figure 7.

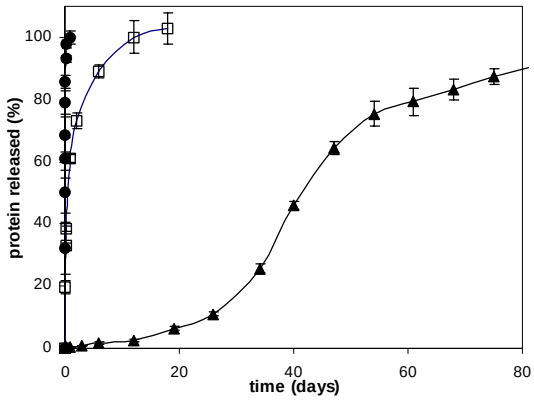


Figure 7: Cumulative release of lysozyme (●), carbonic anhydrase (□) and BSA (▲) from films of 1000PEG(T/S)66PB(T/S)34 containing 45 mol% succinate (n=3; ± s.d.).

Both the release rate and the release profile were dependent on the size of the protein. The release rate decreased with increasing protein size. The lysozyme release was complete within 5 hours, carbonic anhydrase was released in 20 days, whereas over 80 days were required for the complete release of BSA. Plots of released amounts of lysozyme and carbonic anhydrase versus square root of time showed a linear relationship, indicating first order release due to diffusion [37]. The fast diffusion of lysozyme through the matrix indicated that its hydrodynamic diameter is smaller than the mesh size of the

polymer matrix [29,38,39]. Being twice the molecular weight of lysozyme, the release of carbonic anhydrase was slower, although the size of the meshes is still large enough to allow diffusion. The hydrodynamic diameter of BSA is too large to pass the meshes in the matrix, therefore no initial release was determined. However, during the release experiment, the matrix was degraded by hydrolyses (Fig. 4A). After 30 days, the molecular weight of the matrix was decreased to such an extent that the mesh size of the matrix had become sufficiently large to allow the diffusion of the BSA molecules. Further degradation of the matrix increased the release rate. The delayed release profile was determined by this combination of matrix degradation and BSA diffusion. A similar release profile of BSA was observed from triblock copolymers based on poly(ortho ester)s and PEG (POE-PEG-POE) [40] microspheres and PEGT/PBT [41] matrices. In conclusion, these preliminary release experiments showed that PEG(T/S)PB(T/S) copolymers can be used as matrix for the release of proteins. Depending on the size of the protein, the release is controlled by diffusion due to the swelling in water and the degradation of the matrix. Only one copolymer composition was evaluated in this study. However, varying the copolymer composition increases the release possibilities as will be shown in future publications.

Conclusion

Multiblock poly(ether-ester)s based on polyethylene glycol, butylene terephthalate and butylene succinate units were synthesized by a two-step polycondensation reaction. The properties of the copolymers, such as crystallinity and swelling, could be tailored by varying the PEG segment length and the ratio of the three building units. A minimum in crystallinity was observed for poly(ether-ester)s containing 50 mol% of terephthalate and 50 mol% of succinate in the hard segments. The swelling in water increased with increasing mol fraction succinate and increasing PEG segment length. Substitution of the aromatic terephthalate units by aliphatic succinate units accelerated the degradation rate of the copolymers. Mass loss and molecular weight analyses showed a more pronounced in-vitro degradation for polymers with 1000 g/mole PEG segments than copolymers containing shorter and longer PEG segments. Model proteins were successfully

incorporated and released from the poly(ether-ester) films. Depending on the size of the protein, a first order release profile (for small proteins, due to diffusion) or a delayed release profile (for large proteins, due to degradation) was observed.

Future studies will focus on the in-vivo degradation and biocompatibility of these PEG(T/S)PB(T/S) copolymers. Furthermore, the release properties of the poly(ether-ester)s will be characterized in more detail in order to develop controlled release systems for pharmaceutically relevant proteins.

References

1. Kumar N, Ravikumar MNV, Domb AJ. Biodegradable block copolymers. Adv Drug Del Rev 2001;53:23-44.
2. Goedemoed JH, Hennink WH, Bezemer JM, Feijen J, Van Blitterswijk CA, De Bruijn JD. Polyetherester copolymers as drug delivery matrices. European Patent Application 97202533.2, 1998.
3. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J Control Rel 2000;64(1-3):179-192.
4. Van Dijkhuizen-Radersma R, Péters FLAMA, Stienstra NA, Grijpma DW, Feijen J, de Groot K, Bezemer JM. Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers. Biomaterials 2002;23:1527-1536.
5. Reed AM, Gilding DK. Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:2 In vitro degradation. Polymer 1981;22:499-504.
6. Deschamps AA, Claase MB, Sleijster MWJ, De Bruijn JD, Grijpma DW, Feijen J. Design of segmented poly(ether ester) materials and structures for tissue engineering of bone. J Control Rel 2002;78(1-3):175-186.
7. Van Dijkhuizen-Radersma R, Hesseling SC, Kaim PE, De Groot K, Bezemer JM. Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation. Biomaterials 2002;23: 4719-4729.
8. Tokiwa Y, Suzuki T. Hydrolysis of copolyesters containing aromatic and aliphatic ester blocks by lipase. J Appl Polym Sci 1981;26:441-448.
9. Heidary S, Gordon III B. Hydrolyzable Poly(ethylene terephthalate). J Environ Polym Degrad 1994;2:19-26.
10. Müller RJ, Kleeberg I, Deckwer WD. Biodegradation of polyesters containing aromatic constituents. J Biotech 2001;86(2):87-95.
11. Ash M, Ash I, editors: Handbook of Pharmaceutical Additives, second edition. Synapse Information Resources, Inc, 2002. 731-732.

12. Schroff RN. Single-point determination of intrinsic viscosity. J Appl Polym Sci 1962;VI:683-686.
13. Nikolic M, Djonlagic J. Synthesis and characterization of biodegradable poly(butylene succinate-co-butylene adipate)s. Polym Degrad Stab 2001;74:263-270.
14. Fakirov S, Gogeva T. Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 1. Poly(ether/ester)s with various polyester: polyether ratios. Makromol Chem 1990;191:603-614.
15. Ki HC, Park OO. Synthesis, characterization and biodegradability of the biodegradable aliphatic-aromatic random copolyesters. Polymer 2001;42(5):1849-1861.
16. Park YH, Cho CG. Synthesis and characterization of poly[(butylene succinate)-co-(butylene terephthalate)]-b-poly(tetramethylene glycol) segmented block copolymer. J Appl Polym Sci 2001;79:2067–2075.
17. Deschamps AA, Grijpma DW, Feijen J. Poly(ethylene oxide)/poly(butylene terephthalate) segmented block copolymers: the effect of copolymer composition on physical properties and degradation behavior. Polymer 2001;42(23):9335-9345.
18. Kim YJ, Park OO. Miscibility and biodegradability of poly(butylene succinate)/poly(butylene terephthalate) blends. J Environ Polym Degrad 1999;7(1):53-66.
19. Nagata M, Kiyotsukuri T, Takeuchi S, Tsutsumi N, Sakai W. Hydrolytic degradation of aliphatic polyesters copolymerized with poly(ethylene glycol)s. Polym Int 1997;42:33-38.
20. Wang M, Zhang L, Ma D. Degree of microphase separation in segmented copolymers based on (poly(ethylene oxide) and poly(ethylene terephthalate). Eur Polym J. 1999;35:1335-1343.
21. Hiemenz PC. Polymer Chemistry. New York: Marcel Dekker; 1984. p 204-219.
22. Seretoudi G, Bikiaris D, Panayiotou C. Synthesis, characterization and biodegradability of poly(ethylene succinate)/poly(ε-caprolactone) block copolymers. Polymer 2002;43:5405-5415.
23. Montaudo G; Rizzarelli P. Synthesis and enzymatic degradation of aliphatic copolyesters. Polym Degrad Stab 2000;70:305-314.
24. Nagata M. Synthesis and enzymatic degradation of poly(tetramethylene succinate) copolymers with terephtalic acid. Polymer 2000;41:4373-4376.
25. Fakirov S, Gogeva T. Poly(ether/ester)s based on poly(butylene terephthalate) and poly(ethylene glycol), 2. Effect of polyether length. Macromol Chem 1990;191:615-624.
26. Cohn D, Younes H. Biodegradable PEO/PLA block copolymers. J Biomed Mater Res 1988;22:993-1009.
27. Albertsson A, Ljungquist O. Degradable Polymers II. Synthesis, Characterisation, and Degradation of an Aliphatic Thermoplastic Block Copolyester. J Macromol Sci Chem 1986;A23(3): 411-422.
28. Pitt CG. Non-microbial degradation of polyesters: mechanisms and modifications. In: Vert M, Feijen F, Albertsson A, Scott G, Chiellini, editors. Biodegradable polymers and plastics. Melksham: Redwood Press Ltd; 1992. p7-17.
29. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. A controlled release system for proteins based on poly(ether ester) block-copolymers: polymer network characterization. J Control Rel 1999;62(3):393-405.
30. Gilding DK, Reed AM. Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:1. Polymer 1979;20:1454-1458.

31. Penco M, Marcioni S, Ferruti P, Dántone S, Deghenghi R. Degradation behaviour of block copolymers containing poly(lactic-glycolic acid) and poly(ethylene glycol) segments. Biomaterials 1996;17:1583-1590.
32. Bezemer JM, Oude Weme P, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. Amphiphilic poly(ether ester amide) multiblock copolymers as biodegradable matrices for the controlled release of proteins. J Biomed Mater Res 2000;52(1):8-17.
33. Göpferich A. Mechanisms of polymer degradation and erosion. Biomaterials1986;17:103-114.
34. Cho K, Lee J, Kwon K. Hydrolytic degradation behavior of poly(butylene succinate)s with different crystalline morphologies. J Appl Polym Sci 2001;79(6):1025-1033.
35. Budavari S, O'Neil MJ, Smith A, Heckelman PE, Kinneary JF, editors. The Merck Index, twelfth edition. Whitehouse Station, NJ, Merck & Co; 1996. 9038, succinic acid, 9306 terephthalic acid.
36. Kellomäki M, Paasimaa S, Grijpma DW, Kolppo K, Törmälä P. In vitro degradation of Polyactive® 1000PEOT70PBT30 devices. Biomaterials 2002;23(1):283-295.
37. Crank J: The mathematics of diffusion, second edition. Oxford University Press, New York, NY, 1975
38. Hennink WE, Franssen O, Van Dijk-Wolthuis WNE, Talsma H. Dextran hydrogels for the controlled release of proteinsJ Control Rel 1997;48:107-114.
39. De Jong S, Van Eerdenburgh B, Van Nostrum CF, Kettenes-van den Bosch JJ, Hennink WE. Physically crosslinked dextran hydrogels by stereocomplex formation of lactic acid oligomers: degradation and protein release behavior. J Control Rel 2001;71:261-275.
40. Wan JP, Yang YY, Chung TS, Tan D, Ng S, Heller J. PEO-PEG-POE triblock copolymeric microspheres containing protein II. Polymer erosion and protein release mechanism. J Control Rel 2001;75:129-141.
41. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. Control of protein delivery from amphiphilic poly(ether ester) multiblock copolymers by varying their water content using emulsification techniques. J Control Rel 2000;66(2-3):307-20.

Chapter 9

Succinate substituted poly(ethylene glycol)/ poly(butylene terephthalate) copolymers: an in-vivo evaluation

R. van Dijkhuizen-Radersma, J.R. Roosma, J. Sohier, F.L.A.M.A. Péters, M. van den Doel, C.A.van Blitterswijk, K. de Groot, J.M. Bezemer.

Abstract

Multiblock poly(ether-ester)s based on poly(ethylene glycol), butylene terephthalate and butylene succinate segments were evaluated for their in-vivo degradation and biocompatibility in order to establish a correlation with the previously reported in-vitro results. Porous polymer sheets were implanted subcutaneously for 32 weeks in rats. The degradation was monitored visually (histology), by molecular weight (GPC) and by copolymer composition (NMR). Substitution of the aromatic terephthalate units by aliphatic succinate units was shown to accelerate the degradation rate of the copolymers. Direct correlation of the in-vivo and in-vitro degradation of the porous implants showed a slightly faster initial molecular weight decrease in-vivo. Besides hydrolysis, probably also oxidation occurs in-vivo due to the presence of radicals, produced by inflammatory cells. In addition, the higher molecular weight plateau of the residue found in-vivo indicated a higher solubility of the oligomers in the extracellular fluid compared to a phosphate buffer. Minor changes in the poly(ether-ester) compositions were noted due to degradation. Microscopically, fragmentation of the porous implants was observed in time. At later stages of degradation, macrophages were observed phagocytozing small polymer particles. Both in-vitro cytotoxicity studies and histology on in-vivo samples proved the biocompatibility of the poly(ether-ester)s.

Introduction

Recently, multiblock poly(ether-ester)s based on poly(ethylene glycol), butylene terephthalate and butylene succinate segments have been designed as a new series of degradable polymers for controlled release applications [1]. These poly(ether-ester)s are a modification of poly(ethylene glycol) terephthalate (PEGT) / poly(butylene terephthalate) (PBT) copolymers, which have been successfully applied as matrix in controlled release systems [2-5]. However, for controlled release application requiring frequently repeated injections, the degradation rate of some PEGT/PBT copolymer compositions might be too slow [6]. Substitution of the aromatic terephthalate units by aliphatic succinate units was shown to increase the degradation rate of the copolymers in-vitro [1]. However, as reported for several polymer systems, the in-vitro degradation can differ considerably from the in-vivo degradation. In most cases the in-vivo degradation is faster than the in-vitro degradation [7-13]. This is attributed to the presence of certain ions or radicals, produced by inflammatory cells [12,14], enzymes [15] and lipids [9] in the body fluid, which may affect the chain cleavage mechanism as well as the dissolution of the oligomers [16]. For poly(lactide-co-glycolide) (PLGA) in particular, the in-vivo degradation is autocatalysed due to accumulation of acidic degradation products [10,16]. In addition, the extension of both the tissue response and the autocatalytic effect can be affected by the size [16-19] and the shape [20] of the implant. On the other hand, for hydrogel-like polymer systems a slower in-vivo degradation has been reported compared to the in-vitro degradation in phosphate buffer [21]. The presence of less fluid near the implant site retards swelling and subsequently inhibits degradation. For copolymers only degrading by hydrolyses in the bulk, the in-vitro degradation will mimic the in-vivo degradation [22-23].

In this paper, we evaluate the in-vivo degradation of the novel poly(ether-ester) copolymers in rats and compare it to the in-vitro degradation. Both the molecular weight and the copolymer composition were monitored. In order to create a large surface area, porous sheets were implanted subcutaneously. Prior to implantation the cytotoxicity of the poly(ether-ester)s was evaluated.

Materials and methods

Materials

The poly(ethylene glycol) (terephthalate/succinate) poly(butylene (terephthalate/ succinate)) (PEG(T/S)PB(T/S)) copolymers were synthesized at Chienna BV (Bilthoven, The Netherlands). The PEG(T/S)PB(T/S) copolymers varied in PEG segment length (600, 1000 and 4000 g/mole), PEG(T/S)/PB(T/S) weight ratio (62/38 – 71/29) and T/S molar ratio (0/100 – 100/0) (composition determined by NMR). The poly(ether-ester)s are indicated as aPEG(T/S)bPB(T/S)c (T/S=d/e), in which a is the PEG molecular weight, b the combined weight percentage PEGT and PEGS and c, the combined weight percentage of PBT and PBS. d/e is the molar T/S ratio in the whole polymer. Chloroform (analytical grade) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Merck (Darmstadt, Germany) was the supplier of sodium chloride (NaCl) . Phosphate buffered saline, (pH 7.4) was obtained from Life Technologies Ltd (Paisley, Scotland).

Preparation of porous PEG(T/S)PB(T/S) implants

For the in-vivo study, porous polymer implants were prepared using a solvent casting / salt leaching procedure. Three gram of PEG(T/S)PB(T/S) copolymer was dissolved in 15 ml chloroform (13.5 wt%). Sodium chloride with a grain size of 212 to 250 μm was added using a NaCl to copolymer weight ratio of 7.5:1. The mixture was homogenized and cast on a glass plate using a casting knife (setting 2000 μm). After controlled evaporation of the chloroform, the resulting film was removed from the glass plate. Extensive washings in demineralised water leached the NaCl out. The porous film was dried on the air at room temperature and subsequently under vacuum at 50^{o}C for 16 hours. Pieces of the porous films (1.6 mm in diameter, 600 – 800 μm in thickness) were packed under vacuum in aluminum pouches and sterilized by gamma irradiation. A minimum irradiation dose of 25 kGy was applied in a JS6500 Tote Box Irratiator at Gammaster B.V. (Ede, The Netherlands). To study the effect of the copolymer composition on the biocompatibility and in-vivo degradation, a series of PEG(T/S)PB(T/S) copolymers were used varying in PEG segment length (600 – 4000 g/mole) and the terephthalate / succinate ratio (100/0 – 0/100 mol%). The amount of soft segment (PEG(T/S)) was more or less constant at 66 wt% (62 - 71 wt%). As it was not

possible to obtain porous films from the 600PEGS71PBS29, grinded powder (< 600 μm) of this copolymer was used in the in-vivo study.

Implant characterization

The morphology of the implants was evaluated prior to implantation by using scanning electron microscopy (SEM). A Philips XL 30 Environmental Scanning Electron Microscope was used to study the surface characteristics of the implants after coating the samples with a thin gold layer.

