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POLY(LACTIDE-CO-GLYCOLIDE) IN CONTROLLED-RELEASE PHARMACEUTICALS – RELEASE MECHANISMS

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Abstract

Controlled-release formulations reduce the frequency of injections and better maintain plasma concentrations within the therapeutic window. Poly(D,L-lactide-co-glycolide) (PLG) is currently the most frequently used biodegradable polymer for this application. In order to be able to control the release rate, it is vital to know the underlying release mechanisms.

A novel method for measuring the diffusion of proteins and other substances through PLG films was developed. Size-exclusion effects were seen when studying the simultaneous diffusion of human growth hormone and glucose through the PLG films.

Divalent cations in the *in vitro* buffer, especially zinc cations, increased the rate of pore formation in PLG films. Encapsulated zinc acetate increased the effective diffusion coefficient of lysozyme through PLG films. The pore forming effect was probably due to Lewis-acid-catalyzed hydrolysis.

Pore closure was increased in an *in vitro* buffer with low pH, when using a low-M_w PLG with a relatively low degree of hydrophobicity, or at high temperature. Pore closure may have been caused by: (i) polymer–polymer interactions driven by the hydrophobic effect, causing separation from water and rearrangement of the polymer chains, or (ii) polymer–water interactions that led to a more homogeneously swollen polymer mass, instead of distinct regions of polymer and pores. Polymer chain mobility is important in both cases. The highest porosity of PLG films was found at pH 5–6, probably due to rapid pore closure below and above these values and due to relatively rapid hydrolysis in this slightly acidic environment. The pH may be low during drug release due to acidic polymer degradation products and inflammatory reactions.

Studies based on diffusion measurements, confocal microscopy, and scanning electron microscopy suggest that there may be considerable transport resistance inside the polymer matrix during the first stage of degradation, while the most significant transport resistance is at the surface at a later stage.

Release mechanisms and processes that have been reported to govern drug release were analyzed and discussed in a review article. Diffusion through water-filled pores is the most common way in which a drug is released (in this thesis called a true release mechanism). Several processes have been found to determine the rate of drug release. Drug release is often preceded by a chain of processes, and the dominant process may change with time and space, and when a parameter is altered. This provides many ways of modifying drug release and solving specific problems during pharmaceutical development.

Key words Diffusion, Controlled release, Release mechanism, Poly(D,L-lactide-co-glycolide), Degradation, Pore formation, Pore closure, Divalent cations, Protein, hGH		
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Populärvetenskaplig sammanfattning

De senaste årens framsteg inom bioteknologin har medfört att det utvecklas allt fler proteinbaserade läkemedel. Detta innebär att vi har fått bättre mediciner mot allvarliga sjukdomar, men tyvärr måste proteiner nästan alltid tas med spruta, och ofta krävs långa behandlingar med dagliga injektioner. Det finns även andra grupper av läkemedelssubstanser som ökar i omfattning, men som sällan går att ge i form av tabletter. Genom ”kontrollerad frisättning av inkapslade aktiva substanser” kan man minska antalet injektioner och på så sätt underlätta för patienter. Den aktiva substansen kapslas in i en polymer och läcker sedan ut. Ofta kapslar man in läkemedlet i mikropartiklar som sedan injiceras, t.ex. under huden. Man injicerar en stor dos, som räcker under en förutbestämd tid, och den aktiva substansen läcker ut ur mikropartiklarna och tas upp i blodet.

Läkemedel i form av kontrollerad frisättning av inkapslade aktiva substanser har flera fördelar jämfört med tabletter eller injektioner av den aktiva substansen i lösning. Koncentrationen i kroppen hålls mer konstant, vilket gör att man undviker alltför höga koncentrationer, som ger biverkningar, och alltför låga koncentrationer, som inte ger tillräcklig effekt. Ett annat exempel där kontrollerad frisättning kan ge fördelar är vaccin. Vaccin måste ofta tas upprepade gånger för att ha effekt, men med kontrollerad frisättning av inkapslat vaccin kan det räcka med en gång. Inte minst för vaccinationsprogram i utvecklingsländer är detta en stor fördel. Ännu en fördel är att man kan utveckla läkemedel som är aktiva på rätt ställe i kroppen, genom att placera mikropartiklar eller någon annan form av implantat, med den aktiva substansen inkapslad, vid det aktuella stället. Man får då en stor effekt lokalt, vilket gör att dosen kan hållas låg. Ett annat sätt är att injicera mycket små partiklar, så kallade nanopartiklar, direkt i blodet. Ytan på dessa partiklar kan modifieras kemiskt så att partiklarna binder till ett visst ställe, t.ex. en receptor som finns på cancerceller, och på så vis få inkapslad cytostatika att läcka ut precis vid tumören.

Den polymer som används mest för att kapsla in aktiva substanser kallas poly(D,L-lactide-co-glycolide), förkortat PLG. Den bryts ner i kroppen till icke-giftiga ämnen, och mikropartiklar eller implantat behöver därför

inte tas bort. PLG är godkänd av läkemedelsmyndigheter runt om i världen.

Det är oerhört viktigt att man kan styra hur snabbt det inkapslade läkemedlet läcker ut, d.v.s. frisättningshastigheten, för att få rätt koncentration i kroppen. De faktorer som påverkar detta är komplexa. I mitt arbete har jag fokuserat på och kommit fram till bl.a. följande:

- En metod för att undersöka diffusion, d.v.s. transport, av en aktiv substans genom PLG. Metoden bygger på en s.k. diffusionscell.
- Salter med två positiva laddningar, s.k. divalenta katjoner, ökar bildningen av porer i polymeren, vilket gör att läkemedlet frisätts snabbare. Speciellt salt av zink visade stark effekt. Salter med sådana joner används ibland för att stabilisera inkapslat protein, och då är den porbildande effekten bra att känna till. Sådana salter finns även i kroppen, och detta kan vara en förklaring till varför inkapslat läkemedel ofta läcker ut snabbare i kroppen än när man studerar mikropartiklar i laboratorium.
- Ett annat viktigt fenomen som jag har sett och sedan studerat är att porer i polymeren kan förslutas, en process som man inte känner till mycket om. Jag fann att porstängning påverkades av pH, polymerens egenskaper och temperaturen. Resultaten pekade på två bakomliggande mekanismer för porstängning, beroende av pH. Porer bildades snabbast vid pH 5–6, medan porer stängdes snabbt vid lägre pH och vid fysiologiskt pH (7.4). Detta kan vara avgörande för frisättningshastigheten, eftersom pH ofta sjunker både i kroppen och i polymeren när den bryts ner.
- För att kunna modifiera frisättningshastigheten kan det vara viktigt att veta var i en polymerpartikel hastigheten är som lägst. Mina resultat pekar på att detta är i det inre av polymermassan under den första tiden efter injicering i kroppen, medan området vid ytan utgör det största motståndet för transport av det inkapslade läkemedlet efter en längre tid.
- Slutligen har jag skrivit en review-artikel, där jag har analyserat de frisättningsmekanismer och faktorer som har rapporterats i litteratur. För den som ska sätta sig in i ämnesområdet, kan denna review-artikel underlätta att förstå det komplexa system av faktorer och mekanismer som ligger bakom kontrollerad frisättning av aktiva substanser inkapslade i PLG.

Resultaten är baserade på experiment utförda framförallt på filmer av PLG. De metoder som användes var bl.a. diffusionsmätning m.h.a. en diffusionscell, svepelektronmikroskopi, konfokalmikroskopi för att mäta pH inuti polymeren och för att lokalisera fluorescerande prober, differential scanning calorimetry, vattenupptags- och massförlustsmätningar genom vägning, samt frisättningsstudier på tillväxthormon inkapslat i PLG-dragerade mikropartiklar.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals. The papers are appended at the end of the thesis.

I. **Fredenberg, S.**, Reslow, M. and Axelsson, A.
Measurement of protein diffusion through poly(D,L-lactide-co-glycolide)
Pharmaceutical Development and Technology, 2004, 10(2), 299-307.

II. **Fredenberg, S.**, Reslow, M. and Axelsson, A.
Effect of divalent cations on pore formation and degradation of poly(D,L-lactide-co-glycolide)
Pharmaceutical Development and Technology, 2007, 12(6), 563-572.

III. **Fredenberg, S.**, Reslow, M. and Axelsson, A.
Encapsulated zinc salt increases the diffusion of protein through PLG films
International Journal of Pharmaceutics, 2009, 370, 47-53.

IV. **Fredenberg, S.**, Wahlgren, M., Reslow, M. and Axelsson, A.
Pore formation and pore closure in PLG films
Journal of Controlled Release, in press, available on line since 19 November 2010.

V. **Fredenberg, S.**, Jönsson, M., Laakso, T., Wahlgren, M., Reslow, M. and Axelsson, A.
Development of mass transfer resistance in poly(lactide-co-glycolide) films and particles – a mechanistic study
International Journal of Pharmaceutics, 2011, submitted.

VI. **Fredenberg, S.**, Wahlgren, M., Reslow, M. and Axelsson, A.
The mechanisms of drug release in poly(lactide-co-glycolide)-based drug delivery systems – a review
International Journal of Pharmaceutics, 2011, submitted.

My contributions to the papers

I. I did essentially all the work.

II. I did essentially all the work, except that Pia Lindberg carried out some of the experiments.

III. I did essentially all the work.

IV. I planned the study. I carried out the SEM-analysis and some of the measurements of the glass transition temperature and wettability. The remaining measurements of the glass transition temperature and wettability were carried out by Charlotte Fröhberg, who also carried out the measurements of water absorption and mass loss. I analyzed the data and wrote the paper.

V. I did essentially all the work, except the manufacturing of the PLG-coated microparticles, which was done by StratoSphere Pharma AB, and the *in vitro* release experiment was carried out by Alf Laurell.

VI. I did essentially all the work.

Related work

Licentiate thesis, 2004
PLG films in controlled release pharmaceuticals – Diffusion and degradation
Department of Chemical Engineering, LTH, Lund University, Lund, Sweden.

Dissertation for Master of Science in Chemical Engineering, 1999
Studies of the release rate from microspheres – *in vitro–in vivo* correlations
Department of Chemical Engineering, LTH, Lund University, Lund, Sweden.

"Life is like a box of chocolate. You never know what you´re gonna get."

(Forest Gump from the film Forest Gump, 1994)

Acknowledgments

In this very moment I am writing the last words of this doctoral project. I have written 300 laboratory reports, six papers, a number of abstracts and this thesis. These last words I write with the elated feeling of completing a long-term project, but also with a sentimental feeling, as it is the end of something I have enjoyed. Ten years have past since I started this project. Ten developing years, both professionally and personally, filled with valuable experiences. I never thought ten years would have past before I completed this project, and I never thought I would have three children during this time. I never thought I would have three children at all as a matter of fact. There are many things I have learned during this time. For example, if you sit several hours in front of a scanning electron microscope studying pores, you only see pores when you close your eyes at night, and you should never ever smell tetrahydrofuran. I have also learned that there are three things you need in order to accomplish something: some level of skill, you need to work for it, but you also need luck. And I have been lucky! During these ten years, I have met many people who have contributed to me being who and where I am today, and I want acknowledge some of them.

First of all **Professor Anders Axelsson**, head supervisor, who has been a real source of inspiration. I have learnt a lot from you, and you have the ability to bring out the best working spirit in me. When I was considering continuing this project after licentiate, being pregnant with my first child, I would probably not have chosen to continue if another professor was the head supervisor.

Dr Mats Reslow, co-supervisor and my former boss at SkeyPharma, continued to be co-supervisor although having a new job after the close-down of SkyePharma. For that I am very thankful. You have contributed to this project with very good ideas, and I have learned a lot. You have my deepest respect and you are a person I trust.

Professor Marie Wahlgren has been co-supervisor the second half of this project, which I am very glad for. You have contributed with knowledge in physical chemistry that has been very useful. You are also a source of inspiration, and I really appreciate all the (immediate) help I

have got, whether it concerned reading long manuscripts or doing battle with Word-documents.

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First of all the "girl-gang", **Jeanette, Annica, Maria, Sara, Suzi, Marie and Jenny**. So many fun moments we have had! You mean a lot to me!
Anna, who has been a close friend since the beginning of the chemical engineering studies, and who is in some ways like a twin sister. All these lunches during my time Lund have really improved the days!
Karin, how many sms:s have we sent each other? Your friendship is one of the most meaningful ever. I could write so much here, but perhaps I´ll send an sms instead☺.

Rozana, Helena and **Sandeep**, former colleagues at SkyePharma. I am very glad that we still see each other, although Sandeep lives far away now. Always a lot of laughter when we meet!
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My **mother** and **father**, without you I would not be where I am to day. You have supported me with different things during my life, which I hold very valuable (not the least baby sitting)! The perhaps most important thing about parents is unconditional positive regard for their children, something you really have shown.

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My dear children, **Filip, Sara** and **Carolina**, the most precious people in my life. You are too young to be able to read this now, but I hope that you one day understand how happy and proud I am of you. How I longed for you, and when I got you, you were just perfect! Every day when I´ve come home during this time when I have written the last papers and the thesis, you have run to the door and in happiness shouted "Mamma"! Sara, you have every day asked me, in your sweet happy voice: "Mamma, har du jobbat klart?" Yes, Sara, in this very moment my work is done!!

Abbreviations

Abbreviations	Full text
Da (g/mol)	Dalton
DDS	Drug delivery system
D_e	Effective diffusion coefficient
DSC	Differential scanning calorimetry
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), a buffering agent
hGH	Human growth hormone
M_w	Molecular weight
NMR	Nuclear magnetic resonance, used for self-diffusion measurements according to Fourier transform pulsed-gradient spin-echo method
PBS	Phosphate buffered saline
PLA	Poly(lactic acid)
PLG	Poly(D,L-lactide-co-glycolide)
PVDF	Polyvinylidene fluoride
SEM	Scanning electron microscopy
T_g	Glass transition temperature
TMR-dextran	Tetramethylrhodamine coupled to dextran

Contents

1 Introduction

1.1 Controlled release of encapsulated drugs

Controlled release formulations are advantageous in many ways regarding drug delivery. Biopharmaceuticals, such as proteins and peptides, is an expanding group of pharmaceuticals claiming increasing market shares [1,2]. These are large hydrophilic compounds with low oral bioavailability, and administration by injection is almost always necessary, which leads to discomfort for the patient. Another growing group of pharmaceuticals often requiring administration by injection is low-molecular-weight hydrophobic drugs, which also have low oral bioavailability [3]. The use of controlled-release formulations enables the frequency of injections to be reduced, which is beneficial to patients, especially those who require daily or long-term treatment.

Another advantage of controlled-release formulations is that they result in a more constant plasma concentration of the drug, which is better kept within the therapeutic window. Frequent administrations often result in rises and falls in the concentration. Too high a concentration can cause unwanted side-effects, while too low a concentration results in the loss of therapeutic effect. This means that lower total doses may be sufficient with the controlled-release formulations [4].

The drug delivery system (DDS) itself may also be designed to give other advantages. Targeted delivery of drugs encapsulated in nanoparticles may be possible by conjugating a molecule with an affinity for a particular target to the particles, such as cancer cells [5]. Additives that enhance the effect of the drug, for example, by increasing the bioavailability or inhibiting drug resistance development, can be co-encapsulated [6]. Particles within a special particle size range can be actively taken up by cells [7,8], which enables intra-cellular drug delivery, especially beneficial for gene delivery [9]. Particles within a special size range may also trigger the immune system, which may make single-shot vaccines possible [10]. This would be of considerable advantage in developing countries.

A number of natural and synthetic polymers can be used for drug encapsulation and delivery. Poly(D,L-lactide-co-glycolide) (PLG) has attracted much interest during the past two decades, and is the most frequently used biodegradable polymer for this application [11].

1.2 Aims

In order to keep the plasma concentration within the therapeutic window, it is vital to be able to control the rate of drug release. It is therefore important to understand the underlying release mechanisms. Many different factors influence drug release, and the interactions between them are complex. The aim of the work presented in this thesis was to increase our understanding of drug release from PLG-based DDSs. Such knowledge increases the chances of developing pharmaceuticals rapidly, which is economically valuable, and to develop pharmaceuticals that are more advantageous for the patient.