To study the effect of the sterilization process, the molecular weight of the porous implants before and after gamma irradiation was determined using Gel Permeation Chromatography (GPC). Samples were eluted in 0.02M sodiumtrifluoroacetate (NaTFA) in hexafluoroisopropanol (HFIP) through a Polymer Labs HFIP gel guard column (50 x 7.5 mm) and two PL HFIP gel analytical columns (300 x 7.5 mm). Flow rate was 1 ml/min and both an Ultraviolet (UV, 254 nm) and a Refraction Index (RI) detector were used. Column temperature was 40ºC and sample concentration was 0.3 mg/ml (50 μl injection). The weight average molecular weights (M_w) and the polydispersity (M_w/M_n) were determined relative to polymethylmethacrylate (PMMA) standards.

Cytotoxicity

The cytotoxicity of the poly(ether-ester)s towards the growth, morphology and metabolism of fibroblasts was evaluated according to EN/ISO 10993-5. Powder of respectively 1000PEGS72PBS28 and 1000PEG(T/S)73PB(T/S)27 (T/S=55/45) was extracted at 37^{o}C for 24 hours in medium. The medium consisted of minimum essential medium (MEM) supplemented with 10% fetal calf serum. Natural rubber was extracted identically for a positive control. As a negative control material, UHMW polyethylene was used. The extracts were added to cultures of mouse lung fibroblasts (L929) and incubated for 48 hours at 37^{o}C. The biological reactivity (cellular degeneration and malformation) was evaluated under a microscope.

Implantation study

Twenty-four wistar rats (300 g, GDL, Utrecht, The Netherlands) were anesthetized by an intramuscular injection of a mixture containing atropine (0.5 ml, 0.5 mg/ml), xylazine (1.5 ml, 20 mg/ml) and ketamine (1.75 ml, 100 μg/ml). The surgical sites were shaved and cleaned with 70% ethanol and iodine. Subcutaneous pockets (8 per animal) were created along the dorso-medial line, in which the samples were inserted. At 2, 8, 16 and 32 weeks after implantation, six rats were sacrificed by CO_2. The implants (n = 6 per condition) were removed with excess surrounding tissue for evaluation. The animal experiment in this study was performed according to the legal guidelines concerning animal welfare ISO 10993 part 2, 1997, European Directive 86/609/EEC and to the Dutch Laboratory Animal Act.

Characterization in-vivo samples

After explantation, the samples remained for 1 day in Karnovsky's fixative. For histological analyses, pieces of the samples were dehydrated in graded series of ethanol and embedded in glycol methacrylate. Subsequently, the samples were sawed using a microtome and stained with hemotoxylin and eosin. The slides were examined under a light microscope (Nikon Eclipse E400) and evaluated for the tissue response.

For the in-vivo degradation, the fixed samples were washed with demineralised water. To remove the tissue, the samples were dissolved in chloroform and filtered by using a 0.45 μm filter on a syringe. After evaporation of the solvent the remaining polymer film was analyzed for molecular weight and copolymer composition. The molecular weight as function of degradation time was determined using GPC as described above. The effect of the in-vitro degradation on the composition of the copolymers was studied by NMR measurements. In order to remove interfering tissue residues, the polymer samples were first precipitated in hexane. Subsequently, proton NMR spectra were recorded on a Bruker ARX-400 operating at ≥ 200 MHZ. $C_2D_2Cl_4$ was used as solvent without internal standard. The copolymer composition was calculated as described elsewhere [1]

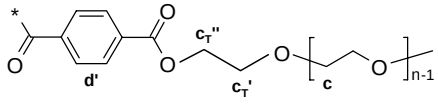
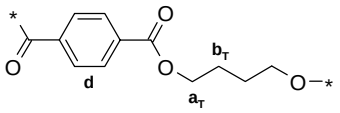
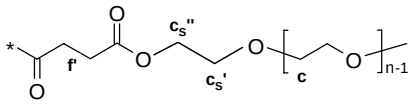
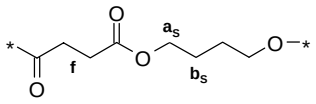
In-vitro degradation

To evaluate the in-vitro degradation, sterilized porous sheets and powder (identical to in-vivo study) were immersed in 50 ml phosphate buffered saline at 37°C in a shaking bath for several time periods. The buffer was refreshed every 2 weeks. After pre-selected degradation periods, parts of the samples were washed with purified water and dried using a vacuum oven at 50°C for at least 16 hours. The in-vitro degradation was monitored by analyzing the changes in molecular weights (GPC) and changes in copolymer composition (NMR) as described for the in-vivo samples.

Results and discussion

The poly(ether-ester) copolymers used in this study can be described as being built up of four different repeating segments (Table 1). Poly(ethylene glycol) terephthalate (PEGT) and poly(ethylene glycol) succinate (PEGS) form the soft hydrophilic blocks, whereas the hard hydrophobic blocks contain butylene terephthalate (BT) and butylene succinate(BS) [1].

Table 1: Segments present in the poly(ether ester) copolymers.

Formula	Name	Abbreviation
	Poly(ethylene glycol) terephthalate	PEGT
	Butylene terephthalate	BT
	Poly(ethylene glycol) succinate	PEGS
	Butylene succinate	BS

Implant characterization

Porous poly(ether-ester) implants were prepared using a solvent casting / salt leaching procedure. Figure 1 shows a cross section of an porous implant of 1000PEG(T/S)66PB(T/S)34 containing 45 mol% succinate in the hard segment.

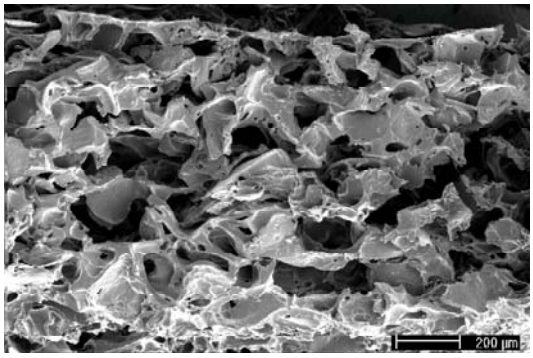


Figure 1: Scanning electron micrograph of porous 1000PEG(T/S)66PB(T/S)34 implant containing 45 mol% succinate in the hard segment.

The weight average molecular weights of the implants prior to and after gamma irradiation as determined by GPC analyses are given in Table 2. The M_w prior to γ irradiation is in the range of 105 - 128 kg/mole. After γ irradiation, the weight average molecular weights of all copolymer compositions increased (15% on average). A similar increase in molecular weight was observed before for sterilized PEGT/PBT microspheres [6].

Table 2: Weight average molecular weight and polydispersity (GPC) of the poly(ether-ester)s implants prior to and after sterilisation as function of the copolymer composition (NMR).

Polymer composition	mol% succinate	M_w (10^{-3} g/mole)	M_w/M_n	M_w (10^{-3} g/mole)	M_w/M_n
		prior to γ irradiation	prior to γ irradiation	after γ irradiation	after γ irradiation
600PEG(T/S)62PB(T/S)38	46	128	2.0	164	2.8
600PEGS71PBS29	100	123	2.5	160	2.8
1000PEG(T/S)62PB(T/S)38	11	105	2.2	127	2.4
1000PEG(T/S)66PB(T/S)34	45	111	2.2	147	2.7
1000PEGS69PBS31	100	121	2.0	123	2.2
1000PEGT71PBT29	0	107	1.7	118	2.1
4000PEG(T/S)64PB(T/S)36	46	113	2.1	137	2.6
4000PEGS66PBS34	100	124	2.1	146	2.5

n.d.: not determined

This was attributed to crosslinking due to recombination of radicals formed by γ irradiation. On the other hand, other polymer systems, like PLGA, showed a decrease in molecular weight after sterilization, as a result of chain scission through radical formation [24]. For the PEG(T/S)/PB(T/S) copolymers, the polydispersity increased from ± 2.1 to ± 2.5 by γ irradation (Table 2). This result suggests the occurrence of both chain cleavage and (partial) crosslinking in these poly(ether-ester)s.

Prior to implantation, the cytotoxicity of the poly(ether-ester)s towards the growth, morphology and metabolism of fibroblasts was evaluated. For these experiments, the fastest degrading copolymer compositions were selected and a high surface area was created by using milled powder. Table 3 shows the results for the poly(ether-ester) samples and the positive and negative control. No biological reactivity was observed in the L929 mammalian cells at 48 hours, post exposure to the polymer extracts. The observed cellular response obtained from the positive control extract and the negative control extract confirmed the suitability of the test system. It was therefore concluded that the poly(ether-ester) copolymers studied were non-cytotoxic.

Table 3: Cellular reactivity in cytotoxicity tests as function of exposure time.

Sample	Reactivity 24 hrs post-exposure	Reactivity 48 hrs post-exposure
Negative control	No cell lysis	No cell lysis
1000PEG(T/S)73PB(T/S)27 (T/S 55/45)	No cell lysis	No cell lysis
1000PEGS72PBS28	No cell lysis	No cell lysis
Positive control	Nearly complete destruction of the cell layers	Nearly complete destruction of the cell layers

Histological evaluation

The effect of the copolymer composition on the in-vivo degradation and the tissue response was examined visually by light microscopy. Figure 2 shows the porous sheets after 2 weeks and 15 weeks of implantation with increasing succinate content at a constant PEG segment length (1000 g/mole) and soft segment amount (65 wt%). The higher the degree of succinate substitution in the polymer, the faster the implant fell apart into small polymer fragments. The size of the polymer fragments decreased in time due to degradation. In the absence of succinate (Fig 2A&B), large polymer fragments are visible after 2 weeks of implantation with limited tissue ingrowth, while after 15 weeks the tissue

ingrowth was complete around the polymer fragments. The implants containing 45 mol% of succinate in the hard segment (Fig 2C&D) also showed partial tissue ingrowth after 2 weeks without capsule formation, whereas after 15 weeks a fibrous capsule was present. The size of polymer fragments of this polymer composition decreased significantly faster in time compared to the copolymer without succinate. In addition, the resulting particles of the partly substituted copolymer were less angular. The implant of the 100% succinate substituted poly (ether-ester) (Fig 2E-G) degraded already in 2 weeks into small particles. The formation of granulation tissue as scaffold for tissue repair was initiated, as expressed by the fibroblast infiltration and the development of blood capillaries [25]. After 15 weeks of implantation, a higher magnification was required to identify the polymer fragments (Fig. 2G). Macrophages started to phagocytose fragments around 10 μm, without the formation of foreign body giant cells, which is in agreement with observation by others [26,27]. All poly(ether-ester) compositions evaluated in this study showed only very mild chronic inflammatory responses followed by a normal foreign body response. In general, the tissue response followed the sequence of local events as described by Anderson et al. [28]. Signs of bioincompatibility, like tissue necrosis and changes in tissue morphology, were not observed. From these results, it was concluded that the poly(ether-ester) implants were well tolerated by the surrounding subcutaneous tissue.

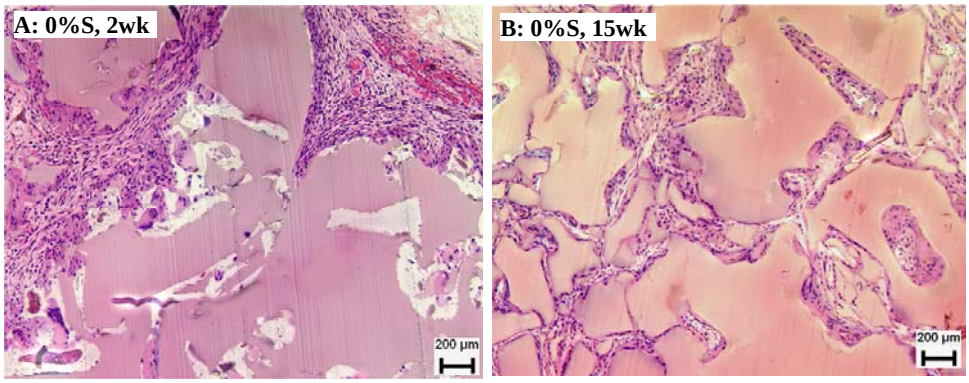
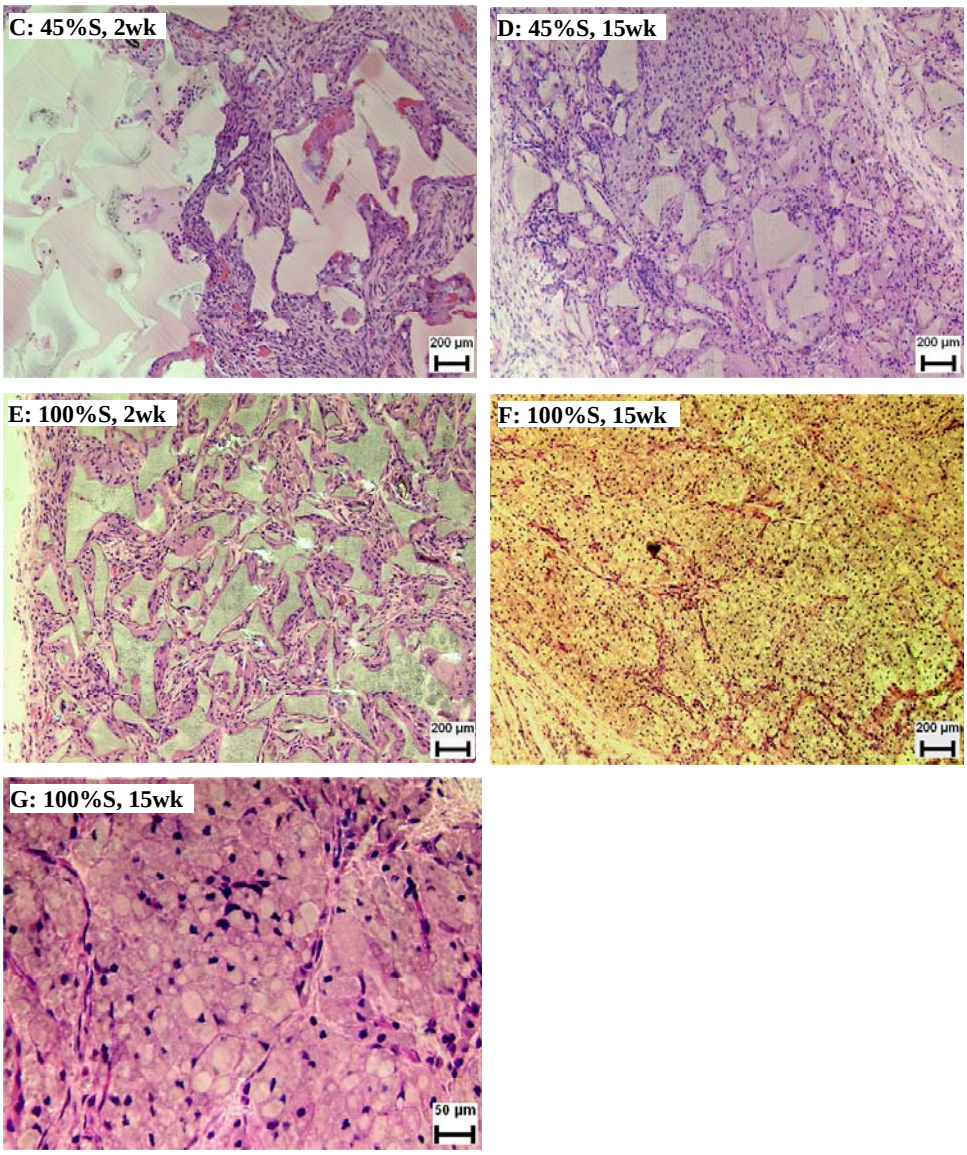


Figure 2: Histology of 1000PEG(T/S)65PB(T/S)35 porous implants containing 0 mol% (A,B), 45 mol% (C,D) and 100 mol% (E,F) of succinate in the hard segment after 2 (A,C,E) and 15 weeks of implantation (B,D,F). Original magnification: 100x. Figure 2G is similar to 2F at a higher magnification (400x). Symbol 'P' indicates the polymer material.



In-vivo and in-vitro degradation

The in-vitro and in-vivo degradation were evaluated in time by monitoring both molecular weight and copolymer composition by GPC and NMR respectively. The animal-to-animal variation was less than 5% r.s.d., calculated from molecular weights of six samples per polymer composition per condition, which is comparable to observations by others [7].

Molecular weight

Figures 3 and 4 show the molecular weight as function of implantation time for poly(ether-ester)s varying in succinate/terephthalate ratio and PEG segment length. The degradation profile -an exponential molecular weight decrease resulting in a plateau value- is independent on the polymer composition. The plateau value reached after 8 to 15 weeks does not mean that degradation has stopped. Further degradation results in soluble low molecular weight oligomers, which are removed from the implantation site, resulting in a more or less constant molecular weight of the residue.

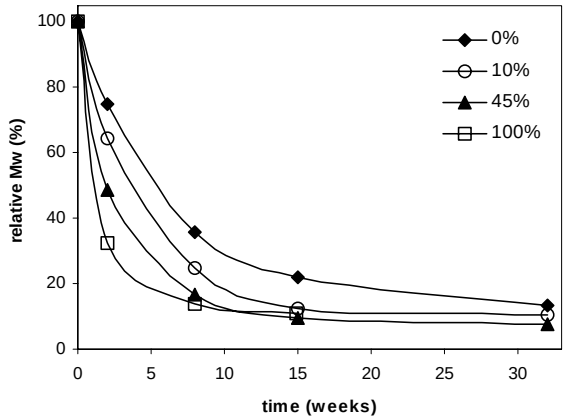


Figure 3: Relative weight average molecular weight of residue as function of degradation time in-vivo for 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (◆), 10 (○), 45 (▲) and 100 (□) mol% succinate.