The studies described in this thesis were mainly performed on PLG films without drugs encapsulated, which is a relatively simple system. The advantage of simple systems is that fewer factors influence the processes underlying drug release. Conclusions can be drawn more easily, and they are applicable also to more complex systems. However, the effect of a studied factor may be insignificant to other effects. The advantage of studying a more complicated system is that the knowledge obtained is directly applicable to that particular system. However, it is more difficult to differentiate between effects of different factors. Although the study of complex pharmaceutical DDSs is necessary for product development,

studying simple systems provides pieces of the puzzle of drug release. Such knowledge is, as mentioned, useful in pharmaceutical development.

One of the challenges of this work was the duration of the experiments performed, as complete polymer degradation requires months, even for a fast-degrading PLG. Therefore, experiments had to be planned thoroughly and run in parallel. This is one of the reasons why a low-molecular-weight, fast-degrading PLG mainly has been used. Such PLGs are also often used in pharmaceutical formulations, and higher-molecular-weight PLGs eventually degrade and become similar to low-molecular-weight PLGs.

This work is focused on *in vitro* studies. The *in vitro* conditions are more simple than the *in vivo*, which gives the same advantage regarding drawing conclusions as discussed above. Although the *in vivo* performance has to fulfill the therapeutic requirements at pharmaceutical development, in this mechanistic study, *in vitro* studies are preferable from a scientific, economical and not the least from an ethical point of view.

The overall aims of this work were to find methods of studying drug diffusion and transport properties, to increase our understanding of release mechanisms and drug transport, and to investigate factors that influence drug release. The work was focused on drug diffusion, pore formation and pore closure, and the influence of zinc and other divalent cations on transport properties.

1.3 Outline of this thesis

Chapter 2 provides background information on PLG in controlled-release formulations, together with a summery of the factors that influence drug release and release mechanisms. This is presented in some detail in Paper 6. The studies performed and the results found are discussed in Chapters 3 to 7, and thus give summarized versions of Papers 1 to 5, respectively. Chapter 8 is devoted to a discussion on release mechanisms, which also are reviewed in Paper 6. Finally, conclusions and suggestions for future work are presented in Chapter 9.

2 PLG – the degradation process and release mechanisms

2.1 PLG in controlled-release formulations and other applications

Many biodegradable polymers have been used as drug carriers. PLG belongs to the group of synthetic biodegradable polymers called polyesters [12]. Other groups are polyorthoesters, polyanhydrides, polyamides, polyalkylcyanoacrylates and polyphosphazenes. Some proteins, for example albumin and gelatin, and some polysaccharides, for example starch and chitosan, are natural biodegradable polymers that have been investigated for use in controlled release [12]. However, PLG is the most frequently used biodegradable polymer in the controlled release of encapsulated drugs, and the work presented in this thesis therefore focuses on PLG. Poly(lactic acid) (PLA) was included in this work as it constitutes a 100:0 PLG, i.e. 100% lactide units and no glycolic units.

PLG is of interest because of its biodegradability and biocompatibility, and the fact that it has been approved for parental use by regulatory authorities around the world. Several products have reached the market. PLG is degraded into its monomers lactic acid and glycolic acid in the presence of water. A schematic of its structure is shown in Figure 1. Another advantage of PLG is that tailoring the drug release profile is possible by selecting PLGs with appropriate properties, such as the

molecular weight (M_w), the lactide:glycolide ratio (L:G), and end-group capping. PLGs with a broad range in such properties are commercially available. The duration of drug release can be controlled from hours [13,14] to several months [15-17]. Blending or co-polymerizing PLG with other materials, or encapsulating PLG microparticles in gels, further extends the possibility of controlling drug release [18-20]. Examples of such materials are polyethylene glycol, [21], polyethylene oxide [22], poly(fumaric-co-sebacic) anhydride [23], poly(ε-caprolactone) [24,25] and poly(L-lysine) [26]. There is also a more hydrophilic derivate of PLG; poly(D,L-lactic-co-hydroxymethyl glycolic acid) (PLHMGA) [27].

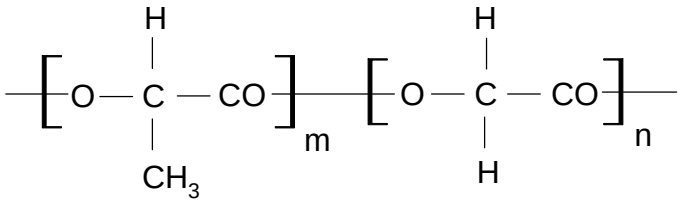


Figure 1. The structure of PLG; showing lactide to the left and glycolide to the right.

The disadvantage associated with PLG is the production of acids upon the hydrolysis of ester bonds, as is the case in many other biodegradable polymers. Proteins may undergo structural changes resulting in biologically inactive forms in acidic environments [28]. Stabilization of acid-sensitive drugs is an area of intense research [29-31], and a number of techniques for counteracting the effect of acidic polymer degradation products can be used. Basic salts, which neutralize the acids, can be co-encapsulated [32]. The cations of such salts are often divalent cations, which have been found to inhibit acylation of peptides [33,34]. PLG can be co-polymerized or blended with more hydrophilic polymers in order to reduce the acidic climate. Small DDSs or hydrophilic cores coated with PLG, which lead to small acid gradients, can also be used.

Among the different forms of PLG-based DDSs, microspheres, also called microparticles, are the most common. Microspheres may take the form of reservoirs, consisting of a drug core and a release-controlling shell [35], or matrix systems, with the drug dispersed throughout the polymer matrix [36]. Other forms of DDSs are nanoparticles, films, cylinders, scaffolds, foams, fibers, and coatings on stents [37-43]. *In situ-*

forming implants or microparticles are DDSs that are formed inside the body upon the injection of a PLG solution [44]. PLG is often mixed with other polymers in such formulations [45]. PLG can be used for wound dressings, and controlled release of antibiotics encapsulated in the wound dressings is possible [46]. DDSs based on PLG can be designed not only for continuous drug release but also pulsed drug release [47,48]. Ryu et al. fabricated a DDS consisting of a slowly degrading bottom layer with holes for drug reservoirs, a rapidly degrading middle layer inhibiting burst release, and a slowly degrading top layer with exit holes with dimensions that governed the drug release [49]. Raiche and Puleo formed a multilayer DDS for pulsed drug release by compressing several drug containing and drug free PLG films with different additives [50].

The process of encapsulating drugs in particles is usually based on emulsion methods [11,51]. The drug is dissolved or suspended in a PLG solution, or an aqueous solution of the drug is emulsified into the PLG solution which, in turn, is emulsified into an outer aqueous phase. The solvent is extracted and evaporated, and the PLG droplets harden into particles. To manufacture films, PLG solutions are often cast onto a plate, and the solvents removed by drying [38]. Spray techniques and compression molding are sometimes used to form particles and cylinders, respectively [52,53]. Drug molecules may also be encapsulated by self-healing, or pore closure, following drug influx upon the immersion of porous particles in a drug solution [54].

Microparticles are normally administered by subcutaneous or intramuscular injection. PLG implants may be surgically placed at the desired location, which gives the advantage of local drug delivery of, for example, antibiotics or anti-cancer drugs [39,55,56]. Nanoparticles can be injected intravenously and targeted delivery can be achieved by conjugating, for example, an antibody with an affinity for a particular receptor to the nanoparticles [5,57]. This technique has proven useful in tumor targeting [6]. Nanoparticles are usually concentrated in tumor vasculature because of the vascular structure surrounding tumors [58], which makes them appropriate for anti-cancer therapy. Active cellular uptake of nanoparticles enables intra-cellular drug delivery [7,8]. This is an advantage in gene delivery [9], and is also useful in the delivery of drugs across the blood–brain barrier [59]. Cellular uptake may also be an advantage in vaccine delivery [60]. Oral delivery to lymphatic tissues via

Peyer's patches is possible due to the uptake of nanoparticles by M-cells [61,62]. There have also been reports of topical delivery using PLG nanoparticles and microparticles [63,64], intranasal delivery [65], ocular delivery [66], and pulmonary delivery of porous microparticles [67]. Drug delivery of magnetic nanoparticles using a magnet has also been reported [68]. As mentioned in Section 1.1, additives may afford the DDS special functions. For example, surface modification with a muco-adhesive additive is useful in oral and nasal delivery, as the retentions time is prolonged [69,70].

The drugs encapsulated in most of the PLG-based products on the market are hormone agonists or antagonists, but there is also a product based on an antipsychotic drug [71]. Numerous drugs have been encapsulated in PLG-based DDSs with demonstrated therapeutic effects *in vivo*, or plasma concentrations considered suitable for therapy. Examples of such drugs are anticancer drugs [41,72], analgesic drugs [13,14], antibiotics [73], anti-inflammatory drugs [74], drugs for gene therapy [75], contraceptives [76] and vaccines [77]. Other examples are drugs against Alzheimers disease [78], diabetes [79], and growth hormone deficiency [4].

PLG has also been found useful in areas other than the controlled release of encapsulated drugs. Scaffolds of PLG are often used in tissue engineering [80,81], for example, in neural repair [82], and PLG has been found to facilitate bone healing [83]. PLG micro- and nanoparticles also have potential within diagnostics by encapsulating substances emitting fluorescence, magnetic resonance and near infrared radiation [84]. Another interesting area of use for PLG is the delivery of vaccines. Vaccines are usually given by repeated injections, first a primer and later a second, and possibly third booster to assure an immune effect. Single-shot vaccines are possible by encapsulating the antigen, or presenting it on the surface of PLG particles, which would lower the cost of vaccination, and thus be of particular interest in developing countries. The booster effect may be achieved by long exposure to the vaccine, as a result of release over a period or a pulsed release, or possibly by the PLG particles acting as an adjuvant, enhancing antibody response [10,77,85]. It is also possible to encapsulate immune potentiators to enhance the immune response [86].

2.2 Factors influencing drug release

The release of drug substances from PLG formulations is affected by a number of physico-chemical processes that enhance or inhibit drug release, for example, water absorption, pore formation and polymer–drug interactions. The properties of the DDS and the properties of the surrounding environment influence these processes. Examples of properties of the DDS are the polymer molecular weight, the encapsulated drug and the size of the DDS.

2.2.1 Processes that enhance or inhibit drug release

The first process that takes place upon immersion in water or administration *in vivo* is water absorption by the polymer. The rate of water absorption, or hydration, of the DDS, is highly dependent on the properties of the polymer, such as the M_w, the lactide:glycolide ratio and end-group capping [87,88], However, hydration is rapid compared to drug release [89,90]. Any volume occupied by water inside the polymer matrix can be regarded as a pore, and water absorption is therefore a pore-forming process. These pores are too small for drug transport during the early stage of this process, but as the number and size of water-filled pores in the polymer increase, a porous connected network is formed allowing drug release [91,92]. Cracks may be formed upon rapid water absorption, leading to burst release.

PLG is hydrolyzed in the presence of water, which results in a lower M_w. Ester bond scission is considered to be random (in contrast to end-group scission), although glycolic units appears to be more susceptible to scission as they pose less sterical hinder [88,93] (see Figure 1). Hydrolysis creates acids, which in turn catalyze hydrolysis [94]. This auto-catalytic phenomenon is known to cause heterogeneous degradation inside PLG matrices [95], i.e. faster degradation at the center of the PLG matrix than at the surface. This effect becomes more pronounced with increasing dimensions of the DDS [96], as the acid gradient increases, but heterogeneous degradation has also been reported in particles and films with dimensions as small as 10 μm [97,98]. Hydrolysis may also be catalyzed by bases [99]. The polymer becomes less hydrophobic with decreasing M_w, and at 1100 Da the oligomers become water soluble [100].

Erosion, i.e. mass loss, of the polymer starts when dissolved polymer degradation products are able to diffuse into the release medium. This process has been reported to start at an average M_w of 15 000 Da [101]. PLG normally undergoes bulk erosion in contrast to surface erosion, as PLG is relatively rapidly hydrolyzed [102]. The dissolution of polymer degradation products and erosion create pores. Small pores, formed by water absorption or polymer erosion, grow as contact with water leads to hydrolysis, and the locally produced acids catalyze degradation and cause polymer dissolution inside the pores, leading to subsequent erosion. Small pores consequently grow, and eventually coalesce with neighboring pores to form fewer, larger pores [89]. A dissolved drug molecule can be released when a pore of sufficient size for drug diffusion leads from the drug molecule to the surface of the DDS, according to the so-called percolation theory.

The dissolved PLG degradation products affect the system until released from the DDS by: (i) catalyzing hydrolysis, (ii) increasing the osmolality, (iii) possibly crystallize and (iv) plasticizing the polymer. The degradation products catalyze hydrolysis as they are acids. There have been reports of pH values of approximately 3, and even as low as pH 1.5, inside particles [103,104]. An acidic microclimate may induce drug–drug interactions such as aggregation [105], and polymer–drug interactions, such as acylation and deamidation [30,106]. These processes, may lead to incomplete drug release or a decrease in the release rate, at least for proteins in their native form. The dissolved monomers and oligomers increase the osmolality inside the DDS, and thus the force driving water absorption. The crystallization of oligomers has been reported [107,108]. Whether oligomers are able to crystallize or not depends on the irregularity of the polymer chain structure, i.e. the blending of L-lactide, D-lactide and glycolide units [109]. Crystallization inhibits hydrolysis and transport through the polymer. However, to the best of the author's knowledge, no study has been performed demonstrating that this process actually governs the rate of drug release. The plasticizing effect of the degradation products decreases the glass transition temperature (T_g) by increasing chain mobility [110].

The original polymer is in the vitreous state, as the T_g is above 37°C. However, upon immersion in water at 37°C, the plasticizing effect of water usually transfers the polymer into the rubbery state [90,111,112]. The transport resistance is lower for PLGs in the rubbery state, and

hydrolysis proceeds more rapidly [113]. The T_g decreases with decreasing M_w [114]. Increased polymer chain mobility may lead to faster drug diffusion through the polymer, and faster hydrolysis [110]. It may also lead to pore closure, also called self-healing, due to rearrangement of the mobile polymer chains [115,116] (Paper IV).

The dissolved degradation products are released by polymer erosion, which means that their effect on the system ceases as they are released. The onset of rapid erosion often coincides with a cessation of the decrease in the average M_w and T_g, as the low-M_w fraction of polymer chains is released, and the effects of dissolved degradation products cease [117]. Transport resistance is thus not only important for the release of the encapsulated drug, but also for the polymer degradation kinetics. Pore formation and pore closure are two very important processes influencing transport resistance. Drug transport occurs mainly through water-filled pores, as the encapsulated drug is often a protein or a peptide, too large and too hydrophilic for transport through the polymer. Small hydrophobic drugs are able to diffuse through the polymer, but they must still be dissolved in water before being released, and high porosity increases the area for dissolution. Drug dissolution may, therefore, govern the rate of drug release [36]. The processes discussed in this section and their effects on other processes and release mechanisms are summarized in Figure 2.

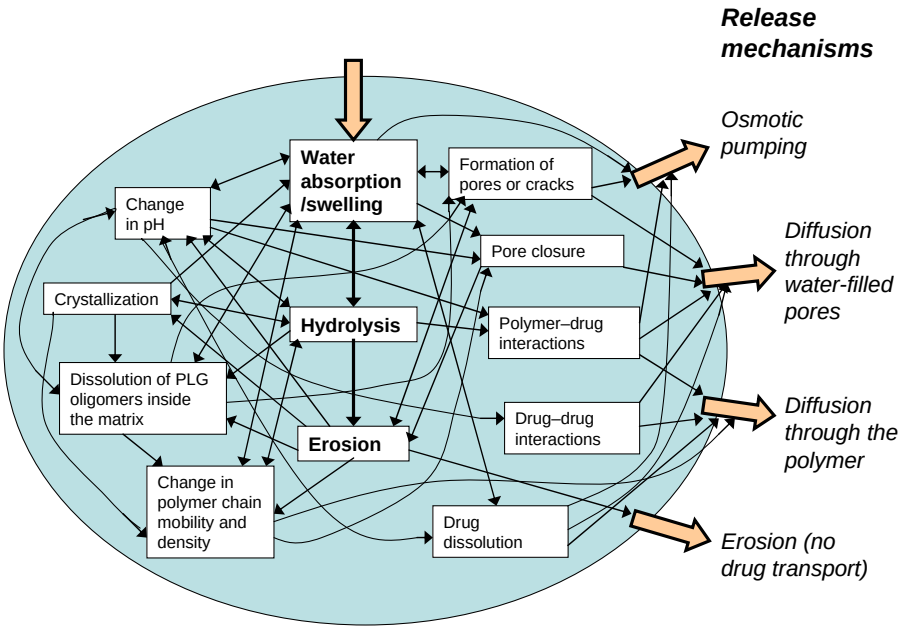


Figure 2. The complex system of physico-chemical processes taking place within PLG matrices, leading to drug release. The influence of processes on drug release and on other processes is illustrated by arrows. Note that some arrows point in both directions.

2.2.2 Properties of the DDS and the surrounding environment that influence drug release

One method of controlling drug release is to select PLGs with the appropriate properties. Low M_w, low L:G ratio and un-capped polymer end groups are properties that make the polymer less hydrophobic and increase the rate of water absorption, hydrolysis and erosion [97,100,118]. The molecular weights of PLGs used in controlled-release formulations are usually relatively low, often less than 50 kDa, and very seldom above 150 kDa. PLGs with molecular weights less than 10 kDa are sometimes used. The L:G ratio ranges from 50:50 to 100:0. The polymer end groups may or may not be capped with a hydrophobic ester group, for example, a stearyl group [119]. The initial T_g and polymer chain mobility are dependent on these properties. Polymers with only the L-lactic acid may be semi-crystalline [88]. As mentioned above, polymer

chain mobility and crystallinity influence the transport properties and degradation kinetics.

The encapsulated drug and additives may affect many of the processes listed in Section 2.2.1. The drug may plasticize the polymer [120], form crystalline regions in the DDS, and affect the rate of water absorption due to its hydrophilicity/hydrophobicity. It may also catalyze hydrolysis or neutralize acids depending whether it is an acid or a base. As the drug dissolves inside the DDS, the osmolality increases and thus the force driving water absorption. The drug load, i.e. the drug-constituting fraction of the DDS, may be important as the space left vacant after drug release will probably constitute pores, facilitating further drug release [121]. The location of the drug, which might be influenced by the physico-chemical properties of the drug, has also been found to influence drug release [122,123,124]. Drug molecules close to the surface are released more easily than those closer to the center. The M_w of the drug molecule and the shape of proteins influence the rate of diffusion [125]. Salts, plasticizing agent and surfactants are common additives. These may influence drug release in the same way as the drug, depending on their hydrophilicity/hydrophobicity, acid/base properties, plasticizing or surface active properties, and by acting as porogens [32,126-28]. Surfactants may disrupt the adsorption of the drug to the polymer [129]. Salts with a basic anion are sometimes encapsulated in order to neutralize acids. Divalent cations, especially zinc salts, are sometimes used to stabilize proteins [4,33,130-132]. Divalent cations, again especially zinc cations, may be pore forming, as they probably catalyze hydrolysis (Papers II and III). Dissolved salts may also shield ion interactions between the drug and the polymer, and between drug molecules.

The characteristics of the DDS, such as the porosity and the polymer density, are important [111,133,134]. Large DDSs result in an increase in the pH gradient, enhancement of the auto-catalytic effect on degradation [104], and an increase in the diffusion distance [135]. The ratio of the surface area to volume affects the release of both the drug and the PLG degradation products [124]. Most of the properties characterizing the DDS are influenced by the manufacturing method [136].

The environment, *in vitro* or *in vivo*, also affects the processes that influence drug release. Increased temperature increases all chemical

reactions, but also increases the mobility of the polymer and, thus, possibly the rate of pore closure. An unstirred surface layer surrounding the DDS may inhibit drug release. Sink conditions are particularly important for hydrophobic drugs with low water solubility [3]. Salts, plasticizing agents and surfactants in the release medium may affect the processes in the same way as if they were encapsulated, but with the exception that high osmolality in the release medium would decrease the rate of water absorption by the DDS [109,137,138]. The pH or buffering capacity is not only important for the rate of degradation [139], but also for the rate of pore formation and pore closure (Paper IV). The *in vivo* environment differs from *in vitro* conditions due to the presence of endogenous enzymes and lipids, and the possibility of non-sink condition, lower pH and immune responses. In one of my previous studies, the solubility of the water-soluble fraction of a low-M_w PLG was found to be higher in serum than in HEPES buffer, which is a common *in vitro* buffer [140]. Adding phospholipids to the HEPES buffer increased this solubility. The conditions must therefore be considered when designing an *in vitro* release method. The assembling of macrophages around the DDS is an immune response, and the phagocytosis of small microparticles and the release of acidic products by these cells may increase the rate of degradation [141]. The formation of a fibrous capsule around injected particles, which may decrease the pH due to acidic degradation products, has also been reported [72]. Faster polymer degradation and drug release, and a shorter drug-release lag phase have been reported *in vivo* [142,143], and were attributed to the differences in the *in vitro* and *in vivo* environment mentioned, and possibly to merging of microparticles [47,144].

Table 1. Properties of the DDS and the surrounding environment that influence the processes occurring in PLG.

Factor	* Process
The polymer	
Molecular weight	wa, h, e, pdi, ddp, pcm
Monomer ratio	wa, h, c, pc, pdi
End-group capping	wa, pH, pc, pdi
Semi-crystallinity	wa, h, pcm
Encapsulated substances	
The characteristics of the drug	wa, pH, diff, dd, pdi, ddi, pcm
Drug load and location	pf, did
The characteristics of additives, such as salts, surfactants and plasticizing substances	wa, h, pH, pcm, pf, pdi, ddi
The DDS	
Size	wa, pH, e, did
Porosity	wa, e, diff
Polymer density	pcm, diff
Shape	wa, e, diff
***In vitro* conditions**	
Temperature	wa, h, e, pcm, dd, diff
Stirring	dd, diff
Composition of the release medium	h, pH, pcm, dd, pdi
pH	pH, dd, pc
Osmolality	wa
***In vivo* conditions**	
Sink condition	dd, diff
Enzymes	h
Lipids	pcm, dd
Immune responses	h, pH

* wa – water absorption, h – hydrolysis, e – erosion, pdi – polymer–drug interactions, ddp – dissolution of polymer degradation products, pcm – polymer chain mobility, c – crystallization, pc – pore closure, diff – diffusion, dd – drug dissolution, ddi – drug–drug interactions, pf – pore formation, did – diffusion distance

The combination of the effects of the processes illustrated in Figure 2 and the effects of the factors that may influence drug release and are summarized in Table 1, leads to a complex picture of drug release. Three things should be borne in mind when discussing drug release and release mechanisms.

- PLGs with different molecular weights, L:G ratios and end-group capping exhibit very different physico-chemical behavior. As PLG is degraded, hydrophobic, high-M_w and slow-degrading PLGs will eventually become more hydrophilic, low-M_w and fast-degrading PLGs. The properties of the polymer and their effects on drug release thus vary with time.
- PLG matrices are heterogeneous systems. The effect of a factor is local. The properties characterizing the DDS may thus vary in position.
- Many processes often precede drug release, and these may affect the system in several ways. The dominant process may vary in time and space.

2.3 Modification of the release profile

Zero-order release is normally the desired release profile. However, drug release is more commonly bi-phasic, and probably even more commonly tri-phasic [145]. An initial burst release is often seen, and this is often attributed to non-encapsulated drug particles on the surface, or drug molecules close to the surface easy accessible by hydration [146]. Other reasons for burst release may be the formation of cracks and the disintegration of particles [147]. One way of counteracting burst release is to disperse micro- or nanoparticles in *in situ*-forming gels [148].

In order to modify the different phases of bi- or tri-phasic release profile to obtain more linear drug release, it helps understanding the processes governing drug release in these phases. In general, drug release is enhanced by co-encapsulation of hydrophilic substances, acidic substances that catalyze hydrolysis and salts that increase the osmolality [19,31,149]. Low-M_w uncapped PLGs with a low L:G ratio generally promote rapid drug release. Other approaches include blending PLG with more hydrophilic polymers, adjusting the manufacturing parameters in order to increase the initial porosity, or making small PLG particles. In contrast, drug release is inhibited by the encapsulation of hydrophobic

substances and bases. However, very basic substances can catalyze degradation. High-M_w capped PLGs with a high L:G ratio degrade slowly, normally leading to slow drug release. Small particles often exhibit a bi-phasic release profile with a relatively rapid second phase [145,150]. Large particles or DDSs often exhibit sigmoidal release profiles [151,152]. The small specific surface area and long diffusion distance in such systems result in slow diffusion until the build-up of the auto-catalytic effect on degradation and erosion becomes pronounced. Combining particles of different sizes has been shown to offer a means of altering the drug release profile, from a Fickian diffusion profile and a sigmoidal profile, to a zero-order profile [153].

2.4 Release mechanisms

Many studies have been carried out with the purpose of improving our mechanistic understanding of the drug release from different PLG-based DDSs, and several physico-chemical processes have been identified as the rate-controlling process in drug release. The two main mechanisms associated with drug release from PLG-based DDSs are diffusion and degradation/erosion. The release rate is often said to be diffusion-controlled initially, and degradation/erosion-controlled during the final stage of drug release [154-159]. Other examples of processes that have been reported to be release mechanisms or to govern drug release, are: dissolution of the drug, diffusion through the polymer matrix, hydrolysis, osmotic mediated events, polymer–drug interactions, drug–drug interactions, pore closure, heterogeneous degradation, the formation of cracks, the deformation of the DDS, polymer relaxation processes, and water absorption/swelling [14,31,36,91,98,115,160-164].

The term "release mechanism" has been defined in slightly different ways. It has been used as a description of the *way* in which drug molecules are transported or released [128,150], and as a description of the process that determines the release *rate*. Most of the examples given above are such rate-controlling processes.

There are only three possible ways for drug molecules to be released from a DDS: (i) transport of the dissolved drug through water-filled pores, (ii) transport through the polymer, and (iii) due to dissolution/erosion of the encapsulating polymer (which does not

require drug transport). Erosion is frequently reported as the main release mechanism, as pores are created and increase the rate of diffusion. However, this implies subsequent drug transport through the pores. There is a difference between erosion leading to drug release without drug transport, and erosion that increases the rate of drug transport. Transport through the polymer phase may occur when the drug is small and hydrophobic [165,166]. The most common type of transport through water-filled pores is diffusion, i.e. random movements of the molecules driven by the chemical potential gradient, which can often be approximated by the concentration gradient. The other type of transport through water-filled pores is convection, which is driven by a force such as osmotic pressure [167]. Drug transport driven by this force is called osmotic pumping [168].

The three basic ways of drug release mentioned above, with two types of transport included in the transport through water-filled pores, result in four possible release mechanisms, if the term "release mechanism" is defined as the *way* in which the drug is released:

- diffusion through water-filled pores,
- diffusion through the polymer,
- osmotic pumping, and
- erosion (no drug transport)

These release mechanisms will be further discussed in Chapter 8.

The most common use of the term release mechanism is in referring to the process that determines the *rate* of release, for example, swelling, drug dissolution or polymer–drug interactions. As mentioned above, erosion can be included in both definitions, but with different meanings. When discussing release mechanisms with the purpose of modifying drug release, it is more informative to describe the process controlling the release rate than to describe the way in which the drug is released. Describing these processes is thus important. However, using these processes as release mechanisms leads to problems. Due to the complexity of the system, it is not always clear which of the processes is dominating, and in a chain of processes that leads to drug release it is not always obvious which is the rate-determining process. For example, the drug may be released by diffusion through water-filled pores, and the rate of pore formation may be the rate-controlling process. Polymer erosion, which is determined by the rate of hydrolysis, *probably* determines the rate of pore formation, although the absorption of water

also results in pores. The release mechanism could then be pore formation, erosion or hydrolysis. Water absorption should perhaps also be mentioned. This is probably one reason why so many different processes have been reported as the release mechanism, which does not help to clarify the complex picture of drug release.

In this thesis, the processes defining the ***way*** in which the drug is released will be called the ***true release mechanisms***, and the processes that control the release ***rate*** will be called ***rate-controlling release mechanisms***. The true release mechanisms are illustrated in Figure 3. Examples of rate-controlling release mechanisms are erosion, which increases rate of diffusion, and polymer–drug interactions, which decreases the rate of diffusion. An encapsulated drug released by a certain true release mechanism, for example diffusion through water-filled pores, is released in this way no matter if the degradation kinetics, the initial porosity or any other factor determines the rate of diffusion. True and rate-controlling release mechanisms should therefore be discussed separately. Knowing the true release mechanisms is useful when trying to identify the rate-controlling release mechanisms.

The true and rate-controlling release mechanisms could be compared to the established terms regarding mechanisms of diffusion, namely intrinsic and apparent diffusion [169]. Intrinsic is the true or inherent mechanism for diffusion, or pure diffusion. The apparent diffusion is the diffusion that can be measured and may depend on other phenomena, such as interaction between the diffusing solute and other materials. There are differences between these couples of terms, through. While diffusion through a porous network, causing an effective diffusion, would fall under the term apparent diffusion, diffusion through a porous network is the *way* in which an encapsulated drug is released and is thus a true release mechanism.

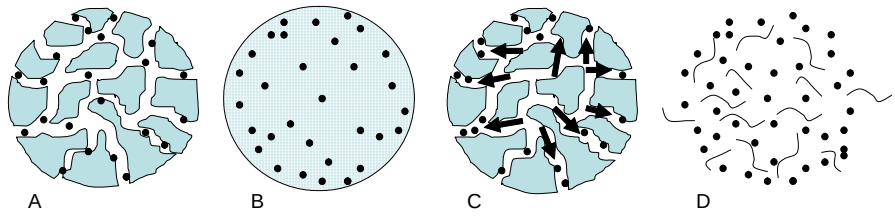


Figure 3. True release mechanisms: (A) diffusion through water-filled pores, (B) diffusion through the polymer, (C) osmotic pumping, and (D) erosion.

3 Diffusion measurements

To be able to study the diffusion of proteins through PLG, a novel method was developed employing a diffusion cell. The method developed for diffusion measurements can be used to compare PLG polymers with different properties, similar polymers, different polymer compositions, the effect of addition of surfactants or other substances, and the diffusion of different drugs. It can also be used to better understand the transport properties. This method is described in Paper I.

The diffusion cell was designed to accommodate pseudo-steady-state diffusion [170], which requires large volumes compared to the volume of the membrane, in this case the PLG film dividing the donor and the receiver compartments. Within the requirements for pseudo-steady-state diffusion, the diffusion area should be large, to allow rapid diffusion, but the compartment volumes should be small, as pharmaceutical drugs are expensive. Stirring should be sufficient to avoid stagnant surface layers, but without causing membrane erosion. Figure 4 illustrates the diffusion cell. Samples can be withdrawn for analyses through the tubes, or a fibre optic probe measuring UV absorption can be placed in the receiver compartment through the tube and connected to a spectrophotometer.

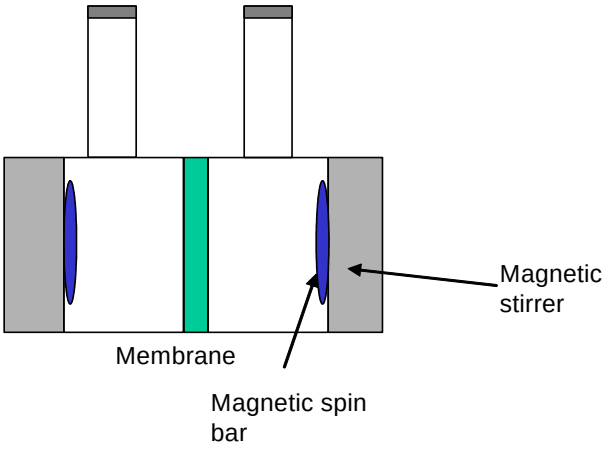


Figure 4. The diffusion cell.