In agreement to previous reported in-vitro experiments [1] and the histology results described above, the degree of substitution of the aromatic groups by aliphatic groups showed a clear effect on the in-vivo degradation. At a constant soft/hard ratio and PEG segment length, the decrease in molecular weight was faster for poly(ether-ester)s with increasing succinate content (Fig. 3). The effect of the PEG segment length on the degradation is shown in Fig 4. For copolymers containing 45 mol% of succinate in the hard segment, the fastest decrease in molecular weight was observed for the copolymers with PEG segments of 1000 g/mole and 600 g/mole, respectively (Fig. 4A). For the copolymer containing 100 mol% of succinate in the hard segment, the fastest degradation was observed for the poly(ether-ester) with 600 g/mole PEG segments, followed by poly(ether-ester)s containing PEG segments of 1000 g/mole and 4000 g/mole (Fig. 4B).

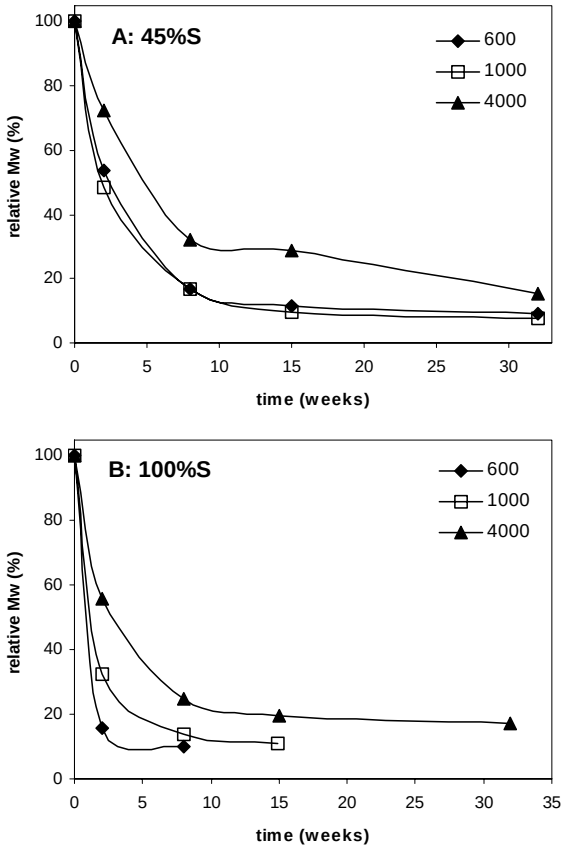


Figure 4: Relative weight average molecular weight of residue as function of degradation time in-vivo for PEG(T/S)65PB(T/S)35 copolymers containing 45 mol% (A) and 100 mol% (B)of succinate with PEG segments of 600 (◆), 1000 (□) and 4000 (▲) g/mole.

Degradation of the polymer and subsequent dissolution of the oligomers is based on a combination of swelling [29-31] and average block length of the copolymer's hard segment [29,32]. A higher swelling makes the ester bonds more accessible for hydrolysis [33,34]. Longer average block lengths of the hard PB(T/S) segments reduces the number of easy accessible hydrolysable ether-ester bonds. At a constant soft/hard ratio and PEG segment length, the swelling of the poly(ether-ester)s increases with increasing amount of succinate [1]. Therefore, the faster degradation observed poly(ether-ester)s with higher aliphatic succinate substitution can be explained by the increase in swelling (Fig. 3). Besides the higher accessibility of the ester bonds due to the higher swelling, the aliphatic esters are more susceptible to hydrolysis than aromatic ester, as a result of steric effects

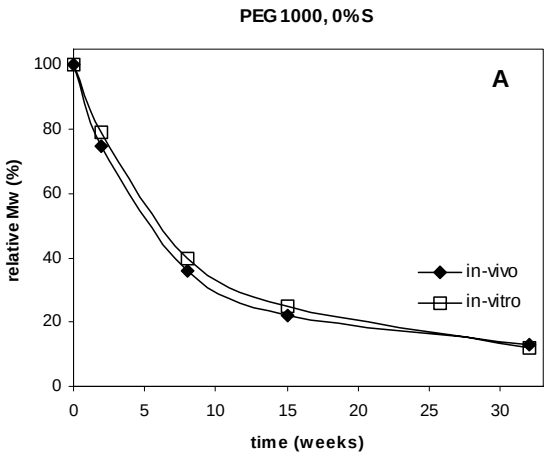
[30,31] and flexibility of the chain backbone [32,35]. The order of degradation rates for copolymers containing various PEG segment lengths is explained by both the swelling and the average block length (Fig. 4). Based on the swelling, the highest degradation rate would be expected for the 4000 g/mole PEG segment containing poly(ether-ester)s as, at a constant amount of succinate, the swelling of the poly(ether-ester) increases with increasing PEG segment length [1]. On the other hand, increasing the molecular weight of the PEG-segments at a constant soft/hard ratio will increase the average block length of the hard PB(T/S) segments, resulting in a slower degradation. Apparently, the combination of swelling and hard block length of copolymers with 600 g/mole and 1000 g/mole PEG segments favours degradation compared to copolymers containing 4000 g/mole PEG segments. The order of degradation rates for these copolymers was also observed in-vitro for dense sheets [1], although the in-vivo degradation was faster than expected for the 600 g/mole PEG segment containing copolymer with 100 mol% succinate (Fig 4B). Being implanted as a powder, the difference in shape [20], surface area and size [17,18,36] may increase the sensitivity of the 600PEGS71PBS29 copolymer towards the tissue response.

The relative molecular weights of the porous implants during the in-vitro and in-vivo degradation time are compared in figure 5 for a selection of poly(ether-ester) compositions. Examination of the plots indicated that, for the 1000PEG(T/S)66PB(T/S)34 copolymers, the in-vitro and in-vivo curves were very similar in profile and time-course, independent on the degree of succinate substitution (Fig. 5 A-C). The same high correlation was observed for the 4000 g/mole PEG segment containing copolymers (data not shown). Greater variability was observed between in-vitro and in-vivo curves for the poly(ether-ester)s with 600 g/mole PEG segments (Fig. 5D for 45 mol% succinate). For most compositions studied, only at early stages of degradation ($t \leq 2$ weeks) a significant deviation was observed between the in-vitro and in-vivo curves. The molecular weights of the in-vivo samples were lower than the values for the in-vitro samples. After 8 to 15 weeks of degradation, the molecular weights reached a plateau value. In general, the plateau value of the in-vivo residue was slightly higher than the in-vitro plateau after 32 weeks of degradation.

The lower molecular weights of the in-vivo samples at early stages of degradation indicated a slightly faster initial degradation. The faster in-vivo degradation

can be attributed to the tissue response [12,14]. Acute inflammation, due to the injury of implantation, and the foreign body reaction, due to the presence of the implant, induce the migration of cells, like polymorphonuclear leucocytes and macrophages to the implant site [26,28]. Reactive species, like super oxide and hydrogen peroxide, produced by these inflammatory cells can oxidize the poly(ether-ester)s [37,38]. Therefore, the in-vivo decrease in molecular weight values is not only due to hydrolytic degradation caused by the extracellular fluid, but probably also by the influence of the of the oxygen free radicals and other species generated by the inflammatory cells [14]. In our in-vitro study in phosphate buffered saline, however, only hydrolysis of the ester bonds occurs resulting in a slightly slower degradation compared to the in-vivo degradation. For the 600 g/mole PEG segment containing copolymers a relatively fast in-vivo degradation was observed compared to the in-vitro results. This might be explained by a higher sensitivity towards chain scission by oxidation of poly(ether-ester)s containing short PEG segments as reported by Deschamps et al. [39].

When the plateau value for the molecular weight has been reached, further degradation results in oligomers, which dissolve and diffuse out of the sample. The higher plateau value of the in-vivo residue compared to the in-vitro plateau after 32 weeks of degradation indicates that segments of higher molecular weight are dissolved in body fluids compared to phosphate buffered saline [16].



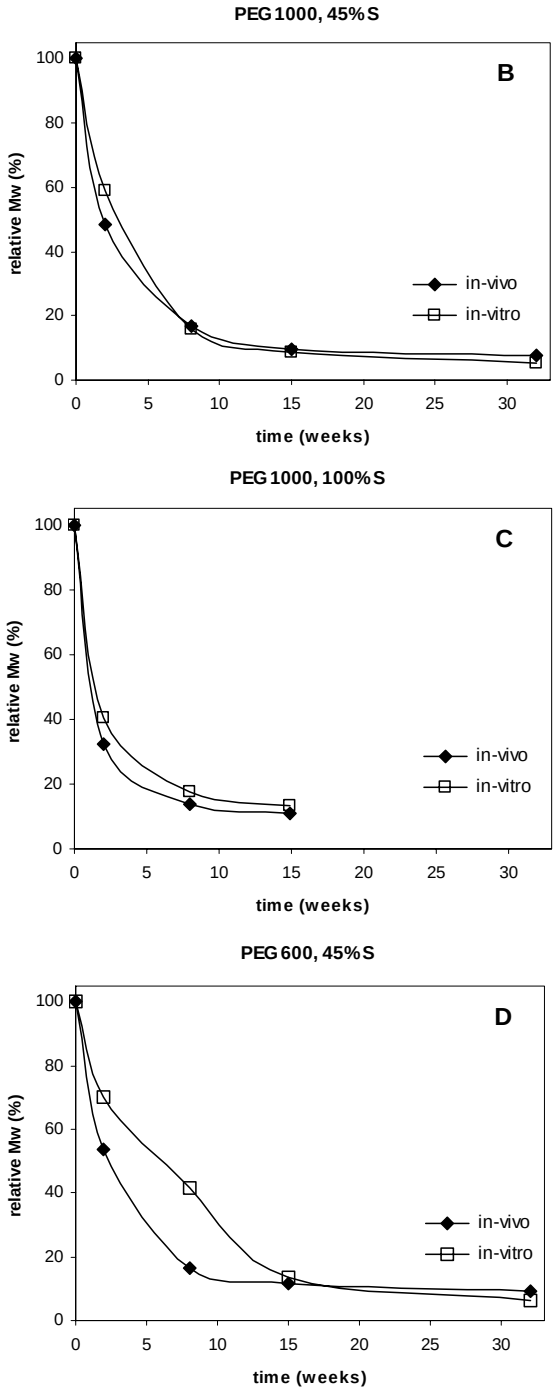


Figure 5: Relative weight average molecular weight of residue as function of in-vitro and in-vivo degradation time for 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (A), 45 (B) and 100 (C) mol% succinate and 600PEG(T/S)62PB(T/S)38 containing 45 mol% succinate (D).

Table 4: Composition of the poly(ether-ester)s implants as function of degradation time.

	t = 0		t = 32 wks in-vitro		t = 32 weeks in-vivo	
PEG length (g/mole)	wt% PEG(T/S)	mol% succinate	wt% PEG(T/S)	mol% succinate	wt% PEG(T/S)	mol% succinate
600	62	46	47	44	43	33
1000	62	11	48	14	48	15
1000	66	45	43	40	44	33
1000	69	100	58*	100*	43*	100*
1000	71	0	61	0	62	0
4000	64	46	45	46	48	44

* t = 15 wks

Copolymer composition

Table 4 gives an overview of the poly(ether-ester) compositions, initially and after 32 weeks of in-vitro and in-vivo degradation. For 1000PEGS67PBS33 no polymer could be retrieved after 32 weeks. The effect of the PEG segment length on the amount of soft segment and the molar percentage of succinate in the hard segment is shown in figure 6 for copolymers with 65 wt% soft segment and 45 mol% succinate as initial composition. Regarding the soft segment content, no difference was observed between the in-vitro and in-vivo results. Independent on the PEG segment length or the degree of succinate substitution, the weight percentage of soft segment decreased significantly from approximately 65 wt% to 45 wt% after 32 weeks (Table 4, Fig. 6). The preferential loss of PEG-rich segments during degradation has also been reported for other polymer systems [33,34,36,40]. This phenomenon was ascribed to the primary cleavage of the ester linkages between the PEG [33,34] and the hard segments and subsequently the higher solubility of the PEG containing oligomers [40,41]. Compared to the 50% decrease of the initial PEG content shown for PEG-PLGA-PEG triblock copolymers [36], however, only a minor decrease in soft segment content was observed in our polymer system. Taking into account the mass loss, as reported elsewhere [1], later stages of the degradation will most likely not result in an inert residue containing only hard segment.

The succinate content in the hard segment decreased slightly during degradation for copolymers initially containing 45 mol% of succinate (Fig. 6B). Succinic acid is soluble in water (77 g/l), whereas terephthalic acid is practically insoluble in water [42]. It is therefore expected that succinate containing degradation products show a higher

solubility and diffusivity, resulting in a lower residual succinate content. This effect was more pronounced in-vivo. Preferential degradation of glycolic acid units observed in PLGA was also ascribed to difference in hydrophilicity of the monomers and was faster in-vivo [16]. As discussed before, the solubility in body fluid is apparently higher than in phosphate buffer. The change in hard segment composition is marginal compared to the mass loss [1] and therefore no hard segment will remain in the residue containing terephthalate groups only.

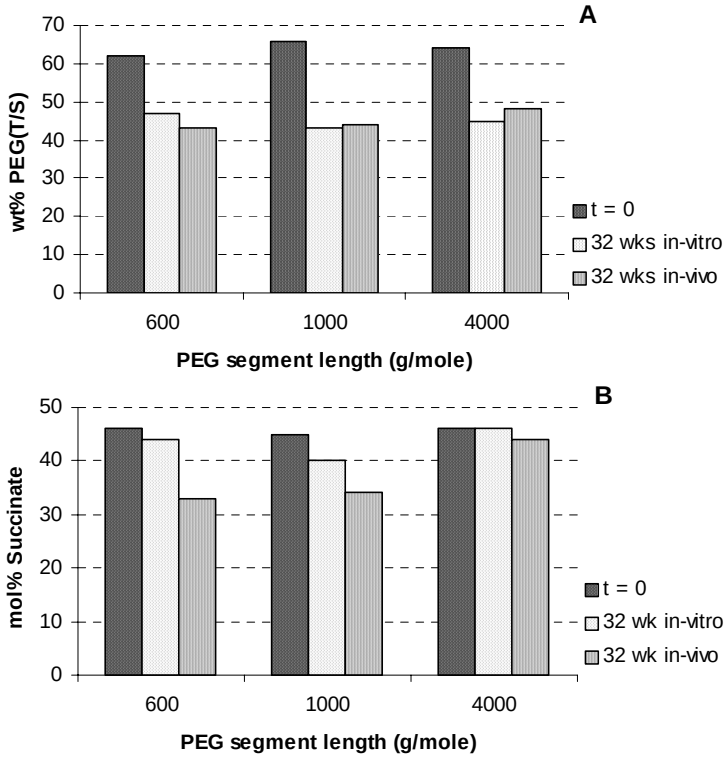


Figure 6: Weight percentage of soft segment (A) and mol percentage of succinate in the hard segment (B) after 32 weeks of degradation in-vitro and in-vivo for copolymers with different PEG segment lengths having PEG(T/S)65PB(T/S)35 and 45 mol% of succinate as initial composition.

Conclusion

The in-vivo degradation, monitored by the molecular weight and copolymer composition, or porous poly(ether-ester) implants confirmed the trends as observed before for in-vitro degradation of dense sheets. Most important, substitution of the aromatic terephthalate units by aliphatic succinate units accelerated the degradation rate of the copolymers. Direct correlation of the in-vivo and in-vitro degradation of the porous implants showed a faster initial degradation in-vivo. Besides hydrolysis, probably also oxidation occurs in-vivo due to the presence of radicals, produced by inflammatory cells. In addition, the higher molecular weight plateau of the residue found in-vivo indicated a higher solubility of the oligomers in the extracellular fluid compared to a phosphate buffer. Minor changes in the poly(ether-ester) compositions were noted due to degradation. The amount of soft segment decreased both in-vitro and in-vivo due to preferential loss of PEG-rich segments. Microscopically, fragmentation of the porous implants was observed in time. At later stages of degradation, macrophages were observed phagocytozing small polymer particles. In addition, both in-vitro cytotoxicity studies and histology on in-vivo samples proved the biocompatibility of the poly(ether-ester)s based on poly(ethylene glycol), butylene terephthalate and butylene succinate segments.

References

1. Van Dijkhuizen-Radersma R, Roosma JR, Kaim P, Métairie S, Péters FLAMA, De Wijn J, Zijlstra PG, De Groot K, Bezemer JM. Biodegradable poly(ether-ester) multiblock copolymers for controlled release applications, J Biomed Mater Res 2003; accepted for publication.
2. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, Feijen J, Van Blitterswijk CA. Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices. J Control Rel 2000;64(1-3):179-192.
3. Bezemer JM, Grijpma DW, Dijkstra PJ, Van Blitterswijk CA, Feijen J. A controlled release system for proteins based on poly(ether ester) block-copolymers: polymer network characterization. J Control Rel 1999;62(3):393-405.
4. Van Dijkhuizen-Radersma R, Péters FLAMA, Stienstra NA, Grijpma DW, Feijen J, de Groot K, Bezemer JM. Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers. Biomaterials 2002;23:1527-1536.

5. Van Dijkhuizen-Radersma R, Wright SJ, Taylor LM, John BA, De Groot K, Bezemer JM. In vitro / in vivo correlation for protein release from poly(ether-ester) microspheres, submitted.
6. Van Dijkhuizen-Radersma R, Hesseling SC, Kaim PE, De Groot K, Bezemer JM. Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation. Biomaterials 2002;23: 4719-4729.
7. Tracy M, Ward KL, Firouzabadian L, Wang Y, Dong N, Qian R, Zhang Y. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in-vitro, Biomaterials 1999;20:1057-1062.
8. Spenlehauer G, Vert M, Benoit JP, Boddaert A. In vitro and in vivo degradation of poly(D,L lactide/glycolide) type microspheres made by solvent evaporation method, Biomaterials 1989;10:557-563.
9. Menei P, Daniel V, Montero-Menei C, Brouillard M, Poulard-Barthelaix A, Benoit JP. Biodegradation and brain tissue reaction to poly(D,L-lactide-co-glycolide) microspheres, Biomaterials 1993;14(6):470-478.
10. Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamade JA, Uyama S, Vacanti JP, Langer R, Mikos AG. In vitro and in vivo degradation of porous poly(DL-lactic-co-glycolic acid) foams, Biomaterials 2000;21:1873-1845.
11. Mainil-Varlet P, Curtis R, Gogolewski S. Effect of in vivo and in vitro degradation on molecular and mechanical properties of various low-molecular-weight polylactides, J Biomed Mater Res 1997;36:360-380.
12. Stoll GH, Nimmerfall F, Acemoglu M, Bodmer D, Bantle S, Müller I, Mahl A, Kolopp M, Tullberg K. Poly(ethylene carbonate)s, part II: degradation mechanisms and parenteral delivery of bioactive agents, J Control Rel 2001;76:209-225.
13. Fulzele SV, Satturwar PM, Dorle AK. Study of the biodegradation and in vivo biocompatibility of novel biomaterials, Eur J Pharm Sci 2003; in press.
14. Ali SAM, Doherty PJ, Williams DF. Molecular biointeractions of biomedical polymers with extracellular exudates ad inflammatory cells and their effects on the biocompatibility, in vivo, Biomaterials 1994;15(10):779-785.
15. Gogolewski S, Janovic M, Perren SM, Dillon JG, Hughes MK. Tissue response and in vivo degradation of selected polyhydroxy acids: Polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate –co-hydroxyvalerate) (PHB/VA), J Biomed Mat Res 1993;27:1135-1148.
16. Therin M, Christel P, Li S, Garreau H, Vert M. In vivo degradation of massive poly(α-hydoxy acids): validation of in vitro findings, Biomaterials 1992;13(9):594-600.
17. Grizzi I, Garreau H, Li S, Vert M. Hydrolytic degradation of devices based on poly(DL-lactic acid) size dependence, Biomaterials 1995;16(4):305-311.
18. Göpferich A, Tessmar J. Polyanhydride degradation and erosion, Adv Drug Del Rev 2002;54:911-931.
19. Lu L, Garcia CA, Mikos AG. In vitro degradation of thin poly(DL-lactic-co-glycolic acid) films, J Biomed Mat Res 1999;46:236-244.
20. Matlaga BF, Yasenchak LP, Salthouse TN. Issue response to implanted polymers: the significance of sample shape, J Biomed Mater Res 1976;10:391-397.

21. Changez M, Koul V, Krishna B, Dinda AK, Choudhary V. Studies on biodegradation and release of gentamcin sulphate from interpenetrating network hydrogels based on poly(acrylic acid) and gelatin: in vitro and in vivo, Biomaterials 2003; in press.
22. Deschamps AA, Van Apeldoorn AA, De Bruijn JD, Grijpma DW, Feijen F. Poly(ether ester amide)s for tissue engineering, Biomaterials 2003;24:2643-2652.
23. Suggs LJ, Krishnan RS, Garcia C.A. Peter SJ, Anderson JM, Mikos AG. In vitro and in vivo degradation of poly(propylene fumarate-co-ethylene glycol) hydrogels, J Biomed Mat Res 1998;42:312-320.
24. Montanari L, Costantini M, Ciranni Signoretti E, Valvo L, Santucci M, Bartolomei M, Fattibene P, Onori S, Faucitano A, Conti B, Genta I. Gamma irradiation effects on poly(DL-lactide-co-glycolide) microspheres. J Control Rel 1998;56:219-229.
25. Anderson JM, Langone JJ. Issues and perspectives on the biocompatibility and immunotoxicity evaluation of implanted controlled release systems. J Control Rel 1999;57;107-113.
26. Anderson JM. In vivo biocompatibility of implantable delivery systems and biomaterials. Eur J Pharm Biopharm 1994;40:1-8.
27. Yamaguchi K, Anderson JM. In vivo biocompatibility studies of medisorb® 65/35 D,L-lactide/glycolide copolymer microspheres, J Control Rel 1993;24:81-93
28. Anderson JA, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Del Rev 1997;28:5-24.
29. Nagata M, Kiyotsukuri T, Takeuchi S, Tsutsumi N, Sakai W. Hydrolytic degradation of aliphatic polyesters copolymerized with poly(ethylene glycol)s. Polym Int 1997;42:33-38.
30. Pitt CG. Non-microbial degradation of polyesters: mechanisms and modifications. In: Vert M, Feijen F, Albertsson A, Scott G, Chiellini, editors. Biodegradable polymers and plastics. Melksham: Redwood Press Ltd; 1992. p7-17.
31. Göpferich A. Mechanisms of polymer degradation and erosion. Biomaterials 1996;17:103-114.
32. Tokiwa Y, Suzuki T. Hydrolysis of copolyesters containing aromatic and aliphatic ester blocks by lipase. J Appl Polym Sci 1981;26:441-448.
33. Reed AM, Gilding DK. Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:2 In vitro degradation. Polymer 1981;22:499-504.
34. Deschamps AA, Grijpma DW, Feijen J. Poly(ethylene oxide)/poly(butylene terephthalate) segmented block copolymers: the effect of copolymer composition on physical properties and degradation behavior. Polymer 2001;42(23):9335-9345.
35. Nikolic M, Djonlagic J. Synthesis and characterization of biodegradable poly(butylene succinate-co-butylene adipate)s. Polym Degrad Stab 2001;74:263-270.
36. Witt C, Kissel T. Morphological characterization of microspheres, films and implants prepared from poly(lactide-co-glycolide) and ABA triblock copolymers: is the erosion controlled by degradation, swelling or diffusion, Eur J Pharm Biopharm 2001;51:171-181.
37. Goedemoed JH, Hennink WH, Bezemer JM, Feijen J, Van Blitterswijk CA, De Bruijn JD. Polyetherester copolymers as drug delivery matrices. European Patent Application 97202533.2, 1998.

38. Deschamps AA, Claase MB, Sleijster MWJ, De Bruijn JD, Grijpma DW, Feijen J. Design of segmented poly(ether ester) materials and structures for tissue engineering of bone. J Control Rel, 2002;78(1-3):175-86.
39. Deschamps AA, thesis University of Twente, The Netherlands, 2002.
40. Jeong B, Bae YH, Kim SW. In situ gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions and degradation thereof, J Biomed Mat Res 2000;50;171-177.
41. Penco M, Marcioni S, Ferruti P, Dántone S, Deghenghi R. Degradation behaviour of block copolymers containing poly(lactic-glycolic acid) and poly(ethylene glycol) segments, Biomaterials 1996;17:1583-1590.
42. Budavari S, O'Neil MJ, Smith A, Heckelman PE, Kinneary JF, editors. The Merck Index, twelfth edition. Whitehouse Station, NJ, Merck & Co; 1996. 9038, succinic acid, 9306 terephthalic acid.

Chapter 10

Controlled release of proteins from succinate substituted poly(ethylene glycol)/poly(butylene terephthalate) multiblock copolymers

R. van Dijkhuizen-Radersma, S. Métairie, J.R. Roosma,
K. de Groot, J.M. Bezemer ,

Abstract

A new series of multiblock poly(ether-ester)s based on poly(ethylene glycol) (PEG), butylene terephthalate (BT) and butylene succinate (BS) segments were introduced as matrices for controlled release applications. The release of two model proteins, lysozyme and bovine serum albumin (BSA) from poly(ether-ester) films were evaluated and correlated to the swelling and degradation characteristics of the polymer matrices. First order and zero order profiles were found for the release of lysozyme, depending on the composition of the polymer matrix. The initial diffusion coefficient was correlated to the swelling of the matrix, which increased with longer PEG segments and lower BT/BS ratios of the polymer. High swelling matrices released the lysozyme according to diffusion controlled first order release profiles. Zero order release profiles were obtained from less swollen matrices due to a combination of diffusion and degradation of the matrix. In contrast to the release of lysozyme, BSA was released from the poly(ether-ester) matrices via delayed release profiles. Both the delay time and the release rate could be tailored by varying the matrix composition. The BSA release rate was mainly determined by the degradation, whereas the delay time was determined by a combination of the swelling and the degradation rate of the polymer matrix.

Introduction

Recently, multiblock poly(ether-ester)s based on poly(ethylene glycol) (PEG), butylene terephthalate (BT) and butylene succinate (BS) segments have been developed as a new series of degradable polymers for controlled release applications [1]. These poly(ether-ester)s are a modification of poly(ethylene glycol) terephthalate (PEGT) / poly(butylene terephthalate) (PBT) copolymers, which have been successfully applied as matrix in controlled release systems both in-vitro and in-vivo [2-7]. However, for controlled release application requiring frequently repeated injections, the degradation rate of some PEGT/PBT copolymer compositions might be too slow [8]. Substitution of the aromatic terephthalate units by aliphatic succinate units was shown to increase the degradation rate of the copolymers [1,9]. In addition, in-vivo studies showed no signs of bioincompatibility for these new materials [9]. Preliminary release studies on a selected PEG(T/S)/PB(T/S) polymer composition showed the complete release of model proteins within hours up to several weeks, depending on the size of the solute [1]. As release mechanism, protein diffusion due to a combination of swelling and degradation of the matrix was proposed but this required further investigation.

The effect of both swelling and degradation of the polymer matrix on solute diffusion has been subject of many studies. Several mathematical models describing solute diffusion through non-degradable hydrogels have been developed and validated using model polymer systems, assuming spherical solutes and neglecting polymer-solute interaction [10-19]. Hydrogels are crosslinked hydrophilic polymers that form a three dimensional structure of water-filled meshes between the polymer chains [10,19-21]. The diffusion of solutes through these meshes depends on the size of the solute in relation to the mesh size of the polymer matrix. Some of the factors affecting the polymer mesh size are chain mobility, chain entanglement, crosslinking density, crystallinity, and equilibrium degree of swelling [16]. Generally, the release of solutes from non-degradable hydrogels follow first order kinetics [e.g. 16,18]. For degradable polymers, however, other release profiles may be obtained, like zero order or delayed release [2,22-31]. Degradation of the matrix may increase the mesh size, which will increase the diffusion coefficient. In addition, the release rate may be altered due to erosion (mass loss) and/or pore formation. Therefore, models describing the release of solutes from

degradable polymers include degradation constants, which depend on the degradation mechanism [23-25,32-36]. In this study, a series of PEG(T/S)/PB(T/S) copolymers are extensively characterized with respect to their release properties. The in-vitro release of model proteins is correlated to both the swelling and the degradation of the poly(ether-ester) matrix. The selected model proteins, lysozyme (14.5 kDa) and bovine serum albumin (65 kDa), cover the important size range of therapeutically relevant proteins like growth hormones, erythropoietin and interferons.

Materials and methods

Materials

A series of multiblock poly(ether-ester)s based on polyethylene glycol, butylene terephthalate and butylene succinate units were synthesized at Chienna BV (Bilthoven, The Netherlands) as described before [1]. The PEG(T/S)PB(T/S) copolymers varied in PEG segment length (600, 1000 and 4000 g/mole), PEG(T/S)/PB(T/S) weight ratio (62/38 – 73/27) and T/S molar ratio (0/100 – 100/0) (composition determined by NMR). The poly(ether-ester)s are indicated as aPEG(T/S)bPB(T/S)c (T/S=d/e), in which a is the PEG molecular weight, b the combined weight percentage PEGT and PEGS and c, the combined weight percentage of PBT and PBS. d/e is the molar T/S ratio in the whole polymer. Dichloromethane (analytical grade) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Phosphate buffered saline (PBS), (pH 7.4) was obtained from Life Technologies Ltd (Paisley, Scotland). Sigma (St. Louis, MO) was the supplier of Lysozyme (Hen egg white, 3x crystallized, dialyzed and lyophilized) and Albumin (Bovine, 98%, initial fraction by heat shock).

Polymer characterisation

Proton NMR spectra were recorded on a Bruker ARX-400 operating at ≥ 200 MHZ at 343 K. $C_2D_2Cl_4$ was used as solvent without internal standard. The PEG(T/S)/PB(T/S) copolymer composition was calculated as described elsewhere [1]. The swelling of the copolymers was determined using films. Poly(ether-ester) films were prepared from solutions of 1 g copolymer in 5 ml dichloromethane. The polymer solutions were cast

onto a glass plate using a casting knife. After slow evaporation of the solvent, the films were removed from the glass plate and vacuum-dried for at least 16 hrs at room temperature. To measure the degree of swelling of the polymers, dry films (surface ± 1.77 cm^2, ± 100 μm in thickness) were weighed and immersed in phosphate buffered saline at 37°C in a shaking bath. After 3 days, the weight of the samples was determined after surface water was removed by blotting the surface with a tissue. The equilibrium volume swelling ratio Q was determined from the equilibrium weight of the swollen samples using a polymer density of 1.2 g/ml for all polymers.

To evaluate the in-vitro degradation, polymer sheets (1 mm thickness) were compression moulded at 100°C – 220°C (depending on melting temperature of copolymer composition) using a PHI press ($9 \cdot 10^3$ kg for 2 min). Samples of the sheets (20 mm x 20 mm) were immersed in 50 ml phosphate buffered saline at 37°C in a shaking bath for 1, 2, 4, 8, 16, and 24 weeks. The buffer was refreshed every 2 weeks. After the degradation periods, the residues of the samples were washed with purified water and dried using a vacuum oven at 50°C for at least 16 hours. The in-vitro degradation was monitored by analysing the changes in molecular weight and mass loss. The molecular weights of the copolymers were determined using Gel Permeation Chromatography (GPC). Samples were eluted in 0.02M sodiumtrifluoroacetate (NaTFA) in hexafluoroisopropanol (HFIP) through a Polymer Labs HFIP gel guard column (50 x 7.5 mm) and two PL HFIP gel analytical columns (300 x 7.5 mm). Flow rate was 1 ml/min and both an Ultraviolet (UV, 254 nm) and a Refraction Index (RI) detector were used. Column temperature was 40ºC and sample concentration was 0.3 mg/ml (50 μl injection). The number average molecular weights (M_n) were determined relative to polymethylmethacrylate (PMMA) standards. The decrease of molecular weight of copolymers based on PEG and PBT due to hydrolytic degradation during incubation in phosphate buffered saline have been described by the following relationship [2]:

$$\frac{1}{\overline{M}_n} = \frac{1}{\overline{M}_{n,0}} + k_d t \qquad (1)$$

in which $\overline{M}_n$ is the number average molecular weight at time t, $\overline{M}_{n,0}$ the initial number average molecular weight and k_d is the degradation constant. By plotting $1/\overline{M}_n$ as function of time, the degradation constant k_d could be calculated from the slope of the

linear part of the curve. The mass loss was determined by weighing the copolymer sheets prior to and after degradation. Based on these data, the mass loss per day could be calculated as a linear relation was found between the mass loss and the degradation period [1].

Preparation of protein loaded films

The release properties of the PEG(T/S)PB(T/S) copolymers were evaluated using protein loaded films. A protein solution (0.6 ml, 55 mg/ml) in PBS was emulsified in a polymer solution (1 g polymer in 5 ml dichloromethane) using ultra-turrax mixing (30 s at 19 krpm, IKA Labor technik T25). The resulting water-in-oil emulsion was cast onto a glass plate using a 0.7 mm casting knife. After slow evaporation of the solvent, the film was removed from the glass plate and freeze dried for at least 16 hours. Lysozyme (14.5 kDa) and bovine serum albumine (BSA) (67 kDa) were used as model proteins.

In-vitro release of proteins

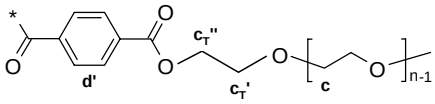
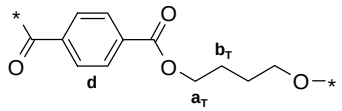
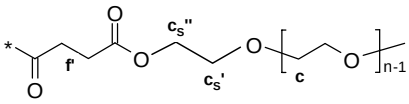
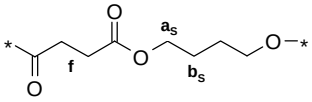
The protein release from the protein loaded PEG(T/S)PB(T/S) films was investigated in triplicate by incubating pieces of the films (± 1.77 cm^2) in 1 ml PBS. Vials were continuously shaken at 37°C and samples of the release medium were taken at various time points. The protein concentration of the buffer was determined using micro bichinchoninic acid (BCA) protein assay on an EL 312e microplate bio-kinetics reader ($\lambda = 570$ nm). The buffer was refreshed after sampling. The thickness of the swollen films was measured using a micrometer.

Results and discussion

Polymer matrix characterization

The poly(ether-ester) copolymers can be described as being built up of four different repeating segments (Table 1). Poly(ethylene glycol) terephthalate (PEGT) and poly(ethylene glycol) succinate (PEGS) form the soft hydrophilic blocks, whereas the hard hydrophobic blocks contain butylene terephthalate (BT) and butylene succinate(BS).

Table 1: Segments present in the poly(ether ester) copolymers.