3.1 Film preparation

A method of making thin films of PLG (7.0 ± 1.0 μm thick) by spraying was developed. Different spray parameters were studied. Analysis using scanning electron microscopy (SEM) confirmed that smooth, non-porous films could be made after optimization of the formulation and the spraying parameters [140]. Briefly, PLG films, containing 2% (w/w) polysorbate 80, were sprayed from solutions in ethyl acetate (1% PLG w/w) onto polyvinylidene filters (PVDF, pore size 0.65 μm, Millipore AB, Sweden) using a Hüttlin spray nozzle. Polysorbate 80 was added in order to mimic a pharmaceutical DDS utilizing microparticles coated with a PLG film [35]. Films without any detergent have been shown to differ in wettability from microparticles, as some of the detergent used in microparticle preparation remains on the microparticles [61,171]. The PVDF filters were mounted on a rotating wheel, and passed through the spray at predetermined intervals. Twelve films were made simultaneously to ensure reproducibility. The films were dried at ambient conditions for 1 day and in a vacuum chamber for 5 days.

3.2 Calculation of the diffusion coefficient

The theory of diffusion measurements using this kind of diffusion cell, and the calculation of the effective diffusion coefficient are described in Paper I. Briefly, the calculation is based on Fick's law:

$$j = -D_e \frac{dC}{dz} \qquad \text{Eq. 1}$$

The mass flux through the film, j (g/(m^{2}s)), is expressed in terms of the effective diffusion coefficient D_e. Pseudo-steady-state diffusion, a condition attained after a short time lag, is often applicable when using this kind of diffusion cell. Solution of Equation 1, together with a mass balance over the two compartments, results in [170]:

$$K = \frac{1}{S\left(\frac{1}{V_A} + \frac{1}{V_B}\right)} \times \frac{\ln\left(\frac{C_{A1} - C_{B1}}{C_{A2} - C_{B2}}\right)}{t_2 - t_1} \qquad \text{Eq. 2}$$

The subscripts A and B denote the donor and receiver compartments, respectively. Subscripts 1 and 2 denote sample numbers. K is a mass transfer coefficient, S is the diffusion area and t denotes time. V is the volume of the compartments, and C is the concentration. To increase the accuracy, many measurements should be made, and at different time points. When the logarithmic concentration ratio in Equation 2 is plotted against time, the value of K can be determined from the slope of the line.

As the PLG films were sprayed onto filters, the total diffusion resistance consisted not only of the resistance afforded by the polymer films, but also that by the filters. The total mass transfer resistance was thus the sum of the mass transfer resistances, according to Equation 3:

$$\frac{1}{K} = \frac{l_{film}}{D_{e\ film}} + \frac{l_{filter}}{D_{e\ filter}} \qquad \text{Eq. 3}$$

where l is the thickness of the film or filter. By rearranging Equation 3, the effective diffusion coefficient in the PLG film can be obtained by

simply subtracting the filter resistance (l_{filter} / $D_{e\ filter}$), which can be determined in the initial experiments.

3.3 Initial experiments

The stirring rate did not affect the diffusion of glucose through dialysis membranes (Spectra®Por, MWCO 3500, Spectrum Laboratories Inc., USA & Canada). These membranes were used in some of the initial studies because the variability between membranes is low. The stirring was sufficient to prevent stagnant layers at the surfaces. The lowest stirring rate tested, 150 rpm, was chosen in the subsequent measurements to minimize effects of erosion on the PLG films.

The reproducibility was evaluated by repeating measurements of glucose diffusion through the dialysis membranes. The standard deviation was 15%, which was considered to be sufficient, bearing in mind the large expected time-dependent variations due to swelling and degradation/erosion, and film-to-film variations expected.

The least number of time-dependent concentration measurements necessary to determine the effective diffusion coefficient accurately was 5. The diffusion coefficient was fairly constant when more than four samples were used.

The influence of a nylon filter (pore size 60 μm, Millipore AB, Sweden), which was clamped together with the PLG film for further protection against erosion due to stirring, was evaluated by comparing the diffusion coefficient with and without this filter. There was no difference in the diffusion rate when using and not using the nylon filter. Diffusion was too rapid to be measured when only the nylon filter divided the donor and receiver compartments, and it was therefore concluded that the nylon filter did not impose any noticeable mass transfer resistance.

Adsorption of the solutes onto PLG films, PVDF filters and nylon filters was studied by measuring the concentration of the solutes in phosphate buffered saline (PBS) before and after incubation of the films and filters at 37°C. Neither glucose nor human growth hormone (hGH) was adsorbed on these materials.

The dependence of D_e on the concentration of hGH up to 30 mg/ml was investigated by measuring the diffusion through the PVDF filter. The diffusion coefficient was independent of the concentration, probably because the ionic strengths of the PBS and HEPES buffers used were sufficiently high ($I_{PBS} \approx 0.15$ M, $I_{Hepes} \approx 0.16$ M). Ionic strengths of 0.1 M and 0.2 M have been shown to prevent the dependence of D_e on the concentration of lysozyme and bovine serum albumin in other studies. [172,173].

The transport resistance through the PVDF filter was calculated based on the effective diffusion coefficient of hGH at a concentration of 30 mg/ml. Similarly, D_e for glucose was determined at a concentration of 5 mg/ml. These values were used in Equation 3.

3.4 Diffusion of hGH through PLG films

The diffusion of hGH through PLG films composed of 75% RG502H (Boehringer Ingelheim Pharma KG, M_w approximately 12 kDa, 50:50 L:G) and 25% of RG576 (M_w approximately 80 kDa, 75:25 L:G) was measured. The PLG films were degraded for 14 days in HEPES buffer (see Table 3 in Section 4.1) with 1 mM $ZnCl_2$ and 0.1% poloxamer 188 NF before diffusion measurements. Diffusion measurements of hGH through a PLG film incubated for 5 days in PBS with 0.1% poloxamer 188 NF and a PLG film incubated for 8 days in a buffer denoted "plasma buffer", pH 7.4, were also carried out. The compositions of the plasma buffer was 60 mM HEPES buffer, salts similar to those present in plasma [174] (see Table 3), and 0.1% poloxamer 188 NF.

The diffusion coefficient of hGH through PLG films degraded for 14 days in HEPES buffer with $ZnCl_2$ was found to be 5.0×10^{-13} m^2/s. The standard deviation was 39%, which was higher than the standard deviation determined from the initial experiments (15%). However, the difference in diffusion coefficients at different stages of degradation, for different proteins, and different compositions of PLG, is expected to be much higher than the variation in repeated measurements. The variation is probably also present in PLG films used for controlled-release formulations, which results in a variation in the formulation. Such variation has been observed for polymer films of other materials used for single units making up a multi-pellet system [175,176]. No diffusion of

hGH was detectable through PLG films degraded for 5 days in PBS, or for 8 days in "plasma buffer". Continuous pores of sufficient size had probably not been formed at these stages of degradation, or the counteracting process of pore closure may have been more rapid.

3.5 Simultaneous diffusion of hGH and glucose

Simultaneous diffusion of hGH and glucose was measured through PLG films degraded for 10 days in HEPES buffer with $ZnCl_2$ and poloxamer NF188 at 37°C. The molecular weights of hGH and glucose are very different, 23 000 and 180 Da respectively. The ratios of the effective diffusion coefficients in the PVDF filters to the diffusion coefficients in water, were almost equal for both solutes (Table 2). This means that the porosity and the tortuosity of the PVDF filters affected the diffusion of these solutes the same way. However, an effect of size exclusion on diffusion though PLG films was observed. The ratio of the D_e in the PLG films to the diffusion coefficient in water, was lower for hGH than that for glucose (Table 2). These results suggest that a fraction of the pores were of insufficient size for diffusion of hGH but large enough for diffusion of glucose. It should be noted that the pore size distribution varies with time.

Table 2. Diffusion coefficients in water, in the PVDF filters, in the PLG films, and ratios of the effective diffusion coefficients to that in water.

	D_{aq} (10^{-10} m^2/s)	$D_{e\ filter}$ (10^{-11} m^2/s)	$D_{e\ film}$ (10^{-14} m^2/s)	$D_{e\ filter}/D_{aq}$	$D_{e\ film}/D_{aq}$
Glucose	* 8.2	22	67	0.24	0.00074
hGH	** 1.3	3.8	4.9	0.29	0.00038
Ratio Glucose:hGH	6.3	5.8	14	0.80	1.9

* [177,178], ** [125]

3.6 Conclusions

A novel method for measuring the diffusion of proteins through PLG films was developed and evaluated. A procedure for spraying thin, smooth, non-porous films of PLG onto PVDF filters was developed. The diffusion of hGH was measured and the reproducibility was

considered sufficient for studies of the diffusive properties of PLG films. The diffusion of hGH through PLG films was measured after porosity was induced using $ZnCl_2$ (Papers II and III), and the results indicated a considerable lag time before continuous pores of sufficient size were formed. An effect of size-exclusion on diffusion through the PLG films was seen when studying the simultaneous diffusion of hGH and glucose.

4 Effect of divalent cations on pore formation and degradation of PLG films

Drug release has been shown to depend on salts that may be used in buffers [92,127]. In order to obtain a good *in vitro–in vivo* correlation it is therefore important to consider the influence of buffer salts when performing *in vitro* experiments. Magnesium and calcium cations are present in the body, but rarely in *in vitro* buffers. Furthermore, basic salts, often with divalent cations, are sometimes co-encapsulated to stabilize the protein due to their acid neutralizing effect [131]. Divalent cations have also been shown to decrease the rate of acylation, and zinc cations have been found to stabilize proteins by forming complexes [33,130]. An investigation of the influence of buffer salts, especially divalent cations, on degradation was carried out. This investigation is presented in Paper II.

4.1 Materials and methods

PLG with an approximate molecular weight of 12 kDa and an L:G ratio of 50:50, (RG502H), was used.

PLG films, approximately 150 μm thick, containing 2% (w/w) polysorbate 80, were cast on glass dishes from polymer and detergent solutions in ethyl acetate. The films were dried at ambient conditions for

10 days and in a vacuum chamber for 7 days. Fluorescent probes attached to dextrans, (Oregon Green, which is pH sensitive, and tetramethylrhodamine, which is pH insensitive, both obtained from Molecular Probes, the Netherlands), were encapsulated in the middle of PLG films intended for pH measurements using confocal microscopy. See Paper II for more details.

Samples from these PLG films were degraded in different buffers at 37°C. The buffer was changed regularly to maintain a constant pH. At predetermined intervals, samples were subjected to analyses of water absorption (gravimetrically), mass loss (gravimetrically), pH of the buffer, and porosity (SEM). Some samples were studied with regard to pH inside the PLG matrix (confocal microscopy). The salt compositions of the buffers are given in Table 3.

Table 3. Buffers used in the experiments. All buffers were adjusted to pH 7.4.

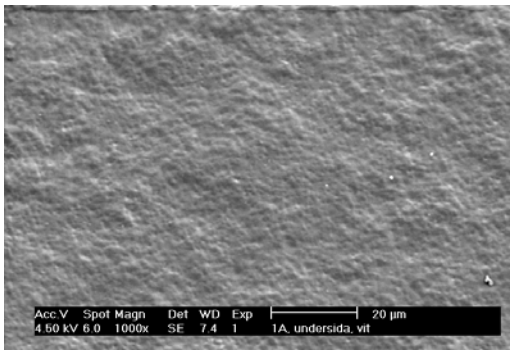
Buffer	**Composition**	**Osmolality (mmol/kg)**
HEPES buffer	37.5 mM HEPES acid, 37.5 mM Na-HEPES, 115 mM NaCl, 15.4 mM NaN_3	345
HEPES buffer + $ZnCl_2$	HEPES buffer, 1 mM $ZnCl_2$	344
HEPES buffer + $MgCl_2$	HEPES buffer, 1 mM $MgCl_2$	342
HEPES buffer + $CaCl_2$	HEPES buffer, 1 mM $CaCl_2$	353
HEPES buffer + Na_2CO_3	HEPES buffer, 1 mM Na_2CO_3	371
PBS	12 mM $NaH_2PO_4 \cdot H_2O$, 18 mM $Na_2HPO_4 \cdot 2H_2O$, 82 mM NaCl, 0.5 mM $CaCl_2$, 3 mM NaN_3	224
"Plasma buffer"	137 mM Na^+, 4.35 mM K^+, 2.1 mM Ca^{2+}, 1.8 mM Mg^{2+}, 0.8 mM SO_4^{2-}, 114 mM Cl^-, 0.7 mM PO_4^{3-}, 3 mM NaN_3, 60 mM HEPES	300

4.2 Results and discussion

4.2.1 Pore formation

The investigation showed that pores were formed faster in the presence of divalent cations. The surface porosity of films degraded for 1 day in

HEPES buffer with and without $ZnCl_2$, and in HEPES buffer with salts similar to those found in plasma (denoted plasma buffer) can be seen in Figure 5.



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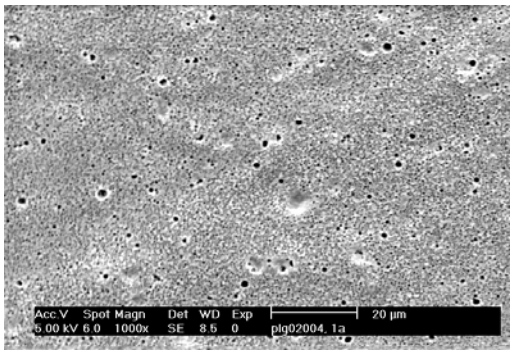

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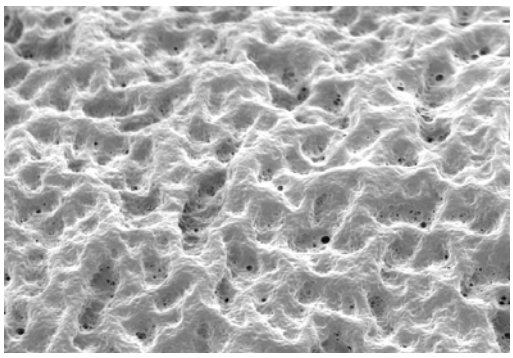


C)

Figure 5. The surfaces of PLG films degraded for 1 day in (A) HEPES buffer, (B) HEPES buffer with $ZnCl_2$, (C) plasma buffer. Magnification 1000x.

Table 4 gives the number of days of degradation required for pores to be visible at the surfaces according to SEM analysis. Pores were formed after only 1 day of degradation in the presence of all the divalent cations, while 7 days of degradation were required in the HEPES buffer without divalent cations. The porosity was greatest in the zinc-containing buffer, and zinc had stronger effect than magnesium and calcium in all of the analyses performed, which will be discussed in the next section. The individual concentrations of Mg^{2+} and Ca^{2+} in the plasma buffer were higher than in the HEPES buffers when these cations were added separately (see Table 3). Samples degraded in plasma buffer were also more porous. Thus, the pore forming effect seems to be dependent on the concentration of divalent cations. The addition of Na_2CO_3 resulted in slower pore formation, probably due to the acid-neutralizing effect of carbonate ions. Samples degraded in PBS became porous after 4–9 days. It was difficult to precisely determine the time required for pores to develop, as different areas differed in porosity. PBS also contained calcium ions, but at a lower concentration than in HEPES buffer with $CaCl_2$. Calcium ions could also have interacted with phosphate ions in PBS, resulting in an even lower concentration of free ions. PBS is the most common *in vitro* buffer. HEPES is another common buffer. These results show that pores were formed faster in a buffer with the same salt composition as that in plasma than in buffers commonly used for *in vitro* studies. Drug release has often been found to be faster *in vivo* than *in vitro* [142,143]. The presence of divalent cations *in vivo* is one possible explanation, among many others, as discussed in Section 2.2.2.

Table 4. The period of degradation before pores were visible at the surfaces. H.b denotes HEPES buffer

Buffer	**H.b**	**H.b + $ZnCl_2$**	**H.b + $MgCl_2$**	**H.b + $CaCl_2$**	**H.b + Na_2CO_3**	**Plasma buffer**	**PBS**
Time (days)	7	1	1	1	9	1	4-9

4.2.2 The explanation of faster pore formation

There are two possible explanations for the faster formation of pores when divalent cations were added to the *in vitro* buffers: (i) faster degradation, and (ii) increased PLG solubility (i.e. increased molecular weight limit for water solubility). Polymer erosion, or mass loss, was

faster in the presence of $ZnCl_2$, after a lag period, as can be seen in Figure 6.

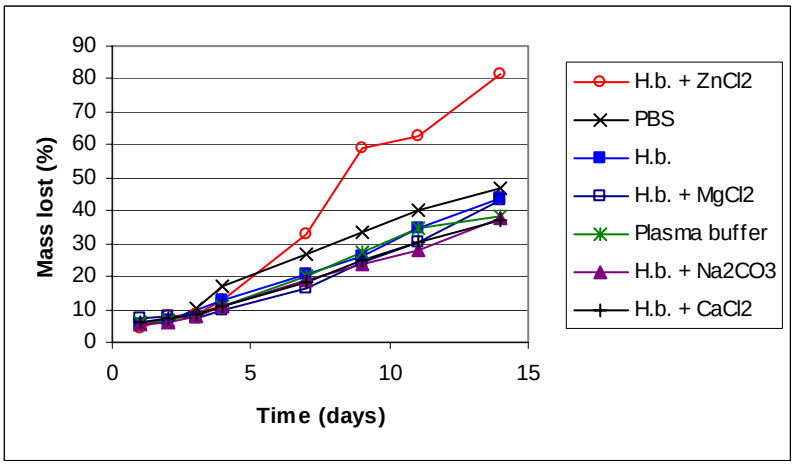


Figure 6. Mass loss of PLG films in the different buffers. H.b. denotes HEPES buffer. The deviation of duplicate samples from the average was about 5%, which means that the experimental error was insignificant.

The faster polymer erosion is an indication of faster hydrolysis. Another indication of faster hydrolysis was that the pH inside the PLG films, measured using pH-sensitive fluorescent probes and confocal microscopy, was lower when the films were degraded in the presence of $ZnCl_2$ (Paper II). It is likely that zinc cations act as a Lewis acid, thereby catalyzing hydrolysis. Zinc is known to be a Lewis acid, and it also had the strongest effect among the cations studied. Lewis acids act to varying degrees with different Lewis bases, depending on the frontier orbitals and the energy of the electrons participating in the bonding [179]. Zinc, which belongs to group IIB of the periodic table, differs in its electron structure from magnesium and calcium, which belong to group IIA. The results indicate increased rate of degradation. The degradation constant is sometimes determined by measuring the decrease in M_w with time. This was done, but no reliable data were obtained. Degraded samples of this low-M_w PLG could not be completely dissolved in any of the solvents or mixtures of water and solvents studied, not even after heating and long-term stirring.

In order to study the influence of the divalent cations on PLG solubility in the buffers, a large amount of PLG powder was incubated in the different buffers for a short period. The term solubility refers here to the water-soluble fraction of PLG. The undissolved PLG powder was removed and the dissolved oligomers were degraded into lactic and glycolic acids. The lactic acid concentration was then analyzed. The solubility of the water-soluble fraction was found to be decreased in plasma buffer and in HEPES buffer with $ZnCl_2$ (Figure 7). The effect of 1 mM $MgCl_2$ or $CaCl_2$ in HEPES buffer was minor, however, the effect was significant in plasma buffer where the ions were combined and present at higher concentrations. Again, zinc cations had the strongest effect. One possible explanation of the decrease in solubility could be that two water-soluble oligomers, or one oligomer and one polymer chain, bind to a divalent cation, and the complex becomes too large to be soluble in water. In another study on the effect of zinc chloride, it was suggested that the interaction between zinc cations and PLG disturbs the solvation sphere and decreases the rate of water absorption [180]. Indeed, in the present study, the rate of water absorption was initially slower when zinc cations were present in the buffer (Figure 8). These results show that the faster rate of pore formation can not be ascribed to increased solubility, or an increase in the water-soluble fraction of the polymer. The explanation of the faster pore formation seems to lie in the rate of polymer degradation.

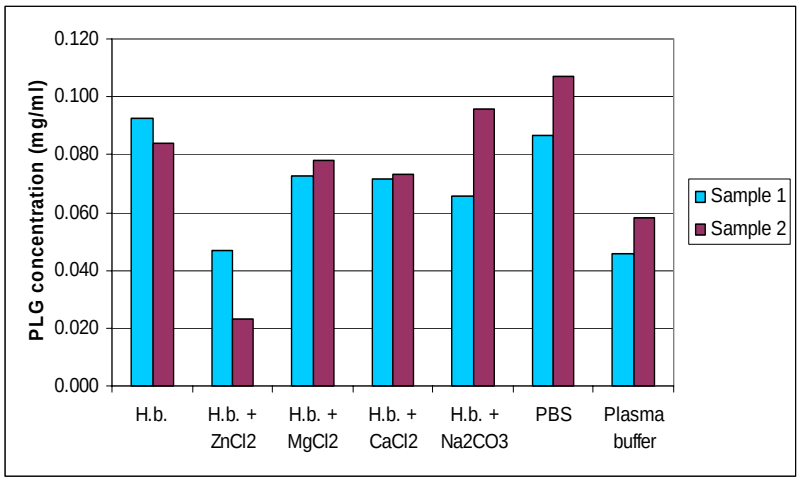


Figure 7. Comparison of the solubility of the water-soluble fraction of PLG in the investigated buffers. Samples 1 and 2 are duplicates.

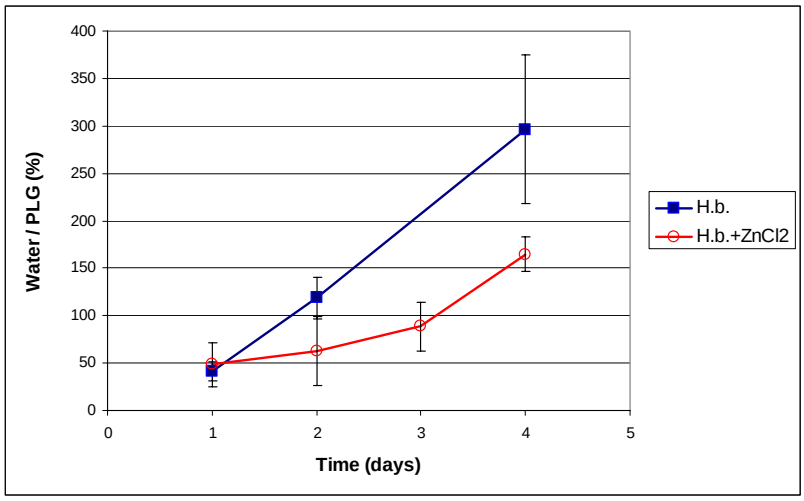


Figure 8. Water absorption of PLG films degraded in HEPES buffer (H.b.) with and without $ZnCl_2$. (n=2).

4.3 Conclusions

Divalent cations increased the rate of pore formation in PLG films. This was probably due to Lewis-acid-catalyzed hydrolysis. Zinc cations had the strongest effect, and polymer erosion was significantly faster, and the pH inside the PLG films lower, when zinc cations were present in the *in vitro* buffer. Salts of divalent cations are sometimes co-encapsulated in PLG formulations in order to stabilize proteins, and their pore forming effect should be borne in mind. The results also show that plasma buffer containing magnesium and calcium cations at the same concentration as in plasma, affected the formation of pores differently from PBS. This is one possible explanation, among several, of the problems associated with *in vitro–in vivo* correlations.

5 Influence of encapsulated zinc salt on diffusivity

In order to further investigate the effect of zinc cations on PLG, zinc acetate was encapsulated in PLG films. Diffusion measurements of lysozyme through degraded PLG films, with and without zinc acetate, were performed. This investigation is presented in Paper III.

5.1 Materials and methods

PLG with an approximate molecular weight of 12 kDa and an 50:50 ratio of L:G was used (RG502H).

Thin PLG films were sprayed onto PVDF filters as described in Chapter 3 (see also Papers I and III). Zinc acetate was co-dissolved in ethyl acetate and constituted 5% (w/w) of the zinc containing PLG films. The films were 7.5-9.5 μm thick, calculated from their weight and a density of 1.3 g/cm^3 [181]. Twelve films were made simultaneously to ensure reproducibility.

Initial experiments on the diffusion of lysozyme were performed. The influence of the concentration of lysozyme on diffusion, and possible adsorption to PLG films or the PVDF filters were investigated. The effective diffusion coefficient in the filter was determined.

Both the films containing zinc acetate (PLG films + Zn^{2+}) and those without (PLG films – Zn^{2+}) were degraded in HEPES buffer, pH 7.4, at 37°C. For thickness measurements, slices of films sprayed simultaneously with those intended for diffusion measurements were cut, clamped in holders, and incubated under the same conditions. The PLG films were placed in the diffusion cell after predetermined periods of degradation, and diffusion measurements were performed. The concentration of lysozyme was measured using a fiberoptic probe measuring UV absorption in the receiver compartment, connected to a Cary 50 Bio spectrophotometer. The film thickness was measured using an optical microscope and imaging software. The diffusion experiments lasted for 24 hours, after which, the PLG films were washed in water and vacuum dried before SEM analysis.

The effective diffusion coefficient through the PLG films was calculated as described in Chapter 3 (see also Papers I and III).

5.2 Results and discussion

5.2.1 Initial experiments

The effective diffusion coefficient (D_e) of lysozyme was found not to depend on the concentration (Paper III), which was in agreement with a previous study [182]. The adsorption of lysozyme to PLG has been reported [183], however, complete release of bioactive lysozyme has also been reported [50]. Only a small amount of lysozyme was adsorbed in the present study, probably due to sufficient ionic strength of the HEPES buffer and the presence of the detergent polysorbate 80 in the films. It has been shown that an ionic strength of 0.1 M is sufficient for ionic shielding of lysozyme [184], and the ionic strength of the HEPES buffer was 0.16. The adsorption of lysozyme has also been found to be inhibited by detergents [185,186]. The adsorption of lysozyme in this study was considered not to influence the calculation of the D_e because: (i) the amount was small, (ii) mathematical correction of the lysozyme concentration due to adsorption resulted in an insignificant change in the diffusion coefficient, and (iii) after an initial lag phase, during which adsorption takes place, the adsorption does not influence the flux, and this lag phase was excluded from the calculations. The D_e in the PVDF filter was determined and used in the calculation of the D_e in the PLG films.

5.2.2 The effect of encapsulated zinc salt on protein diffusivity

The D_e of lysozyme through PLG films + Zn^{2+} increased significantly after a lag period of 14 to 18 days (Figure 9). After 14 days of degradation there was a detectable, but very slow, diffusion of lysozyme through the PLG film in one of three replicates, but it was too small to be calculated. Fourteen days of degradation may thus be the time required for pores sufficiently large for diffusion to form a connected network. No detectable diffusion of lysozyme through the PLG films – Zn^{2+} was seen after 35 days of degradation.

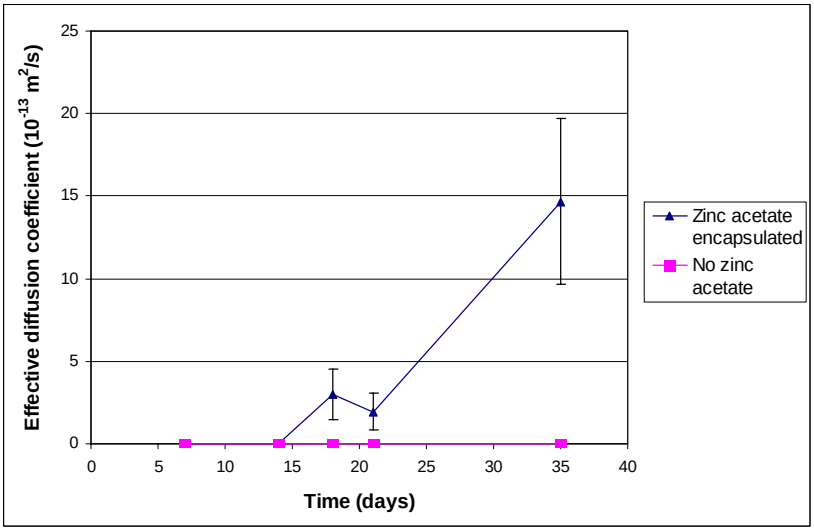
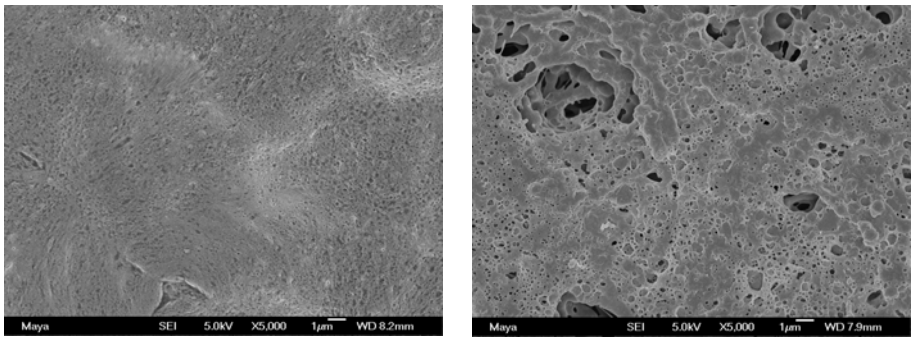


Figure 9. Encapsulated zinc acetate increased the effective diffusion coefficient of lysozyme after 14 to 18 days of degradation. There was no measurable diffusion at 7 and 14 days of degradation. (n=3).

The porosity of the surfaces of PLG films after diffusion measurements as seen using SEM, was in line with the effective diffusion coefficients. The PLG films + Zn^{2+} were porous from day 18, as can be seen in Figure 10. The PLG films – Zn^{2+} contained cavities from day 18, but no continuous pores.



A B

Figure 10. The porosity of the surface of PLG films after diffusion measurements after 35 days of degradation. (A) PLG film – Zn^{2+}, (B) PLG film + Zn^{2+}. Magnification 5000x.

5.2.3 The explanation of increased diffusivity in PLG films containing zinc acetate

This effect of zinc cations on the D_e was expected, based on the results presented in Chapter 4. The divalent cations were dissolved the *in vitro* buffer in that study, while in this present study they were encapsulated in the PLG films. Encapsulated salts could create pores in two additional ways besides the catalytic effect: by acting as porogens (i.e. dissolving and diffusing from the films) and/or as osmotic agents (i.e. increasing the rate of water absorption). However, the porosity in Figure 10B seems to be more than 5%, which was the content of zinc acetate in the PLG films. Water absorption was actually initially faster in PLG films + Zn^{2+} (Figure 11). However, the amount of water in both types of films was far above the amount needed for hydrolysis to proceed, which means that increased rate of hydrolysis in PLG films + Zn^{2+} should not have been caused by initial faster water absorption. After a lag period of 14 to 18 days, the same lag period as in the diffusion measurements, the thickness of PLG films + Zn^{2+} decreased. After 35 days of degradation, there was little PLG film left. This supports the theory of zinc cations catalyzing PLG degradation.

The lag period would probably be longer for higher-M_w PLGs, as a longer time would be needed for a significant amount of the PLG chains to be degraded to water-soluble oligomers. The effect of zinc cations also depends on the number of polymer–zinc cation interactions, and

thus depends on the concentration of free cations and the availability of such salts. The effect could also be counteracted by a basic anion. There have been some reports on the controlled release of drugs co-encapsulated with zinc salts. An increase in the rate of release of protein in the presence of zinc or other divalent cations has been reported, although studying this was not the objective of these investigations [130,187]. A slower release rate has also been reported [188]. However, as the authors noted, this may be due to the different drugs used, which were probably distributed differently in the particles. Encapsulated salt can affect drug release in several ways, as mentioned in Section 2.2.2. The effect on drug release may therefore be different in different situations. The results presented in Chapter 4 and in this chapter strongly indicate that zinc cations have a catalytic effect on PLG degradation.

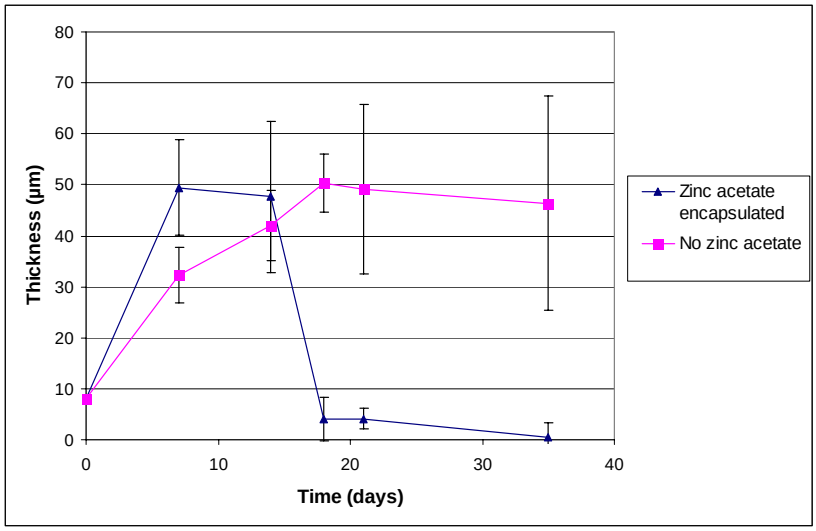


Figure 11. The thickness of the PLG films with and without zinc acetate. (n=3).