Formula	Name	Abbreviation
	Poly(ethylene glycol) terephthalate	PEGT
	Butylene terephthalate	BT
	Poly(ethylene glycol) succinate	PEGS
	Butylene succinate	BS

The PEG(T/S)PB(T/S) copolymer matrices used in this study were characterised for their copolymer composition, swelling and degradation rate (Table 2). The poly(ether-ester)s varied in PEG segment length, amount of soft segment (wt% PEG(T/S) and degree of aliphatic substitution (mol% S). Similar to other amphiphilic block copolymers [37], the volume equilibrium swelling ratio Q increased with increasing length of the hydrophilic (PEG) segment. At a constant PEG segment length and soft/hard segment ratio, the swelling increases with increasing degree of substitution of the aromatic terephthalate groups by aliphatic succinate groups. The aliphatic succinate containing blocks are less efficient in forming physical crosslinks in the water-swollen hydrogels than the hard segments based on aromatic polyesters [1]. In addition, the less hydrophobic nature of the aliphatic segments in comparison with the aromatic polyesters, might also contribute to the efficiency of cross-linking. For controlled release applications, the swelling of the matrix is an important characteristic, as it correlates with the space between the polymer chains [10]. In general, a higher swelling results in a larger mesh size, which facilitates a faster diffusion of a solute through the matrix [10].

Table 2: Equilibrium swelling ratio, degradation rate constant and BSA release characteristics of the poly(ether-ester)s as function of the copolymer composition.

Polymer composition (NMR)	mol% succinate (NMR)	Swelling ratio Q	Degradation rate constant k_d (10^4 (mol·kg^{-1}·day^{-1})	BSA release rate constant k_r (%/day)	Delay time BSA release (days)
600PEG(T/S)62PB(T/S)38	46	1.94 ± 0.19	3.8	1.0	68
1000PEGT65PBT35	0	1.65 ± 0.08	n.d.	n.d.	>100
1000PEG(T/S)62PB(T/S)38	11	1.76 ± 0.02	0.7	n.d.	>100
1000PEG(T/S)66PB(T/S)34	45	2.22 ± 0.11	5.2	3.0	29
1000PEGS67PBS33	100	2.52 ± 0.07	7.3	12.8	2
1000PEGT71PBT29	0	1.80 ± 0.13	0.9	n.d	>100
1000PEG(T/S)73PB(T/S)27	45	2.74 ± 0.08	n.d.	6.6	9
4000PEG(T/S)64PB(T/S)34	46	3.21 ± 0.07	3.8	1.2	38

n.d.: not determined

Degradation of the polymer matrix also affects the diffusion rate as cleavage of the polymer chains enlarges the mesh size. Figure 1 shows a typical example of the reciprocal number average molecular weight as function of time for various poly(ether-ester) compositions. The degradation rate constant k_d as calculated from the slope of these type of graphs is given in Table 2. At a constant PEG segment length and soft/hard ratio, the degradation rate increases with increasing amount of succinate in the hard segment. As copolymers with higher succinate substitution show a higher swelling, the ester bonds are more accessible for hydrolysis [38,39]. In addition, the aliphatic esters are more susceptible to hydrolysis than aromatic ester, as a result of steric effects [40,41] and flexibility of the chain backbone [42,43]. By varying the PEG segment length at a constant soft/hard ratio, the highest value for k_d is observed for the 1000 g/mole containing poly(ether-ester)s. Apparently, copolymers with 1000 g/mole PEG segments have the optimal combination of swelling and hard block length for the fastest decrease in molecular weight [1,9].

Besides decrease in molecular weight, degradation of the polymer matrix may also result in mass loss. Figure 1 shows the mass of residues as function of degradation time for poly(ether-ester)s with varying amount of succinate. The mass loss rates (%/day) calculated from the slope of these graphs are given for various compositions in Table 3. Similar trends were observed for the mass loss rate as for the degradation rate constant k_d. A higher succinate substitution at a constant soft/hard ratio results in a faster mass loss.

At 45 mol% succinate in the hard segment, the fastest mass loss is observed for the 1000 g/mole PEG segment containing poly(ether-ester)s. Mass loss is determined by the decrease molecular weight and the subsequent dissolution of the low molecular weight oligomers. Besides the faster decrease in molecular weight, oligomers dissolution is favoured by a high succinate/terephthalate ratio and by the presence of 1000 g/mole PEG segments [1].

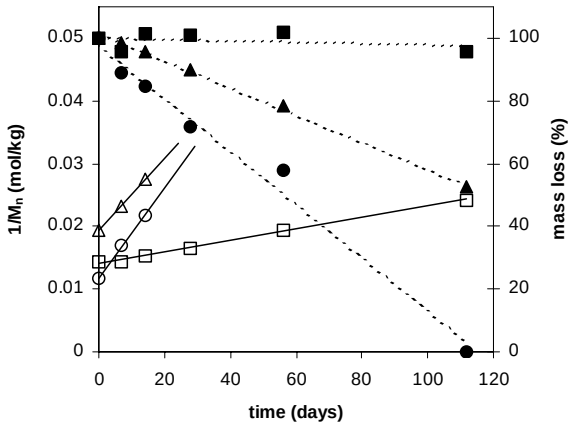


Figure 1: Reciprocal number average molecular weight (open symbols) and mass (filled symbols) of residue as function of degradation time for 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (■), 45 (●) and 100 (▲) mol% succinate.

Lysozyme release

The release profiles of lysozyme (14.5 kDa) from PEG(T/S)/PB(T/S) copolymers varying in amount of succinate are given in Figure 2. Poly(ether-ester)s containing 45 mol% and 100 mol% of succinate in the hard segment show a complete release of lysozyme within 5 and 0.5 hours, respectively (Fig. 2A). The shapes of the release curves indicate first order release kinetics. This was confirmed by plotting the cumulative released amount of lysozyme versus the square root of time, which resulted in a straight line. The fast diffusion of lysozyme through these matrices is mainly determined by the swelling. A higher degree of succinate substitution resulted in a higher equilibrium swelling ratio and subsequently in a faster diffusion. The lower swelling poly(ether-ester)s, containing 0 mol% and 10 mol% of succinate, showed a constant release of lysozyme during 40 days (Fig. 2B.). The zero order release profiles indicate a release, which is not solely

determined by diffusion. Similar release profiles have been described for PEGT/PBT copolymers with various soft/hard rations and PEG segment lengths [2]. This was attributed to a time dependent diffusion coefficient.

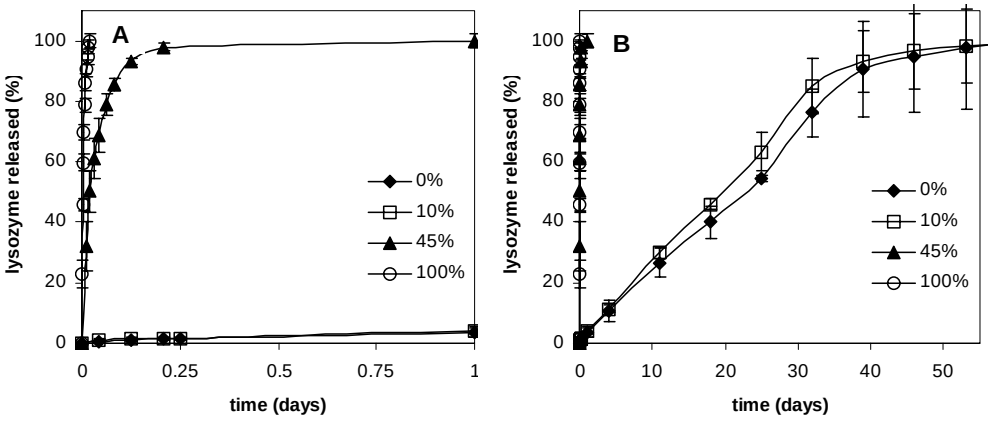


Figure 2: Cumulative release of lysozyme from films of 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (♦), 10 (□), 45 (▲) and 100 (○) mol% succinate (n=3; ± s.d.).

The initial lysozyme diffusion coefficient is mainly determined by the swelling of the matrix. In agreement to the free-volume theory [10], a linear relation ($r^2 = 0.99$) is observed between the logarithm of the diffusion coefficient and 1/(Q-1) of a variety of PEG(T/S)PB(T/S) copolymers (Fig. 3). For high swelling matrices (Q > 2.5), the diffusion coefficient deviates from this linear correlation. These matrices contain long PEG segments (≥ 2000 g/mole). As the soft/hard ratios are constant, the increasing molecular weight of the PEG segments increases the average hard segment length and thereby facilitates phase separation [1,37]. The hydrophobic domains are not permeable for lysozyme and are therefore barriers for lysozyme diffusion through the matrix. This may explain why the observed diffusion coefficients for poly(ether-ester)s containing long PEG segments are lower than expected based on the free volume theory.

The sustained release of lysozyme from poly(ether-ester) matrices, as shown in Figure 2, is not only determined by the initial diffusion coefficient. Due to degradation of the polymer matrix in time, the diffusion rate through the matrix increases [2]. In case the higher diffusion coefficient in time compensates for the decrease in protein concentration in the matrix, a linear release profile can be obtained (Fig 2B). This release mechanism of

lysozyme was previously confirmed for PEGT/PBT copolymers by evaluation of the effect of the polymer molecular weight on the lysozyme release [2]. In that particular study it was demonstrated that the diffusion coefficient of lysozyme through PEGT/PBT copolymers increased with decreasing polymer molecular weight. To study the effect of degradation of the PEG(T/S)/PB(T/S) matrices on the release characteristics in more detail, experiments have been performed using BSA (67 kDa) as model protein. Being almost twice the size of lysozyme (4.1 nm and 7.2 nm [44], respectively), BSA requires a larger mesh size of the matrix to facilitate diffusion.

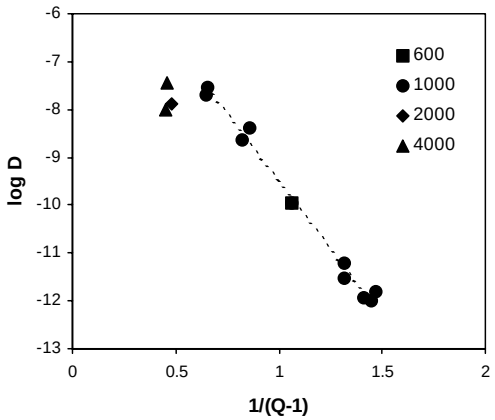


Figure 3: Diffusion coefficient D of lysozyme as function of swelling ratio Q for PEG(T/S)/PB(T/S) copolymers containing PEG segments varying from 600 up to 4000 g/mole.

BSA release

The release of BSA from films of various poly(ether-ester) compositions is shown in Figures 4–6. Instead of a first or zero order release profile as observed for lysozyme, delayed release profiles were found for BSA, independent on the copolymer composition. Several other authors have reported delayed release of proteins from degradable polymer matrices, like dextran hydrogels [27,29,45], poly(lactic-co-glycolic acid) (PLGA) [28,46], poly(ether-ester)s based on PEG and PBT [5], poly(ortho ester)s (POE) [31,47] and their triblock copolymers with PEG (POE-PEG-POE) [30]. Being a degradable hydrogel, the mechanism behind the delayed protein release from our poly(ether-ester)s shows similarity with that described for the dextran hydrogels. This release behaviour can be attributed to a combination of matrix degradation and protein diffusion [27,29,45]. As

the hydrodynamic diameter of BSA is too large to pass the initial meshes present in the hydrogel matrix, no initial release could be determined. However, during the release experiment, the matrix degraded [1]. After a certain time period (depending on the matrix composition), the molecular weight of the matrix decreased to such an extent that the mesh size of the matrix became sufficiently large to allow the diffusion of the BSA molecules [27,29,37,45]. Further degradation of the matrix increased the release rate. Both the release rate and the delay time are given in Table 2 as function of the copolymer composition. The delay time was taken as the time to release 15% of the incorporated protein [27]. The release rate constant k_r was defined as the slope of the cumulative BSA release between 20 and 80% (Fig. 4-6).

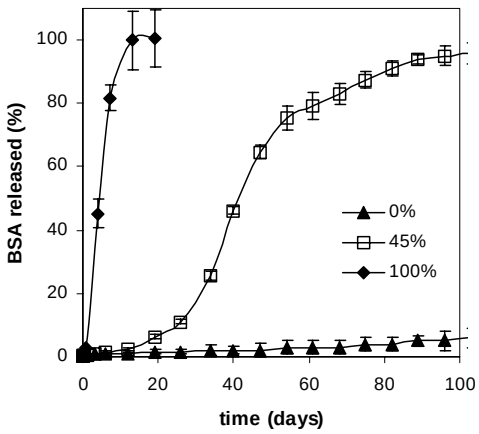


Figure 4: Cumulative release of BSA from films of 1000PEG(T/S)65PB(T/S)35 copolymers containing 0 (▲),45 (□) and 100 (♦) mol% succinate (n=3; ± s.d.).

As can be seen from Figure 4, an higher amount of succinate in the poly(ether-ester) resulted in a shorter delay time and a faster release. The matrix of the poly(ether-ester) containing only butylene succinate units in the hard segment (100% substitution) starts releasing after 2 days, whereas for the matrix containing only butylene terephthalate units in the hard segments (0% substitution) the release of BSA starts after more than 100 days. The copolymer containing 45 mol% of succinate in the hard segment showed intermediate behaviour. For this series of poly(ether-ester)s, the delay time can be directly correlated to the swelling and the degradation rate. The higher swelling of the highly succinate substituted poly(ether-ester)s resulted in an initial mesh size of the

matrix closer to the size of the BSA molecule. In addition, due to the fast degradation of these matrices the mesh size increases fast towards the required size to allow diffusion of the protein (Table 2).

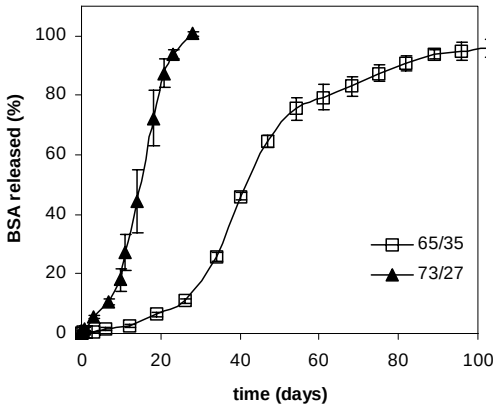


Figure 5: Cumulative release of BSA from films of PEG(T/S)PB(T/S) copolymers containing 45 mol% succinate, PEG segments of 1000 g/mole and soft/hard ratio of 65/35 (□) and 73/27 (▲) (n=3; ± s.d.).

The combined effect of swelling and degradation rate on the delay time and release rate is also observed for varying soft/hard ratios at a constant PEG segment length (1000 g/mole) and succinate percentage in the hard segment (45 mol%) (Fig. 5). An increase in soft segment from 65 to 73 weight percentage resulted in a shorter delay time (9 versus 29 days) and a faster release rate constant k_r (6.6 versus 3.0) (Table 2). This can again be attributed to the higher swelling and faster degradation of poly(ether-ester)s containing higher amounts of soft segment [37-39]. Surprisingly, the higher swelling of the 1000PEG(T/S)73PB(T/S)27 containing 45 mol% succinate did not result in a shorter delay time and a faster release of BSA compared to the 1000PEGS67PBS33 containing 100 mol% succinate (Table 2). This is probably caused by a faster degradation of the 1000PEGS67PBS33.

By varying the PEG segment length at a constant soft/hard ratio and constant amount of succinate, the combined effects of swelling and degradation of the matrix on the release profile become clearer (Fig. 6). The delay time increases in the following order: 600 > 4000 > 1000 g/mole PEG segments. The release rate increases in the opposite order, although the values for the 600 and 4000 g/mole PEG segment containing

polymers differ marginally. The shortest delay time and the highest release rate found for the 1000 PEG-segment containing copolymer is in good agreement with the observation that these copolymers have the highest rate of degradation [1]. So, with increasing degradation rate, the delay time decreases and the release rate increases. The 600 and 4000 g/mole PEG segments containing copolymers have almost similar degradation rates (Table 1). These copolymers also show comparable release rates for BSA, but differ in delay time to a large extent. The latter can be attributed to the difference in swelling of the matrix, resulting in a different initial mesh size. Apparently, the release rate of BSA through the matrix is mainly affected by the degradation rate of the matrix, whereas both the degradation rate and the swelling affect the delay time.

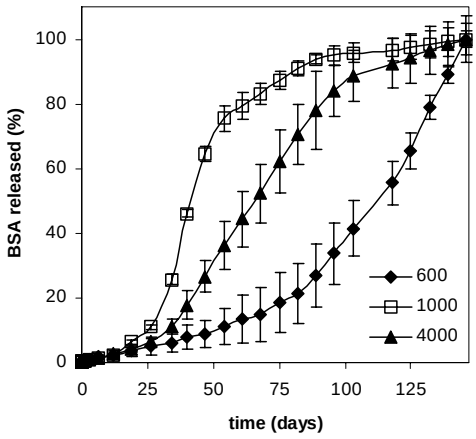


Figure 6: Cumulative release of BSA from films of PEG(T/S)65PB(T/S)35 containing 50 mol% succinate with PEG segments of 600 (♦), 1000 (□) and 4000 (▲) g/mole (n=3; ± s.d.).

In figure 7, a relation between the degradation rate constant k_d of the polymer matrix and the release rate constant k_r for BSA release is given. As stated before, an increasing rate of matrix degradation resulted in a faster release of BSA. The relation observed between swelling, degradation rate and delay time for BSA release from poly(ether-ester) matrices is evaluated in more detail below. A large number of models have been proposed to describe the release from both non-degradable [10,13,15,16,19] and degradable [23-25,32-36] polymer systems, but, to our best knowledge, none of these models dealt with delayed release profiles. Most of the existing models describe the effect of parameters like swelling and degradation on the (initial) diffusion coefficient.