5.3 Conclusions

Encapsulated zinc acetate increased the diffusion coefficient of lysozyme through PLG films after 14 to 18 days of degradation. PLG films + Zn^{2+} also became porous, while PLG films – Zn^{2+} only developed cavities on the surface. The fact that PLG films + Zn^{2+} rapidly became thinner after the same lag period as in the diffusion measurements support the theory

that zinc cations catalyze PLG degradation. The pore forming effects and the probable subsequent increase in release rate should be considered when using such salts as protein stabilizers or pH neutralizers. Although the effect of zinc could be counteracted by the presence of basic anions, and encapsulated salt can affect drug release in many ways, zinc salts may be used as release-modifying agents, as well as protein stabilizers or pH neutralizers.

6 Pore formation and pore closure

Encapsulated proteins or peptides diffuse through water-filled pores [128]. The release rate is thus very dependent on the porosity of the polymer, and it is important to understand both pore ***formation*** and pore ***closure*** when modifying the release from PLG-based formulations. Pore closure has been observed previously [189], but it is unfortunately not often mentioned when discussing release mechanisms. Although some studies on pore closure have been performed, the phenomenon of pore closure is far from understood.

Pore formation and pore closure probably take place simultaneously. Both processes are affected by a number of factors, as discussed in Section 2.2. One such factor is pH. The rate of both pore formation and pore closure may thus vary with pH, which may decrease significantly from the normal physiological value (7.4), due to acidic polymer degradation products and inflammatory reactions *in vivo*.

The purpose of this study was to gain a better understanding of the phenomenon of pore closure, and to identify the mechanisms behind this process. The effects of pH and the temperature of the release medium, and the properties of the polymer were investigated. In order to study the effect of pH on the porosity in PLG matrices, simultaneous pore formation and pore closure were studied at different pH values. This study is presented in Paper IV.

6.1 Material and methods

Three different PLGs were used in this study, all of them obtained from Boehringer Ingelheim Pharma KG (Germany), namely: RG502H (50:50 L:G, with an approximate M_w of 12 kDa), RG504H (50:50 L:G, approximate M_w 45 kDa) and RG756 (75:25 L:G, approximate M_w 80 kDa).

Polymer films (about 150 μm thick), containing 2% (W/W) polysorbate 80, were cast on glass dishes from solutions in ethyl acetate. PVDF filters were encapsulated in films intended for analyses requiring mechanical support. Pores were created in samples intended for studies on pore closure, while those intended for studies on combined pore formation and pore closure at different pH were not subjected to any pore-forming pre-treatment, and were thus smooth and non-porous.

Pores were created in samples of PLG with molecular weights of 12 and 45 kDa by incubation in HEPES buffer with 1 mM $ZnCl_2$, pH 7.4, at 37°C, for 2 days and 4 days, respectively. Pores are formed close to the surface after a few days when $ZnCl_2$ is included in HEPES buffer (Paper II). The M_w of the 80 kDa PLG was too high for $ZnCl_2$-induced erosion to take place without significantly degrading the polymer. Instead, NaCl particles were encapsulated in these PLG films (10% w/w), and pores were formed upon the release of these particles during degradation in HEPES buffer for 4 days. $ZnCl_2$ was added to the HEPES buffer during these 4 days, with the purpose of avoiding unknown effects of $ZnCl_2$ at comparison of the different PLGs. Pores were found on one of the surfaces of the films, as the NaCl particles settled to the bottom during drying. After the pore forming pre-treatment, which was 2 days for the 12 kDa polymer and 4 days for the other PLGs, the samples were incubated in HEPES buffer without $ZnCl_2$, and the analysis of pore closure started.

The porous samples were incubated in HEPES buffer. Samples of different PLGs were incubated at a pH of 3.0 or pH 7.4, and at temperatures of 9°C, 37°C or 45°C. Table 5 presents the experimental design. Pore closure (SEM), water absorption (gravimetrically), polymer mass loss (gravimetrically), the change in wettability (contact angle) and the change in T_g (differential scanning calorimetry (DSC)) were studied.

Table 5. Experimental design for the investigation of pore closure

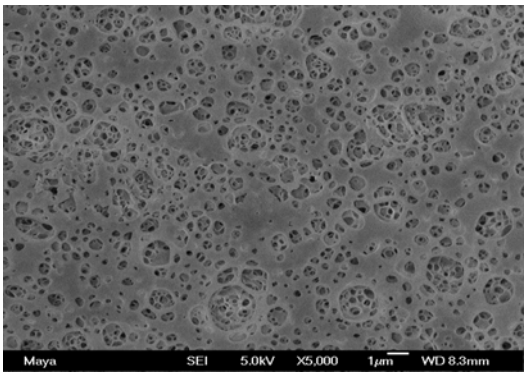
PLG	M_w (kDa)	Relative degree of hydrophobicity	Temperature (°C)	pH of the release medium
RG502H	12	Low	37	7.4
RG502H	12	Low	37	3.0
RG502H	12	Low	9	7.4
RG502H	12	Low	45	7.4
RG504H	45	Average	37	7.4
RG756	80	High	37	7.4

Non-porous samples were used to study simultaneous pore formation and closure. These were degraded in HEPES buffer at 37°C, and at pH 3.0, 5.0, 6.0, or 7.4. These samples were analyzed with regard to porosity, water absorption, and mass loss.

6.2 Results and discussion

6.2.1 The effect of pH on pore closure

Pore closure was faster at pH 3.0 than at pH 7.4 during the 26 days of observation, although pore closure began within two days at both pH values. The pores were completely closed at pH 3.0, but not at pH 7.4 (Figure 12). These results are in agreement with a previous study [146]. Pore formation is often believed to be enhanced at low pH, due to the well-known acid-catalyzed hydrolysis [89,97]. These results show that pH may affect the polymer in more than one way, and pore closure probably occurred more rapidly than pore formation in this case.



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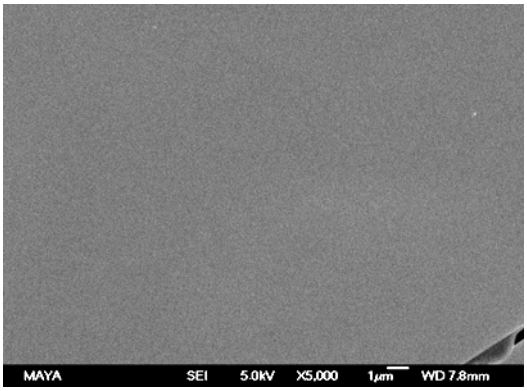

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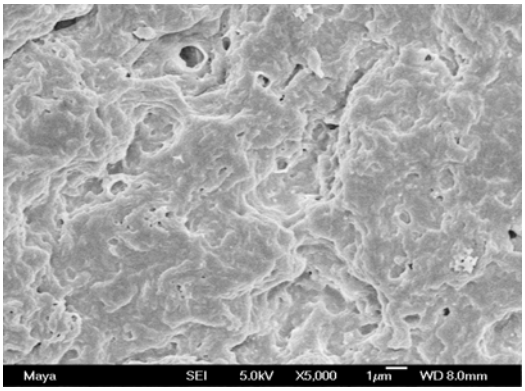


C)

Figure 12. The porosity of the surface of 12 kDa polymer films after (A) pore forming pre-treatment, and after additional five days of degradation (B) at pH 3.0, and (C) at pH 7.4.

Water absorption was slower at the lower pH (Figure 13), which was somewhat unexpected, as acid-catalyzed hydrolysis reduces the M_w of PLG, which in turn makes the polymer chains more hydrophilic [100]. The samples degraded at pH 7.4 became highly swollen, degraded and sometimes fell apart during the last period of the analyses, which caused the fluctuations seen in Figure 13. The results from the pore closure and water absorption analyses can be explained by the low degree of dissociation of the terminal carboxyl acids of the polymer chains at pH 3.0, which makes the polymer less charged and more hydrophobic. Measures of wettability confirmed this (Paper IV). It is likely that this hydrophobic effect was the driving force behind the separation of polymer and water, during which polymer chains were rearranged and pores were closed. The T_g of samples degraded at pH 3.0 decreased significantly with time, which resulted in highly mobile polymer chains. This will be discussed further in Section 6.2.3.

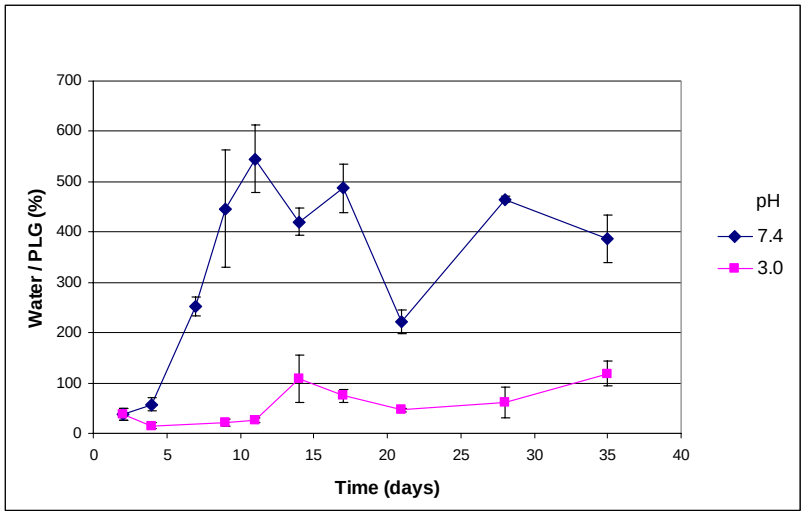


Figure 13. Water absorption at different pH values. The polymer samples were subjected to pore forming pre-treatment in the presence of $ZnCl_2$ at pH 7.4 at 37°C during the first two days. (n=3).

6.2.2 The effect of the properties of the polymer and the temperature on pore closure

Pore closure was faster with a low-M_w polymer with a relatively low degree of hydrophobicity. Pores began to close within two days in the 12

kDa polymer which had the lowest hydrophobicity of the three PLGs studied. In the 45 kDa polymer, with an average hydrophobicity, pores began to close within 7 days, although no clear pore closure was seen until 19 days. In the 80 kDa polymer, with the highest hydrophobicity, the pores did not close at all. The hydrophobicity depends not only on the M_w, but also on the L:G ratio, and whether or not the polymer chains are end-capped. As expected, a low-M_w, and less hydrophobic polymer absorbed more water (Paper IV).

Pores were closed faster as the temperature was increased (Paper IV). Pores were not closed at all at 9°C, but began to close within two days at both 37 and 45°C. However, the pores closed more rapidly during the next 13 days at 45°C. Thereafter, the samples at 45°C were too degraded to be analyzed. Faster pore closure at higher temperature was expected, and is in agreement with a previous report [115]. Increasing the temperature increases the mobility of the polymer chains. In contrast to the case at low pH, a correlation was found between the rate of pore closure and the rate of water absorption at pH 7.4.

6.2.3 The mechanisms governing pore closure

Pore closure has been observed in several studies, and has been found to be related to the mobility of the polymer chains [122]. Examples of factors that have been found to induce or affect pore closure, and also polymer chain mobility, are polymer degradation, plasticizing agents, and increased temperature [115,138,190-192]. The collapse of porous microparticles, and thus pore closure, has been observed when the (constant) incubation temperature had reached the so-called critical softening point, which was 10-20°C higher the decreasing T_g [163].

In the present study, pore closure could not be detected during the experimental period for the 80 kDa polymer, probably due to high-M_w and rigid polymer chains, nor for the 12 kDa polymer at 9°C, probably also due to low polymer chain mobility. The lack of pore closure in the high-M_w PLG demonstrates the importance of the initial porosity when such PLGs are used. At low initial porosity, the release would be very slow due to slow water absorption and degradation. However, if the initial porosity is high, the pores will not close, and the drug release may be faster than when using a low-M_w PLG. This should be considered when selecting the properties of the polymer.

At least two different physico-chemical processes could underlie the closure of pores observed at the two different pH values studied. At pH 3.0, the samples contracted into lumps. Very little water was absorbed, and the samples became more hydrophobic. This was attributed to the less dissociated carboxyl acids at low pH, resulting in a less charged and more hydrophobic polymer. This hydrophobic effect could cause the contraction and separation of polymer and water. The attraction of two hydrophobic polymer areas separated by a water-filled pore releases the surface-bound water and increases the entropy, resulting in a more energetically stable system. In addition, the decrease in the surface energy resulting from the separation of water and hydrophobic polymer is more energetically favorable. There thus appears to be a polymer–polymer interaction driven by the hydrophobic effect. It is likely that polymer chains were rearranged and that pores were closed during the contraction. As mentioned above, the T_g decreased significantly at low pH. This is a result of a low M_w and highly mobile polymer chains, which facilitate rearrangement. The 80 kDa polymer would probably also gain in terms of energy by contracting and separating from water, however, the polymer chains were too rigid.

At pH 7.4, a correlation was found between the rates of pore closure and water absorption. The samples swelled instead of contracting, and there seemed to be a polymer–water interaction instead of a polymer–polymer interaction. It is likely that the mobile polymer chains diffused and spread, resulting in pore closure. Instead of distinct regions of polymer and pores, a more swollen homogeneous polymer network was formed.

6.2.4 The effect of pH on simultaneous pore formation and pore closure

Pores did not form at pH 3.0, while samples incubated at pH 5–6 had the most porous surfaces (Figure 14). Pore closure could be seen at pH 5, 6 and 7.4 after different periods of time. Water absorption was faster at high pH and slower at low pH due to the degree of dissociated carboxyl acids, as discussed in Section 6.2.3.

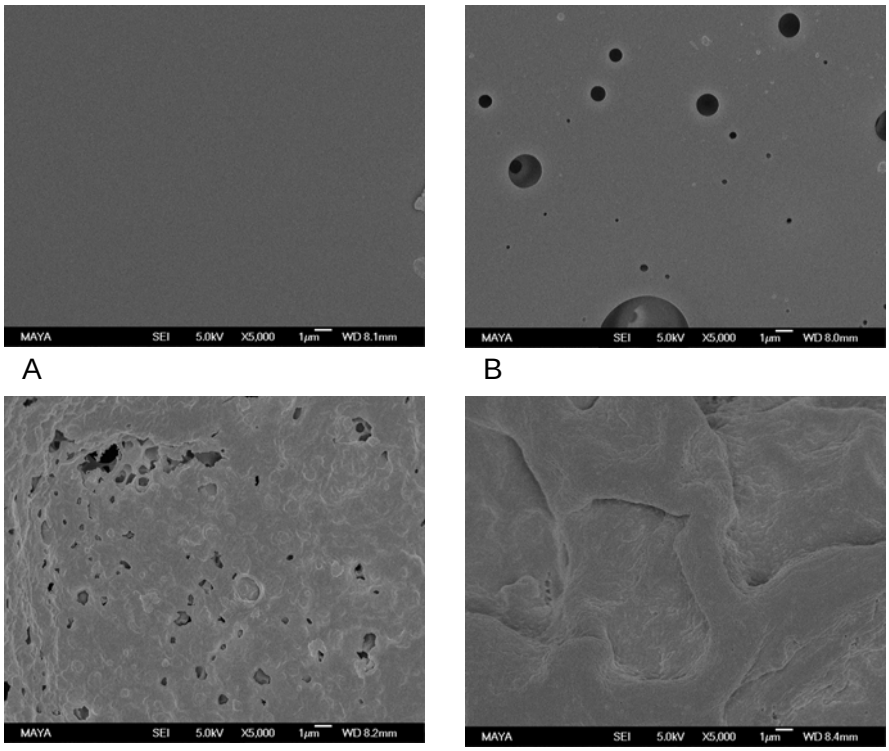


Figure 14. Pore formation after 10 days of incubation at (A) pH 3.0, (B) pH 5.0, (C) pH 6.0, and (D) pH 7.4.