For delayed release profiles, however, no initial diffusion occurs. Instead of the diffusion coefficient, we will focus on the mesh size of the matrix in time. Initially no protein is released from the matrix, indicating an initial mesh size smaller than the size of the solute. After a certain time period, the BSA release starts meaning that the size of the polymer meshes has increased up to a so-called critical mesh size. In fact, the release is a combination of the probability of presence of a volume sufficiently large for solute passage and the probability of finding an opening equal to or larger than the size of the solute. If we assume that the critical mesh size for BSA is identical for all poly(ether-ester)s used in this study, the delay time of a specific polymer composition can be defined as the time period to reach the critical mesh size. This time period depends on the initial mesh size (at t = 0) and the speed of increase of the mesh size in time. At a molecular scale, the mesh size is determined by the distance between crosslinks and/or entanglements of the polymer chains, which are related to the polymer volume fraction (1/Q) [10,13,19]. Several relations between the mesh size and the swelling ratio Q have been described in literature varying from a power of ½ [12], ¾ [17] up to a linear relation [12,37]. The power depends on the polymer volume fraction [10]. For polymer volume fractions (1/Q) exceeding 0.1, which is the case in our study, a linear relation can be expected between the swelling ratio and the initial mesh size [10].

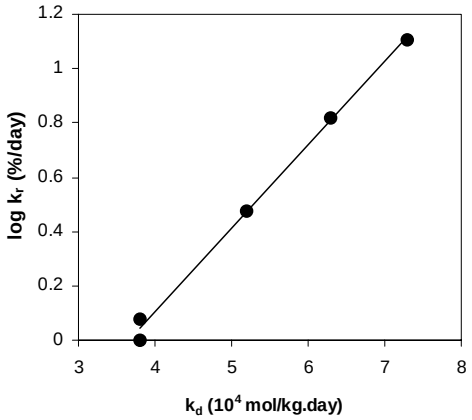


Figure 7: BSA release rate constant k_r versus degradation constant k_d of the PEG(T/S)PB(T/S) matrix.

Swelling experiments showed that the equilibrium swelling ratio of the polymer matrix was reached within minutes and remained constant during the release period (data

not shown). The time dependent increase of the initial mesh size is therefore not related to swelling changes in time, but related to the molecular weight decrease of the polymer matrix. There are, however, no models describing the relationship between the molecular weight of degradable matrices and the mesh size. Only for well-characterized non-degradable model polymer networks with defined (chemical) crosslinking density, a correlation between the molecular weight between crosslinks and the mesh size has been established [18,21]. In our polymer system, a complicated network consisting of physical crosslinks formed by hydrophobic interactions and entanglements determines the mesh size [1]. The precise effect of the decreasing molecular weight on the physical crosslinks and entanglements and thus on the mesh size is not apparent. Bezemer et al. found a linear relation between the diffusion coefficient (of lysozyme) and $1/Mn^2$ for poly(ether-ester)s based on PEG and PBT [2]. A decrease in molecular weight of the matrix by degradation will thus enhance the diffusion coefficient. Although the mechanism behind this relation is not fully understood, it can be expected that a comparable relation is valid for our polymer system. According to several models, the diffusion coefficient is proportional to the mesh size [10,19]. Therefore, a positive relation can be expected between the degradation rate constant k_d and the mesh size increase in time. The delay time is inversely proportionate to the relation of the mesh size, as the delay time will decrease with increasing initial mesh size and increasing mesh size growth. The delay time, indicating the time to reach the critical mesh size required for BSA diffusion is thus dependent on a combination of Q (for initial mesh size) and k_d (for increase in mesh size).

Franssen et al. found a linear relation between delay time and degradation rate for the release of IgG from enzymatically-degrading dextran hydrogels [29]. For these matrices, surprisingly, no significant effect of the swelling on the delay time was found. Apparently, the contribution of the initial mesh size to the observed release profile was small as compared to the rate of mesh size increase due to the degradation process [29].

So far, the effect of erosion (mass loss) has not been considered in our model describing the effect of swelling and degradation on the delay time. Heller et al showed that delayed release profiles for BSA obtained with poly(ortho ester)s could be directly correlated to the mass loss [31,47]. Therefore, we calculated the amount of mass lost during the time period required for the complete release of BSA (Table 3). Depending on

the poly(ether-ester) composition, the mass loss during release varied between 11% and 34%. This result indicates that the release of BSA in our system is not predominantly determined by the mass loss, although the effect should not be completely neglected.

Table 3: Time to reach complete BSA release and mass loss characteristics of the poly(ether-ester)s as function of the copolymer composition.

Polymer composition (NMR)	mol% succinate (NMR)	Time at 100% BSA release (days)	Mass loss rate (%/day)	Mass loss at 100% BSA release (%)
600PEG(T/S)62PB(T/S)38	46	146	0.09	13
1000PEG(T/S)62PB(T/S)38	11	n.d.	0.05	n.d.
1000PEG(T/S)66PB(T/S)34	45	100	0.34	34
1000PEGS67PBS33	100	13	0.88	11
1000PEGT71PBT29	0	n.d.	0.02	n.d.
1000PEG(T/S)73PB(T/S)27	45	28	n.d.	n.d.
4000PEG(T/S)64PB(T/S)34	46	132	0.12	15

n.d.: not determined

In conclusion, due to swelling and degradation of the matrix delayed release profiles were obtained for BSA from poly(ether-ester)s matrices. Both the delay time and the release rate of BSA can be tailored by selecting the copolymer composition. Delayed release profiles may be of particular interest for vaccination purposes, which require repeated boosters after certain time periods [46]. Future studies will therefore focus on the preparation and characterisation of poly(ether-ester) microspheres using therapeutically relevant proteins. In addition, the in-vivo release of these proteins from these polymer matrices should be evaluated.

Conclusion

A new series of multiblock poly(ether-ester)s based on poly(ethylene glycol) (PEG), butylene terephthalate (BT) and butylene succinate (BS) segments were evaluated for their controlled release properties. The release profiles for model proteins from poly(ether-ester) films could be controlled by changing the copolymer composition. First order and zero order profiles were found for the release of lysozyme, depending on the

composition of the polymer matrix. The initial diffusion coefficient was correlated to the swelling of the matrix, which increased with longer PEG segments and lower terephthalate/succinate ratios. In contrast to the release of lysozyme, BSA was released from the poly(ether-ester) matrices via delayed release profiles. Both the delay time and the release rate could be tailored by varying the matrix composition. The BSA release rate was mainly determined by the swelling, while the delay time was determined by a combination of the swelling and the degradation rate of the polymer matrix.

Future studies will focus on the preparation and characterisation of poly(ether-ester) microspheres using therapeutically relevant proteins. Both the in-vitro and in-vivo release of these proteins as well as the integrity and activity of the released protein will be subject of these studies.

References

1. R. van Dijkhuizen-Radersma, J.R. Roosma, P. Kaim, S. Métairie, F.L.A.M.A. Péters, J. de Wijn, P.G. Zijlstra, K.de Groot, J.M. Bezemer, Biodegradable poly(ether-ester) multiblock copolymers for controlled release applications, J. Biomed. Mater. Res. 67A (2003) 1294-1304.
2. J.M. Bezemer, R. Radersma, D.W. Grijpma, P.J. Dijkstra, J. Feijen, C.A. van Blitterswijk, Zero-order release of lysozyme from poly(ethylene glycol)/poly(buthylene terephthalate) matrices, J. Control. Release 64 (2000) 179–192.
3. J.M. Bezemer, R. Radersma, D.W. Grijpma, P.J. Dijkstra, C.A. van Blitterswijk, J. Feijen, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 1. Influence of preparation techniques on particle characteristics and protein delivery, J. Control. Release 67 (2000) 233-248.
4. J.M. Bezemer, R. Radersma, D.W. Grijpma, P.J. Dijkstra, C.A. van Blitterswijk, J. Feijen, Microspheres for protein delivery prepared from amphiphilic multiblock copolymers: 2. Modulation of release rate, J. Control. Release 67 (2000) 249-260.
5. J.M. Bezemer, D.W. Grijpma, P.J. Dijkstra, C.A. van Blitterswijk, J. Feijen, Control of protein delivery from amphiphilic poly(ether-ester) multiblock copolymers by varying their water content using emulsification techniques, J. Control. Release 66 (2000) 307-320.
6. R. van Dijkhuizen-Radersma, F.L.A.M.A. Péters, N.A. Stienstra, D.W. Grijpma, J. Feijen, K. de Groot, J.M. Bezemer, Control of vitamin B_{12} from poly(ethylene glycol)/poly(buthylene terephthalate) multiblock copolymers, Biomaterials 23 (2002) 1527-1536.
7. R. van Dijkhuizen-Radersma, S.J. Wright, L.M. Taylor, B.A. John, K.de Groot, J.M. Bezemer, In vitro / in vivo correlation for ^{14}C-methylated lysozyme release from poly(ether-ester) microspheres, Pharm. Res. 21 (2004) 484-491.

8. R. van Dijkhuizen-Radersma R, S.C. Hesseling, P.E. Kaim, K. de Groot, J.M. Bezemer, Biocompatibility and degradation of poly(ether-ester) microspheres: in-vitro and in-vivo evaluation, Biomaterials 23 (2002) 4719-4729.
9. R. van Dijkhuizen-Radersma, J.R. Roosma, J. Sohier, F.L.A.M.A Péters, M. van den Doel, C.A. van Blitterswijk, K.de Groot, J.M. Bezemer, Biodegradable poly(ether-ester) multiblock copolymers for controlled release applications: an in-vivo evaluation, submitted.
10. N.A. Peppas (Ed.), Hydrogels in Medicine and Pharmacy, Vol I, CRC Press, Boca Raton, FL, 1986
11. C.L. Bell, N.A. Peppas, Water, solute and protein diffusion in physiologically responsive hydrogels of poly(methacrylic acid-g-ethylene glycol), Biomaterials 17 (1996) 12-03-1218.
12. T. Canal, N.A. Peppas, Correlattion between mesh size and equilibrium degree of swelling of polymeric networks, J. Biomed. Mater. Res. 23 (1989) 1183-1193.
13. W.E. Hennink, H. Talsma, J.C.H. Borchert, S.C. De Smedt, J. Demeester, Controlled release of proteins form dextran hydrogels, J Control. Release 39 (1996) 47-55.
14. B. Amsden, An obstruction-scaling model for diffusion in homogeneous hydrogels, Macromolecules 32 (1999) 874-879.
15. N.A. Peppas, C.T. Reinhart, Solute diffusion in swollen membranes Part I. A new theory, J. Membr. Sci. 15 (1983) 275-287.
16. R.W. Korsmeyer, N.A. Peppas, Effect of the morphology of hydrophilic polymeric matrices on the diffusion and release of water soluble drugs, J. Membr. Sci. 9 (1981) 211-227.
17. S.R. Lustig, N.A. Peppas, Solute diffusion in swollen membranes. IX Scaling laws for solute diffusion in gels, J. Appl. Poly. Sci. 36 (1988) 735-747.
18. C.T. Reinhart, N.A. Peppas, Solute diffusion in swollen membranes Part II. Influence of crosslinking on diffusive properties, J. Membr. Sci. 18 (1984) 227-239.
19. B. Amsden, Solute diffusion within hydrogels. Mechanisms and models, Macromolecules 31 (1998) 8382-8395.
20. W.E. Hennink, C.F. van Nostrum, Novel crosslinking methods to design, Adv. Drug Dev. Rev. 54 (2002) 13-26.
21. N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, Eur. J. Pharm. Biopharm. 50 (2000) 27-46.
22. T. Tarvainen, T. Karjalainen, M. Malin, S. Pohjolainen, J. Tuomines, J. Seppälä, K. Järvinen, Degradation of and drug release from a novel 2,2,-bis(2oxazoline) linked poly(lactic acid) polymer. J. Control. Release 81 (2002) 251-261.
23. R. Wada, S.H. Hyon, Y. Ikada, Kinetics of diffusion-mediated drug release enhanced by matrix degradation, J. Control. Release 37 (1995) 151-160.
24. V. Lemaire, J. Bélair, P. Hildgen, Structural modelling of drug release from biodegradable porous matrices based on a combined diffusion/erosion process, Int. J. Pharm. 258 (2003) 95-107.
25. A. Charlier, B. Leclerc, G. Couarraze, Release of mifepristone from biodegradable matrices: experimental and theoretical evaluations, Int. J. Pharm. 200 (2000) 115-120.
26. J.A. Cadée, C.J. de Groot, W. Jiskoot, W. den Otter, W.E. Hennink, Release of recombinant human interleukin-2 from dextran-based hydrogels, J. Control. Release 78 (2002) 1-13.

27. O. Franssen, L. Vandervennet, P. Roders, W.E. Hennink, Degradable dextran hydrogels: controlled release of a model protein from cylinders and microspheres, J. Control. Release 60 (1999) 211-221.
28. D.M. Schachter, J. Kohn, A synthetic polymer matrix for the delayed or pulsatile release of water-soluble peptides, J. Control. Release 78 (2002) 143-153.
29. O. Franssen, O.P. Vos, W.E. Hennink, Delayed release of a model protein from enzymatically-degrading dextran hydrogels, J. Control. Release 44 (1997) 237-245.
30. J.P. Wan, Y.Y. Yang, T.S. Chung, D. Tan, S. Ng, J. Heller, POE-PEG-POE triblock copolymeric microspheres containing protein II. Polymer erosion and protein release mechanism, J. Control. Release 75 (2001)129-141.
31. J. Heller, J. Barr, S.Y. Ng, K. Schwach-Abdellaoui, R. Gurny, Poly(ortho esters): synthesis, characterization, properties and uses, Adv. Drug Deliv. Rev. 54 (2002) 1015-1039.
32. A.R. Tzafriri, Mathematical modelling of diffusion-mediated release from bulk degrading matrices, J. Control. Release 63 (2000) 69-79.
33. N. Faisant, J. Siepmann, J. Richard, J.P. Benoit, Mathematical modelling of drug release from bioerodible microparticles: effect of gamma-irradiation, Eur. J. Pharm. Biopharm. 56 (2003) 271-279.
34. M. Polakovič, T. Görner, R. Gref, E. Dellacherie, Lidocaine loaded biodegradable nanopheres II. Modelling of drug release.
35. N. Faisant, J. Siepmann, J.P. Benoit, PLGA-based microparticles: elucidation of mechanism and a new, simple mathematical model quantifying drug release, Eur. J. Pharm. Sci. 15 (2002) 355-366.
36. J. Siepmann, N. Faisant, J.P. Benoit, A new mathematical model quantifying drug release from bioerodible microparticles using Monte Carlo simulations, Pharm. Res. 19 (2002) 1885-1893.
37. J.M. Bezemer, D.W. Grijpma, P.J. Dijkstra, C.A. van Blitterswijk, J. Feijen, A controlled release systems for proteins based on poly(ether ester) block-copolymers: polymer network characterization, J. Control. Release 62 (1999) 393-405.
38. A.M. Reed, D.K. Gilding, Biodegradable polymers for use in surgery poly(ethylene oxide)/poly(ethylene terephthalate) (PEO/PET) copolymers:2 In vitro degradation, Polymer 22 (1981) 499-504.
39. A.A. Deschamps, D.W. Grijpma, J. Feijen, Poly(ethylene oxide)/poly(butylene terephthalate) segmented block copolymers: the effect of copolymer composition on physical properties and degradation behavior, Polymer 42 (2001) 9335-9345.
40. C.G. Pitt, Non-microbial degradation of polyesters: mechanisms and modifications. In: Vert M, Feijen F, Albertsson A, Scott G, Chiellini, editors. Biodegradable polymers and plastics. Melksham: Redwood Press Ltd (1992) 7-17.
41. A. Göpferich, Mechanisms of polymer degradation and erosion, Biomaterials 17 (1996) 103-114.
42. Y. Tokiwa, T. Suzuki, Hydrolysis of copolyesters containing aromatic and aliphatic ester blocks by lipase, J. Appl. Polym. Sci. 26 (1981) 441-448.
43. M. Nikolic, J. Djonlagic, Synthesis and characterization of biodegradable poly(butylene succinate-co-butylene adipate)s, Polym. Degrad. Stab. 74 (2001) 263-270.
44. C. Tranford, Physical chemistry of macromolecules, New York, Wiley , 1961.
45. W.E. Hennink, O. Franssen, W.N.E. van Dijk-Wolthuis, H. Talsma, Dextran hydrogels for the controlled release of proteins, J. Control. Release 48 (1997) 107-114.

46. J.L. Cleland, A. Lim, L. Barrón, E.T. Duenas, M.F. Powell, Development of a single-shot subunit vaccine for HIV-1: Part 4. Optimizing microencapsulation and pulsatile release of MN rgp120 from biodegradable microspheres, J. Control. Release 47 (1997) 135-150.
47. A. Rothe-Weinhold, K. Schwach-Abdellaoui, J. Barr, S.Y. Ng, H.R. Shen, R. Gurny, J. Heller, Release of BSA from poly(ortho ester) extruded thin strands, J. Control. Release 71 (2001) 31-37.

Summary

With rapid advances in genomic research and biotechnology, an increasing number of pharmaceutical proteins and peptides become available for a variety of diseases. However, the efficient delivery of these drugs is hampered by their large size and (biological) instability. Consequently, to obtain a desired therapeutic effect, proteins and peptides are usually administered parenterally by repeated subcutaneous or intramuscular injection. To overcome the problem of patient compliance, extensive research is performed on the development of controlled release systems. A promising approach is the encapsulation of the protein or peptide drug within a polymer matrix, to protect the drug from rapid clearance and to provide a sustained release. Biodegradable amphiphilic multiblock copolymers have gained increasing interest for drug delivery applications. From the literature reviewed in *chapter 2*, it was concluded that this type of polymers has great potential as matrix for controlled drug release. The presence of hydrophilic and hydrophobic blocks offers the possibility to tailor material properties like swelling and degradation rate, which are important factors for drug release. In addition, the reviewed multiblock copolymers show good biocompatibility. In particular poly(ether-ester) multiblock copolymers based on poly(ethylene glycol)-terephthalate (PEGT) and poly(butylene terephthalate) (PBT) have been the subject of many studies to evaluate the release behavior. Complete in-vitro release within several minutes up to months could be obtained for molecules of various sizes depending on the copolymer composition. In the research reported so far, PEGT/PBT copolymers have been shown to be biocompatible and degradable as medical device and suitable as matrix for the in-vitro release of model proteins. However, the development of a controlled release system based on these poly(ether-ester)s requires additional research. The objective of this thesis was to answer the following predefined questions:

What is the tissue response towards PEGT/PBT microspheres? All in-vitro and in-vivo studies up to now investigating the biocompatibility and degradability of

PEGT/PBT copolymers dealt with larger implants. Biocompatibility of biomaterials, however, depends to a large extent on the size and the shape of the implant. For controlled release applications, easily injectable microspheres are preferred. Injection of microspheres results in the implantation of a high surface area at a low volume biomaterial, which may show a more intense tissue response. In *chapter 3*, therefore, microspheres of a hydrophobic and a hydrophilic PEGT/PBT copolymer were evaluated for their in-vitro and in-vivo biocompatibility and degradation. The microspheres prior to and after sterilization were tested for in-vitro cytotoxicity. The in-vivo biocompatibility of the PEGT/PBT microspheres was evaluated subcutaneously and intramuscularly for 24 weeks in rabbits. The in-vivo degradation of the microspheres was studied microscopically and compared to the in-vitro degradation. The in-vitro and in-vivo studies showed the biocompatibility of the microspheres of both the hydrophobic and the hydrophilic PEGT/PBT copolymer. Extracts of these microspheres showed no cytotoxic reactivity in the in-vitro cytotoxicity test. Sterilization of the microspheres by gamma irradiation did not affect the cytotoxicity. PEGT/PBT microspheres injected subcutaneously and intramuscularly in rabbits showed a mild tissue response in vivo, in terms of the inflammatory response, the foreign body reaction and the granulation tissue response. Although an in-vitro degradation experiment showed a decrease in molecular weight due to hydrolysis, the in-vivo degradation of the microspheres was slower than expected from previous studies dealing with large implants. For controlled release applications requiring frequently repeated injections, the degradation rate of the evaluated PEGT/PBT copolymer compositions might be too slow.

Can PEGT/PBT copolymers be used as matrix for the sustained release of small molecules? Previously reported research on PEGT/PBT copolymers as matrix for controlled release applications focused on the release of high molecular weight model proteins. A lot of pharmaceuticals, however, are small molecules like peptides. Therefore, the suitability of PEGT/PBT matrices for the release of a small water-soluble model was investigated. In chapter 4, model compound vitamin B_{12} was incorporated in films of a series of PEGT/PBT copolymers using a water-in-oil emulsion method. The copolymer properties, like permeability, could be varied by increasing the PEG-segment length from 300 up to 4000 g/mole and by changing the wt% of PEGT. From permeation and release experiments, the diffusion coefficient of vitamin B_{12} through PEGT/PBT films of

different compositions was determined. The diffusion coefficient of vitamin B_{12} was strongly dependent on the composition of the copolymers. Although an increased wt% of PEGT (at a constant PEG-segment length) resulted in a higher diffusion coefficient, a major effect was observed at increasing PEG-segment length. By varying the copolymer composition, a complete release of vitamin B_{12} in 1 day up to a constant release for over 12 weeks was obtained. The release rate could be effectively tailored by blending copolymers with different PEG-segment lengths. The swelling and the crystallinity of the matrix could explain the effect of the matrix composition on the release behavior. It was concluded that PEGT/PBT copolymers were suitable as matrix for sustained release of a model compound vitamin B_{12}, but additional research is required for the release of relevant peptides.

Chapter 5 describes the development of a controlled release system for a therapeutically active peptide, calcitonin. As this peptide is known for its physical instability, special attention is paid to stabilization of calcitonin in the matrix. Salmon calcitonin loaded PEGT/PBT films were prepared from water-in-oil emulsions. The initial calcitonin release rate could be tailored by the copolymer composition, but incomplete release of calcitonin was observed. FTIR measurements indicated aggregation of calcitonin in the matrix, which was not due to the preparation method of the matrices, but due to the instability of calcitonin in an aqueous environment. Release experiments showed the susceptibility of calcitonin towards the composition of the release medium, in particular to the presence of metal ions. With increasing amount of sodium ions, a decrease in the total amount of released calcitonin was observed due to enhanced aggregation. The calcitonin had to be stabilized in the matrix to prevent aggregation. Incorporation of sodium dodecyl sulphate (SDS) as a stabilizer in PEGT/PBT matrices increased the percentage of calcitonin released, but could not avoid aggregation on a longer term.

So far, the in-vitro release of calcitonin from PEGT/PBT films was evaluated. In *chapter 6*, the results of in-vitro and in-vivo studies on calcitonin-loaded PEGT/PBT microspheres are reported. The in-vitro study focused on the encapsulation and calcitonin release, whereas in-vivo the pharmacodynamic effect and biocompatibility of the microspheres was investigated. Salmon calcitonin was successfully encapsulated in poly(ether-ester) microspheres using a water-in-oil-in-water emulsion. The presence of

sodium chloride in the outer water phase increased the encapsulation efficiency, but decreased the total amount of calcitonin released from the microspheres. The in-vitro release could be tailored effectively by varying the copolymer composition. The in-vivo study in rats showed the release of active calcitonin by a decreased calcium concentration and an increase in parathyroid hormone plasma level. Pathology showed no treatment-related findings and no indication for systemic toxicity.

Is the in-vitro release from PEGT/PBT matrices predictive for the in-vivo release? Besides the limited in-vivo study described in chapter 6, all experiments so far using these poly(ether-ester)s as matrix for controlled drug release have been performed in-vitro. However, for many release systems, the in-vivo release behaviour is very often not predicted by the in-vitro release. Therefore an in-vitro / in-vivo correlation for sustained release from PEGT/PBT microspheres should be obtained. In *chapter 7*, radiolabelled lysozyme, as a model, was encapsulated in PEGT/PBT microspheres via a water-in-oil-in-water emulsion. Three microsphere formulations varying in copolymer composition were administered subcutaneously to rats. The blood plasma was analysed for radioactivity content representing released lysozyme at various time points post-dose. The in-vitro release was studied in phosphate buffered saline (PBS). The encapsulation efficiency, calculated from the radioactivity in the outer water phase of the emulsion, varied from 60% to 87%. Depending on the PEG segment length and wt% PEGT, the lysozyme was released completely in-vitro within 14 to 28 days without initial burst. ^{14}C-methylated lysozyme could be detected in the plasma over the same time courses. The in-vitro/in-vivo correlation coefficients obtained from point-to-point analysis were greater than 0.96 for all microsphere formulations. In addition, less then 10% of administered radioactivity remained at dose site at 28 days for the microsphere formulations, indicating no notable retention of the protein at the injection site. From this study it was concluded that the in-vitro release in PBS and the in-vivo release in rats showed an excellent congruence independent of the release rate of ^{14}C-methylated lysozyme from PEGT/PBT microspheres.

Is the degradation of PEGT/PBT sufficiently fast for controlled release applications and can the degradation rate be modified, with preservation of the release characteristics? As concluded from the study described in chapter 3, the degradation rate of some PEGT/PBT copolymer compositions might be too slow for controlled release

applications requiring frequent injection. A known method to increase the degradation rate of aromatic polyesters is by (partial) substitution of the aromatic groups by aliphatic groups. *Chapter 8* describes the synthesis and characterisation of PEGT/PBT multiblock copolymers in which part of the butylene terephthalate (BT) units were replaced by butylene succinate (BS) units. The multiblock poly(ether-ester)s based on PEG, BT and BS units were synthesized by a two-step melt polycondensation reaction. The copolymers were characterized with respect to their composition (NMR), thermal properties (DSC) and swelling. The main focus was on the degradation kinetics and release properties of the copolymers. The crystallinity and swelling could be tailored by the PEG segment length and the ratio of the building units. With increasing mol fraction succinate in the hard (BT-BS) segment, the swelling increased. The in-vitro degradation was found to occur by molecular weight decrease and mass loss. Substitution of the aromatic terephthalate units by aliphatic succinate units increased the degradation rate of the copolymers. Polymers with PEG segments of 1000 g/mole showed a more pronounced degradation than copolymers containing shorter and longer PEG segments. Model proteins were successfully incorporated and released from the poly(ether-ester) films. Depending on the size of the protein, the release mechanism was based on diffusion of the protein and degradation of the matrix. Based on the in-vitro degradation and preliminary release results, it was concluded that this new series of poly(ether-ester)s has good potential as matrix for controlled drug release. However, additional experiments should evaluate the effect of the substitution on the biocompatibility and in-vivo degradation.

In *chapter 9*, therefore, the multiblock poly(ether-ester)s based on poly(ethylene glycol), butylene terephthalate and butylene succinate segments were evaluated for their in-vivo degradation and biocompatibility in order to establish a correlation with the in chapter 8 reported in-vitro results. Porous polymer sheets were implanted subcutaneously for 32 weeks in rats. The degradation was monitored visually (histology), by molecular weight (GPC) and by copolymer composition (NMR). Substitution of the aromatic terephthalate units by aliphatic succinate units was shown to accelerate the degradation rate of the copolymers. Direct correlation of the in-vivo and in-vitro degradation of the porous implants showed a slightly faster initial molecular weight decrease in-vivo. Besides hydrolysis, probably also oxidation occurs in-vivo due to the presence of

radicals, produced by inflammatory cells. In addition, the higher molecular weight plateau of the residue found in-vivo indicated a higher solubility of the oligomers in the extracellular fluid compared to a phosphate buffer. Minor changes in the poly(ether-ester) compositions were noted due to degradation. Microscopically, fragmentation of the porous implants was observed in time. At later stages of degradation, macrophages were observed phagocytozing small polymer particles. Both in-vitro cytotoxicity studies and histology on in-vivo samples proved the biocompatibility of the poly(ether-ester)s.

As a follow-up of the preliminary release results reported in chapter 8, an extensive study was performed to characterize the new series of multiblock poly(ether-ester)s in more detail with respect to their release properties. In *chapter 10* the release of two model proteins, lysozyme and bovine serum albumin (BSA) from poly(ether-ester) films were evaluated and correlated to the swelling and degradation characteristics of the polymer matrices. First order and zero order profiles were found for the release of lysozyme, depending on the composition of the polymer matrix. The initial diffusion coefficient was correlated to the swelling of the matrix, which increased with longer PEG segments and lower BT/BS ratios of the polymer. High swelling matrices released the lysozyme according to diffusion controlled first order release profiles. Zero order release profiles were obtained from less swollen matrices due to a combination of diffusion and degradation of the matrix. In contrast to the release of lysozyme, BSA was released from the poly(ether-ester) matrices via delayed release profiles. Both the delay time and the release rate could be tailored by varying the matrix composition. The BSA release rate was mainly determined by the degradation, whereas the delay time was determined by a combination of the swelling and the degradation rate of the polymer matrix.

Samenvatting

De snelle ontwikkelingen in de biotechnologie hebben geleid tot een groeiend aantal geneesmiddelen op basis van eiwitten en peptiden. De toepassing van deze therapeutische eiwitten en peptiden wordt echter beperkt door hun grootte en biologische instabiliteit. In tegenstelling tot conventionele medicijnen, kunnen eiwitten niet effectief worden toegediend via klassieke toedieningsvormen zoals tablet of drankje. Om het gewenste therapeutische effect te bereiken worden deze nieuwe medicijnen bij voorkeur toegediend via een onderhuidse of intramusculaire injectie. Aangezien eiwitten in het algemeen slechts kort in het lichaam aanwezig zijn, bijvoorbeeld doordat ze worden afgebroken door enzymen, zijn herhaaldelijke injecties noodzakelijk. Om patiënt-vriendelijkere toedieningsvormen te ontwikkelen wordt uitgebreid onderzoek verricht naar gecontroleerde afgiftesystemen. Een veelbelovende benadering is het verpakken van eiwitten of peptiden in een polymere ('plastic') matrix, om het medicijn te beschermen tegen snelle afbraak en een langdurige afgifte van het medicijn te verzorgen. Zo kan gedurende een lange periode op de juiste plaats in het lichaam de hoeveelheid actief eiwit of peptide op het gewenste niveau gehandhaafd blijven. In het ideale geval is de snelheid waarmee het medicijn uit het afgiftesysteem vrijkomt nauwkeurig te reguleren, bijvoorbeeld door de vorm van het afgiftesysteem of de eigenschappen van het polymeer. Het afgiftesysteem kan geformuleerd worden als kleine nano- of microdeeltjes, injecteerbare gelen of als implantaat. Veel afgiftesystemen zijn gebaseerd op biodegradeerbare polymeren. Tijdens of na het vrijkomen van het eiwit of peptide degradeert ('verdwijnt') de drager, waardoor een operatie om het afgiftesysteem te verwijderen kan worden vermeden.

Degradeerbare polymeren die gebruikt worden in een afgiftesysteem moeten aan hoge eisen voldoen. Zo moet het polymeer biocompatibel ('lichaamsvriendelijk') zijn, binnen een redelijke termijn degraderen (afhankelijk van de toepassing) en mogen de vrijkomende degradatieproducten niet toxisch zijn. Verder dient het polymeer een

optimale omgeving voor het ingepakte medicijn te creëren om de gecompliceerde structuur en daarmee de activiteit van het eiwit of peptide te bewaren. Bovendien moet de afgiftesnelheid van het medicijn nauwkeurig geregeld kunnen worden door de karakteristieken van het polymeer te variëren, zodat het gewenste therapeutisch effect kan worden bereikt. Een veel bestudeerd biodegradeerbaar materiaal is poly(lactide-co-glycolide). De laatste jaren wordt de geschiktheid van dit polymeer als matrix voor gecontroleerde afgifte van eiwitten echter in twijfel getrokken. Zo is aangetoond dat ingepakte eiwitten in deze matrices niet stabiel zijn en bovendien biedt het polymeer gelimiteerde mogelijkheden om het afgifteprofiel in te stellen. Om deze problemen te overbruggen, wordt onderzoek verricht naar afgiftesystemen gebaseerd op nieuwe polymeren zoals bijvoorbeeld amfifiele multiblok-copolymeren. Zoals beschreven in *hoofdstuk 2,* blijkt uit de literatuur dat dit type polymeer goede mogelijkheden biedt als matrix voor gecontroleerde medicijnafgifte. Door de aanwezigheid van hydrofiele en hydrofobe blokken kunnen materiaal eigenschappen die van belang zijn voor medicijn afgifte, zoals degradatiesnelheid en zwelling, nauwkeurig worden beïnvloed. Bovendien zijn de beschreven multiblok-copolymeren biocompatibel. Een groot aantal studies is uitgevoerd naar de mogelijkheden van poly(ether-ester) multiblok-copolymeren gebaseerd op hydrofiele poly(ethyleen glycol)-tereftalaat (PEGT) segmenten en hydrofobe poly(butylene tereftalaat) (PBT) blokken als matrix voor gecontroleerde afgifte. In-vivo (‘in het lichaam’) studies hebben aangetoond dat implantaten gebaseerd op deze PEGT/PBT copolymeren biocompatibel zijn en in het lichaam degraderen. Bovendien bleek uit in-vitro (‘in het laboratorium’) studies dat PEGT/PBT geschikt zijn als matrix voor gecontroleerde afgifte van eiwitten. Voor de ontwikkeling van een gecontroleerd afgifte systeem op basis van deze poly(ether-ester)s is echter aanvullend onderzoek nodig. In dit proefschrift worden een viertal onderzoeksvragen beantwoord.

Hoe reageert het weefsel op PEGT/PBT microbolletjes? Alle in-vitro en in-vivo onderzoeken naar de biocompatibiliteit en degradatie van PEGT/PBT copolymeren hebben zich tot dusver gericht op grotere implantaten. De biocompatibiliteit van een materiaal wordt echter voor een groot deel bepaald door de grootte en de vorm van het implantaat. Voor gecontroleerde afgifte toepassingen hebben kleine microbolletjes die gemakkelijk te injecteren zijn de voorkeur. Het relatief grote oppervlak van geïnjecteerde microbolletjes zouden een sterkere weefselreactie kunnen veroorzaken

in vergelijking met grotere implantaten. In *hoofdstuk 3* zijn daarom de in-vitro en in-vivo biocompatibiliteit en degradatie onderzocht van microbolletjes van verschillende PEGT/PBT copolymeren. De microbolletjes zijn voor en na sterilisatie getest op hun in-vitro cytotoxiciteit. De in-vivo biocompatibiliteit van de PEGT/PBT microbolletjes is gedurende 24 weken onderzocht in konijnen. De in-vivo degradatie van de microbolletjes is microscopisch bestudeerd en vergeleken met de in-vitro degradatie. De in-vitro en in-vivo experimenten toonde aan dat de microbolletjes biocompatibel waren onafhankelijk van de PEGT/PBT copolymeer compositie. Extracten van de microbolletjes vertoonden geen cytotoxische reactie in de in-vitro test. Bovendien bleek sterilisatie van de microbolletjes door middel van gamma bestraling geen effect te hebben op de cytotoxiciteit. De onderhuids en intramusculair geïnjecteerde microbolletjes veroorzaakten een milde weefselreactie, uitgedrukt in ontstekingsreactie, vreemd-lichaamsreactie en granulatieweefselreactie. De in-vitro degradatie test liet een daling in het molecuulgewicht van de PEGT/PBT copolymeren zien als gevolg van hydrolyse. De in-vivo degradatie was echter langzamer dan verwacht op basis van eerder experimenten met grotere implantaten. Voor gecontroleerde afgifte toepassingen waarbij herhaalde injecties noodzakelijk zijn, is de degradatiesnelheid van de bestudeerde PEGT/PBT copolymeer composities mogelijk te langzaam.