Pore formation and closure probably took place simultaneously, and it is likely that the resulting porosity was dependent on the rates of both these processes. At pH 3.0, pore closure was probably so dominant that pores were not seen. The polymer–polymer interaction, driving pore closure at pH 3, should decrease in significance with increasing pH. Pore closure was also rapid at pH 7.4, as shown in Figure 12. The polymer–water interaction, causing pore closure at pH 7.4, should instead decrease in significance with decreasing pH. Pore formation should thus be pronounced between pH 3.0 and 7.4, as hydrolysis, which leads to pore formation, is relatively fast due to acid catalysis. This probably explains the optimal pore formation at pH 5–6, seen in Figure 14.

The effect of pH on pore formation and pore closure may be important, as pH may decrease significantly from the physiological value (7.4).

Acidic degradation products of PLG may decrease the pH inside PLG DDSs and of the release medium *in vitro* [94,103,193]. Inflammatory reactions and the formation of a fibrous capsule surrounding PLG microspheres may decrease the local pH *in vivo* [72,141]. The microclimate inside the polymer matrices and in the surrounding environment affects the processes that influence drug release in many ways, and there have been reports of both increased and decreased rate of drug release at low pH [78,194]. This result could explain the slower release. It should be noted that the effect of the pH on pore formation and pore closure is probably dependent on the M_w and the mobility of the polymer chains.

6.3 Conclusions

Pore closure could affect the rate of drug release, as the encapsulated drugs are usually large biopharmaceuticals, which are released through water-filled pores. In this study, pore closure was increased in a release medium with low pH, with a low-M_w PLG of relatively low degree of hydrophobicity, or at high temperature. At pH 3.0, the PLG samples contracted, absorbed hardly any water, became more hydrophobic, and T_g decreased significantly. At pH 7.4, the samples instead swelled and absorbed a large amount of water. Pore closure may have been caused by at least two different physico-chemical processes in these two cases, namely a polymer–polymer interaction, mainly driven by the hydrophobic effect, and a polymer–water interaction, which leads to a more homogeneously swollen polymer mass. Polymer mobility is an important factor in both cases. Pore closure was not detected in the high-M_w PLG, which demonstrates the effect of initial porosity in such systems. Pores form slowly and do no close in high-M_w PLGs.

The pH affects pore formation and pore closure in different ways. The highest porosity was found at pH 5–6, probably due to rapid pore closure below and above these values, and due to relatively rapid hydrolysis in this slightly acidic environment. The effect of pH on both pore formation and pore closure may be important, as pH may decrease significantly from the physiological value. This effect may also cause pore structure heterogeneity in PLG matrices, as the microclimate may vary in position. The effect of pH should thus be borne in mind when evaluating experimental results.

7 Regions of high transport resistance

Drug release is often described as diffusion-controlled or erosion-controlled [154,155]. However, more detailed information may be required in order to efficiently modify drug release, for example, the region offering the most significant transport resistance.

In the study presented in Chapter 5, no diffusion of lysozyme through PLG films without encapsulated zinc acetate could be detected, although the films had lost approximately 27% of their original weight, and had absorbed a large amount of water. Although the adsorption of lysozyme to PLG was found to be small, the diffusion of hGH (with the opposite charge to lysozyme at pH 7.4), and glucose (a small hydrophilic, non-charged and non-interacting molecule) was also measured in order to rule out the properties of lysozyme being the cause of the result. *In vitro* release of hGH encapsulated in PLG-coated microspheres was studied to compare the results with a more relevant pharmaceutical DDS. The purpose of this investigation was to study diffusion through PLG, to explain the unexpectedly slow diffusion, and to show the occurrence of regions with high transport resistances. This study is presented in Paper V.

7.1 Materials and methods

PLG with an approximate M_w of 12 kDa and an 50:50 ratio of L:G was used (RG502H).

7.1.1 Measurements of diffusion of hGH and glucose through thin PLG films

Thin PLG films (7.0 ± 1.0 μm thick), containing 2% (w/w) polysorbate 80, were sprayed from solutions in ethyl acetate onto PVDF filters. These thin films were degraded for 21 days in HEPES buffer, pH 7.4, at 37°C before being placed in the diffusion cell. The diffusion of hGH and glucose was measured for 24 hours. Samples were removed from the receiver compartment and replaced with fresh buffer. The concentration of hGH was measured using HPLC with UV detection, and the concentration of glucose was measured using high-pH, anion-exchange chromatography, coupled to pulsed amperometric detection, with an ED40 electrochemical detector. The film thicknesses were measured on slices of films sprayed simultaneously as the films used for diffusion measurements. The slices were clamped in special holders and degraded under the same conditions as the films for diffusion measurements. An optical microscope and imaging software were used for thickness measurements.

7.1.2 *In vitro* release of hGH encapsulated in PLG coated microspheres

Microspheres were coated with the same PLG as used in the films. The hGH-encapsulated coated microspheres were a kind gift from StratoSphere Pharma AB (Sweden). The microspheres were incubated in HEPES buffer, pH 7.4, at 37°C and placed on a tilting board. Triplicate samples were prepared for each measurement. The pH of the HEPES buffer was checked regularly, and the buffer in the samples intended for analyses after 21 days of degradation was changed after 21 days, at which time the pH had decreased from 7.4 to 6.8.

7.1.3 Water absorption and mass loss

Thick PLG films (about 150 μm), containing 2% (w/w) polysorbate 80, were cast on glass dishes from solutions in ethyl acetate. Thin films were

prepared by spraying, as described in Section 7.1.1. The films were degraded in HEPES buffer, pH 7.4, at 37°C. At predetermined intervals, the films were analyzed gravimetrically with regard to water absorption and, after drying, mass loss.

7.1.4 Diffusion of lysozyme through thick PLG films

The thick films were degraded for 35 days in HEPES buffer, pH 7.4, at 37°C, before being placed in the diffusion cell. Some films were subjected to pore forming treatment by adding 1 mM $ZnCl_2$ to the HEPES buffer for the last 3 days of the 35-day degradation period. In the study regarding divalent cations (Paper II), it was found that pores only formed at the surface during this short period of treatment with $ZnCl_2$ in the buffer, in contrast to if zinc acetate was encapsulated in the thick films (Paper V). The concentration of lysozyme was analyzed using a UV measuring probe inserted into in the receiver compartment of the diffusion cell, and connected to a spectrophotometer. Diffusion was measured for 4 days, after which the films were washed, dried and subjected to porosity analysis using SEM.

7.1.5 Visualization of fluorescent probes in PLG films

Thick films of PLG were degraded in HEPES with 1 mM $ZnCl_2$, pH 7.4, at 37°C for 2 days. The zinc salt was added to counteract potential high surface transport resistance. The samples were then degraded for another 19 days in HEPES buffer with tetramethylrhodamine attached to 10 kDa dextran (TMR-dextran). The location of TMR-dextran molecules inside the PLG films was studied using confocal microscopy. The stability of TMR-dextran and background fluorescence were checked in initial experiments.

7.2 Results and discussion

7.2.1 Diffusion of hGH and glucose through thin PLG films

No diffusion of hGH or glucose through thin PLG films degraded for 21 days could be detected. The slowest diffusion of hGH measurable, using this method, was 960 times slower than the rate of diffusion in water at 37°C, and for glucose it was 3100 times slower. Compared to the rate of diffusion through the PVDF filters used for mechanical

support of the thin PLG films, the slowest diffusion measurable was 280 slower for hGH and 850 times slower for glucose. The PLG films contained approximately 10 times more water than PLG mass due to water absorption (swelling measured using microscopy, data not shown), and they had lost approximately 27% of their polymer mass. The rate of diffusion of glucose and proteins through a hydrogel containing this amount of water is usually much more rapid than the detection limit with this method. The amount of hGH and glucose adsorbed to the PLG films or filters was found to be negligible (Paper I).

These diffusion measurements showed that the lack of detection of lysozyme diffusion (Chapter 5 and III) was not caused by the properties of lysozyme. The shielding effect of the buffer and the presence of polysorbate 80 in the films were probably sufficient to prevent ionic and hydrophobic interactions between lysozyme and PLG, as discussed in Chapter 5. The fact that glucose, a small hydrophilic non-charged molecule, also diffused slowly shows that the transport resistance in the PLG films was very high. Lysozyme was used in the subsequent diffusion measurements.

7.2.2 *In vitro* release of hGH encapsulated in PLG-coated microspheres

The encapsulated hGH was completely released after 35 days in HEPES buffer at 37°C (Figure 15). The average D_e was estimated to be roughly in the order of 10^{-16} m^2/s, using Fick's law applied to membrane systems [167]. This is about one million times lower than the diffusion coefficient in pure water at 37°C (1.3×10^{-10} m^2/s). The microspheres were coated with the same PLG as used in the PLG films, and the thickness of the coating layer (approximately 15 μm) was similar to the thickness of the thin PLG films (7 μm). The swelling and mass loss data from thin films should therefore be applicable to the coating layer. After 35 days of incubation, the PLG coating probably contained about 10 times more water than polymer mass, and had lost approximately 40% of its polymer mass. This slow diffusion is surprising considering the amount of water and the degree of degradation, although the estimated D_e was an average over time.

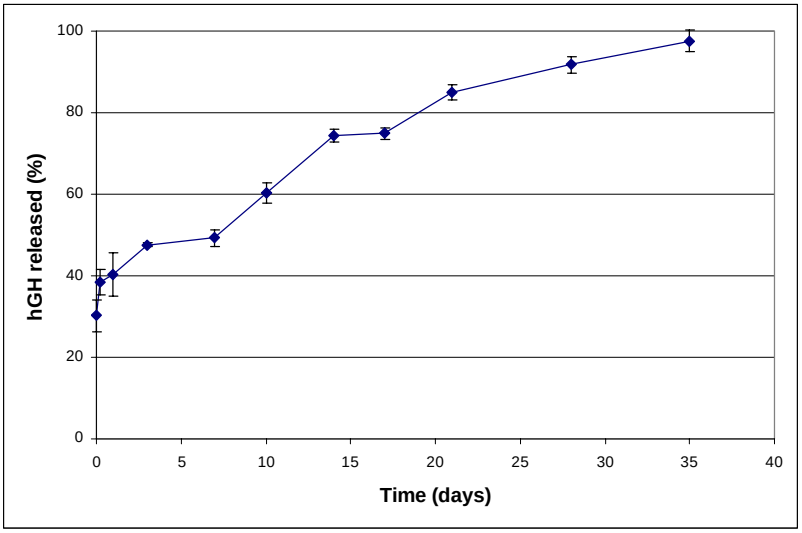


Figure 15. In vitro *release of hGH encapsulated in PLG-coated microspheres (n=3). The manufacturing method used by StratoSphere Pharma AB was modified in order to be able to compare this result to other measurements. The burst release was not important in this study, and it can be controlled by the formulation- and manufacturing process parameters.*

7.2.3 Transport resistance at the surface

Pore closure is one possible explanation of the slow diffusion through PLG films and coating layers. Pore closure has mainly been observed at the surfaces of microspheres and films [122,146]. Diffusion through thick PLG films, of which some had been subjected to a pore-forming treatment to increase the surface porosity, was measured. These thick PLG films had lost 78% of their initial weight after 35 days of degradation before diffusion measurements.

The diffusion of lysozyme was significantly faster through PLG films with increased surface porosity (Figure 16). It was difficult to measure the thickness of the $ZnCl_2$-treated films precisely. However, the inaccuracy in these measurements would not account for the difference in the diffusion coefficients. This indicates that there was considerable transport resistance at the surfaces, which could explain the slow or undetectable diffusion discussed in Sections 7.2.1 and 7.2.2. The surface could therefore be the region of greatest transport resistance, and changes in the surface of DDSs, for example, the formation of cracks or

the detachment of small pieces of microparticles, may thus influence the release rate significantly.

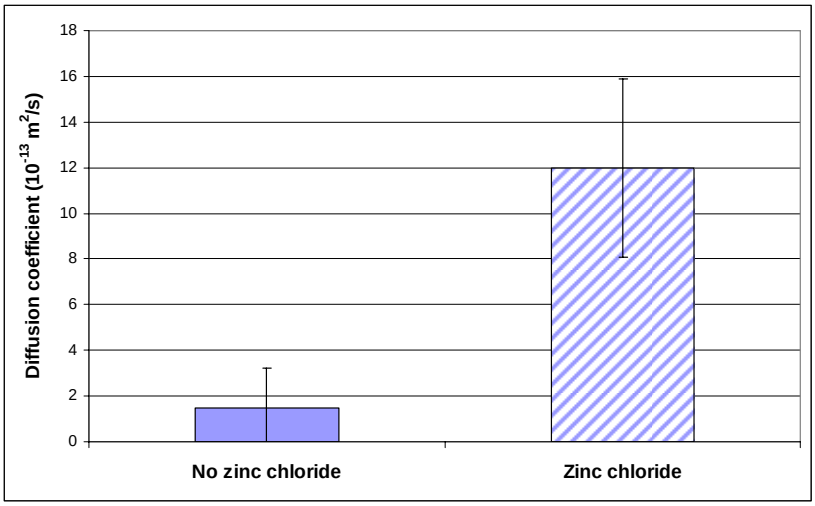
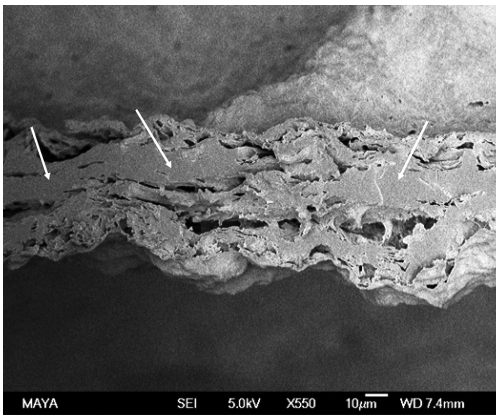


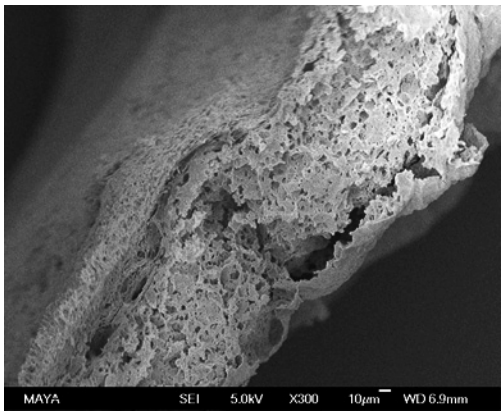
Figure 16. A significant increase in D_e was observed when the surfaces of the PLG films were made more porous by the presence of $ZnCl_2$ in the buffer during the last 3 days of the 35-day degradation period. (n=3).

7.2.4 Transport resistance inside the PLG films

SEM analysis of the cross sections of PLG films sometimes showed non-porous areas (Figure 17A), at least when using this low-M_w, relatively hydrophilic PLG. These non-porous areas have mainly been seen after a relatively short period of degradation, up to approximately 21 days. Longer degradation periods usually result in porous appearances (Figure 17B). These SEM images (and all other SEM images in this thesis) are representative.



A)



B)

Figure 17. Cross sections of two PLG films. (A) Non-porous areas were visible after 21 days of degradation (arrows). (B) The cross section was porous after 35 days of degradation.

TMR-dextran did not diffuse homogeneously inside the PLG films. There were areas approximately up to 20 μm in width, into which the probes seemed unable to diffuse (Figure 18). The existence of these dark areas indicates that there may be large regions through which there is very little or no diffusion. Upon inspecting the image very closely, very small channels exhibiting fluorescence could be seen in some of these dark areas. Initial experiments and instrument settings confirmed that the source of the fluorescence was the probes. The TMR-dextran remained stable during the experimental period. As TMR-dextran is a non-ionic hydrophilic substance, it is expected to spread throughout the whole

water-filled space in the polymer, unless the pores are too small. Interactions between PLG and TMR-dextran are considered unlikely. The dark areas can thus not be explained by anything other than TMR-dextran not being able to diffuse into these regions.