Zijn PEGT/PBT copolymeren geschikt als matrix voor langdurige afgifte van kleine moleculen? Het onderzoek naar de toepassing van PEGT/PBT copolymeren als matrix voor gecontroleerde afgifte systemen hebben zich tot nu toe gericht op de afgifte van grote modeleiwitten. Veel nieuwe medicijnen zijn echter kleine moleculen zoals peptiden. Daarom is onderzocht of PEGT/PBT copolymeren ook gebruikt kunnen worden voor de afgifte van kleine water-oplosbare moleculen. In *hoofdstuk 4* is vitamine B_{12} als model ingekapseld in PEGT/PBT films via een emulsiemethode. De eigenschappen van de copolymeren zoals de doorlaatbaarheid konden worden gevarieerd door de lengte van de PEG blokken (van 300 tot 4000 g/mol) en de hoeveelheid PEGT. Met behulp van doorlaatbaarheids- en afgifte-experimenten kon de diffusiecoëfficient van vitamine B_{12} door de PEGT/PBT films worden bepaald. De diffusiecoëfficient bleek sterk afhankelijk van de compositie van de copolymeren. Zowel de toename in de hoeveelheid PEGT als in de lengte van het PEG blok resulteerde in een grotere diffusiecoëfficient. De afgifte van vitamine B_{12} was compleet binnen 1 dag tot 12 weken, afhankelijk van de compositie van

het copolymeer. De afgifte snelheid kon nauwkeurig worden ingesteld door de copolymeren met verschillende PEG-bloklengtes te mixen. Het effect van de samenstelling van de matrix op de afgifte kon worden verklaard aan de hand van de zwelling en de kristalliniteit van de matrix. De conclusie van dit onderzoek was dat PEGT/PBT copolymeren geschikt zijn als matrix voor langdurige afgifte van het model vitamine B_{12}, maar dat aanvullend onderzoek nodig is naar de afgifte van relevante peptiden.

Hoofdstuk 5 beschrijft de ontwikkeling van een gecontroleerd afgifte systeem voor het therapeutisch actieve peptide calcitonine. Omdat dit peptide als instabiel bekend staat, is er in het bijzonder aandacht besteed aan het stabiliseren van de calcitonine in de matrix. Calcitonine beladen PEGT/PBT films zijn gemaakt vanuit water-in-olie emulsies. De initiële calcitonine afgiftesnelheid kon worden beïnvloed door de copolymeer samenstelling. De totale hoeveelheid calcitonine die vrij kwam bleek echter incompleet. FTIR-analyses duidden erop dat de calcitonine in de matrix was geaggregeerd. Deze aggregatie werd niet veroorzaakt door de bereidingswijze, maar was het gevolg van de instabiliteit van calcitonine in een waterig milieu. Afgifte experimenten toonden de gevoeligheid van calcitonine voor de samenstelling van het afgifte medium en voor metaal ionen in het bijzonder. Een toenemende hoeveelheid natrium ionen in het afgifte medium resulteerde in een afname van de totale hoeveelheid vrijgekomen calcitonine als gevolg van toenemende aggregatie. De calcitonine moest daarom in de matrix worden gestabiliseerd om aggregatie te voorkomen. Het inkapselen van natrium dodecyl sulfaat als stabilisator in de PEGT/PBT matrices zorgde voor een toename in de hoeveelheid calcitonine die werd afgegeven, maar kon op langere termijn geen aggregatie voorkomen.

Tot dusver is de afgifte van calcitonine bestudeerd in-vitro vanuit PEGT/PBT films. In *hoofdstuk 6* worden de resultaten van in-vitro en in-vivo studies naar calcitonin-beladen PEGT/PBT microbolletjes gerapporteerd. De in-vitro studie richtte zich op de inkapseling en de afgifte van calcitonine, terwijl in-vivo het farmacodynamische effect en de biocompatibiliteit van de microbolletjes werd onderzocht. Zalm calcitonine werd met succes ingekapseld in poly(ether-ester) microbolletjes via een water-in-olie-in-water emulsie. De aanwezigheid van natriumchloride in de buitenste waterfase vergrootte de inkapselingsefficiëntie, maar resulteerde in een afname van de totale hoeveelheid calcitonine die uit de microbolletjes werd afgegeven. De in-vitro afgifte kon precies

worden geregeld door de copolymeer samenstelling te variëren. Uit de in-vivo studie in ratten bleek dat de vrijgekomen calcitonine nog actief was, omdat de calcium concentratie in het bloedplasma daalde waardoor het parathyroid hormoon niveau steeg. Pathologie toonde geen geen indicatie voor systematische toxiciteit.

Wordt de in-vivo afgifte vanuit PEGT/PBT matrices voorspelt door de in-vitro afgifte? Behoudens de in hoofdstuk 6 beschreven in-vivo studie zijn alle experimenten met deze poly(ether-ester)s als matrix voor gecontroleerde afgifte van medicijnen uitgevoerd in het laboratorium. Voor een groot aantal afgifte systemen is de in-vitro afgifte echter niet voorspellend voor de in-vivo afgifte. Daarom dient er een zogenaamde in-vitro/in-vivo correlatie te worden verkregen. In *hoofdstuk 7* is radioactief gelabeld lysozyme als model ingekapseld in PEGT/PBT microbolletjes via een water-in-olie-in-water emulsie. Drie formuleringen variërend in copolymeer compositie zijn aan ratten toegediend middels een onderhuidse injectie. Vervolgens is het bloedplasma op verschillende tijden geanalyseerd op radioactiviteit als maat voor de hoeveelheid vrijgekomen lysozyme. De in-vitro afgifte is gemeten in een met fosfaat gebufferde zoutoplossing (PBS). De inkapselingsefficiëntie, berekend uit de radioactiviteit in de buitenste waterfase, varieerde van 60 tot 87%. Afhankelijk van de PEG-bloklengte en de hoeveelheid PEGT, kwam alle lysozyme uit de microbolletjes vrij binnen 14 tot 28 dagen met een min of meer constante snelheid. De aanwezigheid van radioactief gelabeld lysozyme kon over dezelfde tijdspanne worden aangetoond. De berekende in-vitro/in-vivo correlatiecoëfficienten waren groter dan 0,96 voor alle formuleringen met microbolletjes. Bovendien bleek dat minder dan 10% van de toegediende radioactiviteit aanwezig was op de injectieplaats, wat erop duidt dat er geen grote hoeveelheid eiwit is achter gebleven. Uit deze studie werd geconcludeerd dat de in-vitro afgifte voor de lysozyme uit PEGT/PBT microbolletjes uitstekend overeenkomt met de in-vivo afgifte in ratten onafhankelijk van de afgifte snelheid.

Is de degradatie van PEGT/PBT snel genoeg voor gecontroleerde afgifte toepassingen en kan de degradatie aangepast worden met behoud van de afgifte eigenschappen? Uit de resultaten van hoofdstuk 3 werd geconcludeerd dat de degradatiesnelheid voor sommige PEGT/PBT composities te laag zou kunnen zijn voor gecontroleerde afgifte toepassingen die herhaaldelijk injecties vereisen. Een bekende methode om de degradatiesnelheid van aromatische polyesters te vergroten is het

(gedeeltelijk) vervangen van de aromatische groepen door alifatische groepen. *Hoofdstuk 8* beschrijft de synthese en karakterisering PEGT/PBT multiblokcopolymeren waarin een gedeelte van de butyleen tereftalaat (BT) groepen vervangen zijn door butyleen succinaat (BS) groepen. De multiblok poly(ether-ester)s werden gesynthetiseerd door een zogenaamde twee-staps polycondensatiereactie in de smelt. Vervolgens zijn de copolymeren onderzocht op hun compositie (NMR), thermische eigenschappen (DSC) en zwelling. De nadruk lag op de degradatie kinetiek en de afgifte eigenschappen van de copolymeren. De kristalliniteit en zwelling konden worden ingesteld door de PEG-bloklengte en de ratio van de verschillende blokken te variëren. Een toename in de hoeveelheid succinaat in de harde (BT-BS) blokken resulteerde in een hogere zwelling. De in-vitro degradatie verliep middels afname van het molecuulgewicht en massa verlies. Het vervangen van de aromatische tereftalaat groepen door de alifatische succinaat groepen versnelde de degradatie van de copolymeren. Polymeren met PEG-blokken van 1000 g/mol bleken sneller te degraderen dan copolymeren met kortere of langere PEG-blokken. Model eiwitten konden met succes worden ingekapseld en afgegeven vanuit poly(ether-ester) films. Het mechanisme achter de afgifte was, afhankelijk van de grootte van het eiwit, gebaseerd op diffusie van het eiwit en degradatie van de matrix. Gebaseerd op de in-vitro degradatie en de resultaten van de voorlopige afgifte experimenten, werd geconcludeerd dat deze nieuwe poly(ether-ester)s veelbelovend zijn als matrix voor gecontroleerde medicijn afgifte. Vervolgexperimenten zijn echter noodzakelijk om het effect van de substitutie met succinaat op de biocompatibiliteit en de in-vivo degradatie te evalueren.

In *hoofdstuk 9* zijn de multiblok-copolymeren gebaseerd op poly(ethyleen glycol), buthyleen tereftalaat en butyleen succinaat onderzocht op hun in-vivo degradatie en biocompatibiliteit om een correlatie met de in-vitro resultaten uit hoofdstuk 8 te leggen. Poreuze polymere films werden onderhuids geïmplanteerd in ratten gedurende 32 weken. De degradatie in de tijd werd visueel bepaald met histologie en gemeten aan de hand van het molecuulgewicht (GPC) en de polymeer samenstelling (NMR). De substitutie van de aromatische terephthalate groepen met de alifatische succinaat groepen resulteerde in een snellere degradatie van de copolymeren. Uit directe correlatie van de in-vivo en de in-vitro degradatie van de poreuze implantaten bleek in-vivo een iets snellere initiële afname van het molecuulgewicht. Behalve hydrolyse vindt in-vivo

waarschijnlijk ook oxidatie plaats door de aanwezigheid van radicalen die door de ontstekingscellen worden geproduceerd. Bovendien bleek dat de plateauwaarde van het molecuulgewicht in-vivo hoger te liggen. Dit duidt op een hogere oplosbaarheid van de degradatie producten in de extracellulaire vloeistof in vergelijking met een fosfaat buffer. De degradatie veroorzaakte kleine veranderingen in de poly(ether-ester) compositie. Met behulp van een microscoop kon de fragmentatie van de poreuze implantaten in de tijd worden aangetoond. In latere stadia van degradatie werden macrofagen zichtbaar die kleine polymere deeltjes fagocyteerden. Zowel in-vitro cytotoxiciteitsstudies als histologie bewezen de biocompatibiliteit van de poly(ether-ester)s.

Als vervolg op de eerste afgifte experimenten uit hoofdstuk 8, is een uitgebreide studie uitgevoerd om de afgifte eigenschappen van deze nieuwe multiblok-copolymer nader te bestuderen. In *hoofdstuk 10* is de afgifte van twee model eiwitten, lysozyme and bovine serum albumine (BSA) geëvalueerd en gecorreleerd aan de zwelling en degradatie van de polymere matrix. Voor de afgifte van lysozyme werden eerste-orde en nulde-orde profielen gevonden afhankelijk van de samenstelling van de polymere matrix. Eerste-orde afgifte duidt op een hoge initiële afgiftesnelheid die steeds verder afneemt in de tijd, terwijl bij nulde-orde de afgifte snelheid constant is. De initiële diffusiecoëfficient kon aan de zwelling worden gecorreleerd, welke toenam naarmate de polymere matrix langere PEG-blokken en lagere BT/BS ratio's bevatte. Uit matrices met een hoge zwelling werd de lysozyme afgegeven volgens een diffusie gecontroleerde eerste-orde afgifteprofiel. Nulde-orde afgifteprofielen werden gevonden voor minder gezwollen matrices als gevolg van een combinatie van diffusie en degradatie van de matrix. In tegenstelling tot lysozyme, werd BSA vanuit de poly(ether-ester) matrices afgegeven via vertraagde afgifte profielen, waarbij het enige tijd duurt voordat de afgifte begint. Zowel de tijdsduur van de vertraging als de afgifte snelheid konden worden geregeld door de matrix compositie te variëren. De BSA afgiftesnelheid bleek met name bepaald door de degradatie, terwijl de tijdsduur van de vertraging werd bepaald door de zwelling en de degradatie snelheid van de polymere matrix.

Dankwoord

Hoewel de voorkant van dit boekje slechts een naam vermeldt, hadden dat er veel meer kunnen zijn. De onderstaande mensen hebben door hun aanwezigheid en hulp een onmisbare bijdrage geleverd.

Zonder mijn beide promotoren, de professoren Klaas de Groot en Clemens van Blitterswijk en assistent promotor Jeroen Bezemer zou er van promoveren geen sprake zijn. Klaas, bedankt voor het vertrouwen. Je positieve en snelle commentaar op het schrijfwerk hebben zeer motiverend gewerkt. En Clemens, bedankt dat je mij de mogelijk hebt geboden om te promoveren. Jeroen is de grote inspirator achter het hele gebeuren. Zonder jou was dit project nooit begonnen laat staan afgemaakt. Veel dank voor alle avond/weekend uurtjes, ondanks je eigen drukte.

Het onderzoek dat in dit proefschrift is beschreven is uitgevoerd in nauwe samenwerking met een groot aantal IsoTis/Chienna collega's van wie een groot aantal terug te vinden is als medeauteur. Ik ben me zeer bewust van mijn luxe positie met alle samenwerkingsverbanden tot in Engeland en Denemarken aan toe. In het bijzonder de (oud)PolyActivers Anne, Fabienne, Jeroen, Jérôme, Joost, Jorg, Klaas S, Monica, Peter K, Peter Z, Robert en Sophie hebben een grote inspanning geleverd. Het boekje voelt dan ook niet als eigen werk maar als dat van iedereen. Ik hoop dat jullie dat ook zo voelen. Verder kijk ik met heel veel plezier terug op onze PolyActive fêtes, barbecues en de unieke werksfeer. Een speciale vermelding is er voor Jeroen en mijn paranifmen Jorg en Monica. Jeroen, mijn mailbox zit stampvol mailtjes getiteld hoi, Rehoi, ReRehoi etc. van jmbezemer. Het is heerlijk om met jou samen te werken. Jorg, je grote aantal coauteurschappen is al veelzeggend, maar als je drie jaar lang iemands password bent moet je wel heel bijzonder en belangrijk zijn. Met Monica heb ik de afgelopen vier jaar van alles bekletst, bepraat en besproken en ben daar nog van aan het afkicken.

Familie en vrienden hebben het hele project met interesse gevolgd en voor de nodige afleiding gezorgd, en niet te vergeten alle goede zorgen en oppasuurtjes (papa, mama, Henk en Coosje heel erg bedankt). Nu kunnen jullie eindelijk lezen wat er schuil gaat achter de onuitspreekbare term poly(ethylene glycol) / poly(butylene tereftalaat). Tenslotte nog een paar speciale bedankjes voor de nauwst-betrokkenen.

Jeroen, met zo'n vriend als jij ben je zo rijk. We hebben alles al besproken en zijn toch nooit uitgepraat, wonderlijk. Nog 25 jaar te gaan tot de tweede beklimming van de Dr. Woodson Mountain.

Lieve Henk, Akke en Hendrik, dankzij en ondanks jullie is het toch gelukt. Nu kunnen we weer zo vaak wandelen als jullie willen. Ik ben zo verschrikkelijk wijs met jullie.

Riemke

Curriculum vitae

Riemke van Dijkhuizen-Radersma werd geboren op 30 november 1973 te Lelystad. Na het behalen van haar VWO diploma in 1992 aan het Lambert Franckens College te Elburg begon zij met de studie Chemische Technologie aan de Universiteit Twente. Na een stage bij het Instituut voor Agrotechnologisch Onderzoek (ATO-DLO) werd deze studie in 1997 afgerond met een doctoraal-opdracht bij de vakgroep 'Polymeerchemie en Biomaterialen' van Prof. Dr. J. Feijen. De afstudeeropdracht was getiteld 'Bereiding en karakterisering van eiwitbeladen PEG/PBT microspheres'. Van augustus 1997 tot oktober 1999 werkte ze bij Organon NV in Product Development Department aan de ontwikkeling van op polymeren gebaseerde contraceptives. In november 1999 trad ze in dienst van IsoTis BV om in nauwe samenwerking met Dr. Ir. J.M. Bezemer gecontroleerde afgifte systemen op basis van PEG/PBT copolymeren te ontwikkelen. Dit proefschrift beschrijft een gedeelte van het onderzoek dat is uitgevoerd binnen de groep PolyActive en de latere afsplitsing Chienna BV. Op 2 juli 2004 hoopt zij hierop te promoveren bij Prof. Dr. K. de Groot en Prof. Dr. C.A. van Blitterswijk aan de Universiteit Twente. Sinds juli 2003 is ze werkzaam als Scientist bij OctoPlus Technologies BV.