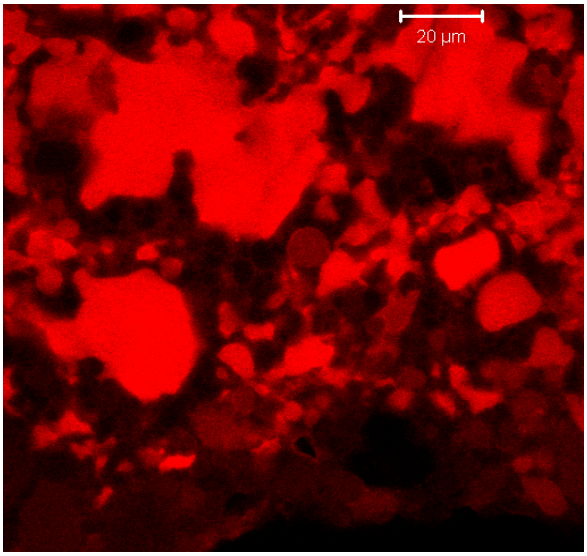


Figure 18. The location of fluorescent probes attached to 10 kDa dextran within a PLG film. The dark areas show regions into which the probes could not diffuse. In contrast to the cross sections shown in Figure 17, this image was obtained along the plane of the PLG film.

The cause of these non-porous areas may be either that pores have not yet been formed, or that the pores have closed. The balance between pore formation and pore closure was found to be dependent of pH, among other factors (Chapter 6 and IV). The microclimate may vary in both space and time, and this is one probable explanation of the heterogeneity of the porosity inside the PLG matrix.

During the work described in this thesis, the porosity of a vast number of PLG samples was studied using SEM. Sometimes, areas of high porosity were found together with non-porous areas within the same sample, and it appeared that a process resulting in pore formation had started to occur at a few locations, and that the effect had spread. Another example of heterogeneity is that pores were found not to develop homogeneously throughout the films. They were first formed close to the surface and the border between the porous and non-porous

region moved towards the center of the films with time (Paper II). It is unlikely that this corresponded to the border of absorbed water in the film, as water absorption was found to be much more rapid, both in this study and by others [87]. The explanation probably lies in the heterogeneous environment, pH being one important factor.

The non-porous regions inside the films may form considerable transport resistance, which could explain the slow or undetectable diffusion discussed in Sections 7.2.1 and 7.2.2. This was supported by the results of similar diffusion experiments to those presented in Section 7.2.3, but carried out after 21 days of degradation (non-porous areas inside the film, according to Figure 17A) instead of 35 days (porous interior, according to Figure 17B). It should be noted that only one measurement of diffusion through a $ZnCl_2$-treated PLG film was carried out. The increase in surface porosity after 21 days of degradation did not have any effect on the diffusion resistance. This suggests that the major transport resistance is inside the polymer matrix during the first part of degradation.

7.2.5 Other explanations

Another possible explanation for the slow or undetectable diffusion described above is inadequate pore size. A size-exclusion effect was seen on diffusion though PLG films in the study presented in Paper I. One may also speculate on the nature of the fluid inside the PLG matrices, which may contain high concentrations of dissolved polymer degradation products and proteins, and thus might be highly viscous. A high concentration of acidic degradation products should result in a very low pH. However, the fact that the pH was found to increase from approximately 3 to between 5 and 6 with time, as more water was absorbed (Paper II) does not support this theory. Neither does the fact that pH inside PLG microsphere was found to be between < 2.8 and 5.8 in another study [103]. High viscosity can not explain the decrease observed in the effective diffusion coefficient compared to diffusion in water, as diffusion though such systems is not that slow [195-197].

7.3 Conclusions

No diffusion of hGH and glucose though thin PLG films that had absorbed a large amount of water was detectable. An estimate of the average D_e of hGH encapsulated in PLG-coated microspheres indicated that the diffusion of hGH was about one million times lower than in pure water. As diffusion was slow even for the small, hydrophilic non-charged glucose molecules, the reason for slow diffusion seemed to lie in the transport properties. Diffusion experiments on thick, heavily degraded PLG films, some of which had been subjected to a treatment that increased the surface porosity, indicated that the greatest transport resistance was at the surfaces. Using fluorescent probes attached to dextran and confocal microscopy, revealed large non-porous regions inside the PLG films after 21 days of degradation. It appears that high transport resistance can be found inside the polymer matrix during the first part of degradation, while the most significant transport resistance is at the surfaces at a later state of degradation. Pores of insufficient size may also cause transport resistance.

8 Release mechanisms for PLG-based DDSs

The definition of the term release mechanism was discussed in Section 2.4, where it was suggested that processes describing the *way* in which the drug is released should be called *true* release mechanisms, while the processes controlling the *rate* of release should be called *rate-controlling* release mechanisms. There are only four true release mechanisms: diffusion through water-filled pores, diffusion through the polymer, osmotic pumping and erosion (i.e. no drug transport). True and rate-controlling release mechanisms, and the techniques used for the study of these mechanisms, are discussed in a review article (Paper VI) and briefly in this chapter.

8.1 Studying release mechanisms

Release mechanisms have been studied in different ways, based on the shape of the release profile [198], mathematical modeling [199] or studies on processes that influence drug release [98]. Drawing conclusions regarding the release mechanism based on the release profile alone is simple and may be accurate. However, many different processes, some with counteracting effects, may result in similar release profiles. Different mechanistic mathematical models have been used to elucidate release mechanisms [133,200,201]. Many models are based on diffusion described by Fick's law. As PLG swells and degrades, the inclusion of an

expression for a non-constant diffusion coefficient is an advantage. Mathematical modeling gives a rapid general view and provides fundamental insight into the dominating release mechanisms, or the processes influencing drug release. However, PLG systems are complex, and the predictive power of mathematical models should be evaluated thoroughly. A third means of investigating release mechanisms is to study the specific processes that influence drug release, for example, polymer erosion, pore closure and polymer–drug interactions. In combination with the drug release profile, such studies provide detailed knowledge on drug release, from which conclusions regarding the true and rate-controlling release mechanisms can be drawn. However, this approach may be more time consuming than mathematical modeling, and the complexity should be considered when drawing conclusions.

8.2 True release mechanisms

8.2.1 Diffusion through water-filled pores

Diffusion through water-filled pores is often the dominating true release mechanism, as (i) the encapsulated drug is usually a protein or a peptide, which is too large and too hydrophilic to diffuse through the polymer, (ii) osmotic pumping in PLG DDSs is rare, and (iii) drug transport is often faster than polymer erosion. Diffusion through pores has been mentioned as the release mechanism countless times [135,136,202-204]. In many studies, this release mechanism has only been used to describe the first stage of the release, before the onset of polymer erosion [37,158,187]. According to the definition of the term release mechanism in this thesis, diffusion is the true release mechanism in those cases during the whole release period, and different processes may determine the rate of drug release instead of the initial porosity, i.e. the rate-controlling release mechanisms. Erosion is the most commonly reported of such processes. However, there are also examples of complete drug release before any significant polymer erosion [78,150,205]. The burst release phase is sometimes said to be diffusion dependent [206].

Diffusion through water-filled pores is very dependent on the porous structure of the polymer, and is therefore dependent on the processes that promote pore formation and pore closure. D_e is dependent on the diffusion coefficient in the fluid in the pores, the porosity and the tortuosity [167]. Pores must also be continuous from the drug molecule

to the surface of the DDS, and sufficiently large for the solute to pass through them. Dead-end pores, too small pores and the degree of connectivity between pores influence the porosity and the tortuosity. Constant effective diffusion coefficients for drugs encapsulated in PLG-based DDSs are more likely to be found in cases of small and initially porous particles consisting of high-M_w, hydrophobic PLGs, which swell and degrade slowly and have low polymer chain mobility. Pore forming processes, i.e. erosion and swelling, will have greater effect on low-M_w, less hydrophobic PLGs, and on large or non-porous particles [134].

8.2.2 Diffusion through the polymer

Diffusion through the polymer is possible for small hydrophobic drugs [14,166]. The polymer/water partition coefficient determines the extent to which the drug is transported in this way. Diffusion through the polymer is normally slower than diffusion through water, and is not particularly dependent on the porosity [207]. However, the drug must be dissolved in water before being released, and a highly porous system provides a large area for dissolution. Drug dissolution in water may be the rate-determining process in the release process [3]. Unstirred surface layers may pose significant transport resistance, and such layers may be saturated due to low water solubility of the drug.

The rate of diffusion through a polymer is very dependent on the physical state of the polymer, and for a small molecule, may increase by several orders of magnitude upon the transition from the vitreous to the rubbery state [113]. The glass transition temperature of PLG in a DDS may also be lower than that of the original polymer due to degradation during the manufacturing process and the plasticizing effects of additives or residual water [112,143]. The plasticizing effect of water usually transfers the polymer into the rubbery state rapidly upon immersion in water at 37°C or administration *in vivo* [90,111]. A very high-M_w PLG may remain in the vitreous state for some time before degradation and water absorption affect the polymer. Drug diffusivity through the polymer is often higher in lower-M_w polymers, as the polymer chains are more flexible [208]. Different mathematical relationships have been found between the diffusivity and polymer M_w. As in the case of diffusion through water-filled pores, the diffusion coefficient will be less variable in high-M_w PLGs with small particles. Degradation will play a greater role in low-M_w PLGs and with large particles. [165].

8.2.3 Osmotic pumping

The most common mode of transport through water-filled pores is diffusion, i.e. random movements of molecules driven by the chemical potential gradient, which can often be approximated to the concentration gradient. The other mode of transport through water-filled pores is convection, driven by some force such as osmotic pressure [167]. Drug transport driven by this force is called osmotic pumping [168]. This release mechanism is more common in DDSs made of other materials than PLG, for example, ethyl cellulose [209]. A semi-permeable barrier and osmotic agents are often used to create an osmotic pressure due to the influx of water. Such DDSs are often designed with special exit holes or pores for the drug and water. There have, however, been reports of osmotic pumping from PLG-based DDSs in very high-M_w, hydrophobic PLGs, in which swelling and polymer erosion are negligible [210,211]. Channels were created during the manufacturing process, or pores were created by dissolving PEG to allow drug transport. The osmotic agents were co-encapsulated. As the rate of water absorption and swelling of these PLGs are minimal, it is possible to maintain a uniform water influx and efflux, and thus osmotic pressure. However, most PLG-based DDSs consist of lower-M_w PLGs, which swell significantly sooner or later, and any osmotic pressure will then be compensated for by the increase in volume. A difference in osmolality inside and outside the DDS may cause the rate of water absorption to increase, resulting in the formation of pores and an increase in the rate of diffusion. Osmotic pressure caused by water absorption may result in rupture of the polymer [212]. However, osmotic pumping is not a common release mechanism in PLG-based DDSs.

8.2.4 Erosion

Erosion as a true release mechanism, i.e. drug release without drug transport, results in identical profiles of drug release and polymer erosion, assuming that the drug is homogeneously distributed throughout the DDS. Identical, or nearly identical, drug release and polymer erosion profiles have been reported, although such reports are rare [213,214]. As mentioned in Section 8.2.1, degradation/erosion is frequently reported as a *rate-controlling* release mechanism, often during the final stage of drug release [99,159,215]. However, there is a

difference between erosion leading to an increase in the rate of drug transport, and erosion resulting in drug release without drug transport. Polymer erosion could cause drug molecules very close to the surface to be released without transport, and the release mechanism would then be erosion. However, as hydration is normally much faster than erosion, it is more probable that the drug will diffuse through pores formed by water absorption. Erosion could be the main release mechanism for low-M_w PLG formulations, in which a significant proportion of the polymer has a molecular weight just above the limit for water solubility. However, as remnants of the polymer are commonly reported after complete drug release [208,216], erosion is rarely the dominating true release mechanism.

8.3 Rate-controlling release mechanisms

Many physico-chemical processes have been found to control the release rate. These can be summarized into the following processes:

- Drug dissolution – mostly with very hydrophobic drugs
- Water absorption – creates pores, but may also enhance pore closure
- Hydrolysis – affects many other processes that influence drug release, for example, porosity and polymer chain mobility and density
- Heterogeneous degradation – may lead to a non-porous surface that controls the drug transport
- Erosion – creates pores, and decreases the impact of dissolved polymer degradation products trapped inside the DDS.
- Pore formation – increases the rate of diffusion
- Pore closure – decreases the rate of diffusion
- Polymer–drug interactions – usually result in a slower release rate, but may also plasticize the polymer and enhance drug release
- Drug–drug interactions – usually result in slower or incomplete drug release
- Formation of cracks – increases the rate of diffusion
- The collapse of the DDS – may result in new surfaces or a decrease in porosity

All of these rate-controlling release mechanisms have been found to be the process governing drug release, or at least to be one of a few processes determining the release rate [31,36,91,98,118,146,162,163,217-219]. Some of these processes may influence drug release in more than one way, as is discussed more thoroughly in Paper VI. The rate of drug release is influenced to different degrees by these processes, depending on the true release mechanism.

8.4 Discussion

As mentioned in Section 2.2.2, three things should be borne in mind when discussing drug release from PLG-based DDSs: (i) PLGs with different properties exhibit very different physico-chemical behavior, and these properties change with time, (ii) PLG matrices form heterogeneous systems, and the process governing drug release may vary in space, and (iii) the effects of different factors on drug release are complex and the dominating factor may change with time.

Some of the rate-controlling release mechanisms presented in Section 8.3 may both enhance and inhibit drug release. Examples are given in Table 6. Different factors may influence these processes in more than one way, as discussed in Section 2.2.2. The complexity of PLG-based DDSs might make it difficult to generalize conclusions drawn under specific conditions.

Table 6. Processes that may increase or decrease the rate of drug release.

Process	Possible effect	Effect on the release rate
Hydrolysis	Auto-catalysis Erosion and pore formation Plasticizing effect of oligomers	Increase
	Crystallization of oligomers Polymer chain mobility and pore closure Drug–drug and polymer–drug interactions	Decrease
Erosion	Pore formation	Increase
	Loss of catalytic effect of acidic degradation products	Decrease
Water absorption	Hydrolysis Pore formation	Increase
	Increased pH Polymer chain mobility and pore closure	Decrease
Collapse of the polymer structure	Cracks and new surfaces	Increase
	Decreased porosity	Decrease

The dominant process may differ between different microparticles in the same system. Particles of different sizes are prone to different degrees of auto-catalytic degradation. Cracks may be formed on some particles but not on others. The release rate from different regions of a microparticle may differ [15], due to the heterogeneous nature of PLG matrices. When a process takes place locally in the matrix, the effect will be local, and as one process may influence others, regions with different characteristics may arise. These effects may then spread throughout the matrix. The porous and non-porous regions inside PLG films discussed in Chapter 7, and the inhomogeneous development of pores through PLG films presented in Paper II are examples of this.

The dominant process may also change during the release period. The impact of one process on drug release may be altered when other processes or the environment are changed. For example, the solubility of the drug, drug–drug interactions, polymer–drug interactions, hydrolysis, pore formation and pore closure, all depend on the pH, which depends

on the rate of hydrolysis, water absorption and transport out of the system.

The fact that all true and rate-controlling release mechanisms discussed in this chapter have been found to be the dominating way of release, or to determine the release rate, is probably due to the complexity of drug release. Among the true release mechanisms, diffusion through water-filled pores is often the dominant one, as discussed in Section 8.2.1. Among the rate-controlling release mechanisms, erosion is probably the most common process to dominate, as faster release rate has been found to coincide with the onset of erosion in many studies [99,159,215]. Among the properties of the DDS and the surrounding environment that influence drug release, the properties of the polymer, especially the M_w, probably have the strongest effect. Different polymer properties result in highly varying rates of water absorption and polymer erosion [140]. The duration of drug release can be varied from hours to several months, depending on the properties of the PLG selected.

The complexity of the system provides many possible ways of designing controlled-release pharmaceuticals. Each arrow in Figure 2, and each effect of the factors listed in Table 1, constitutes a potential way of modifying drug release. There is probably no PLG-based DDS that is suitable for every drug and every application. However, the chances of successfully developing a suitable DDS for each separate drug and application should be very good. Knowledge of PLG systems increases those chances. Although studies on specific DDSs are necessary for product development, general, mechanistic and non-specific research provides pieces of the puzzle of drug release, facilitating pharmaceutical development.

9 Conclusions and future work

Poly(D,L-lactide-co-glycolide) has been a subject of intense research for at least two decades, due to its suitability as a drug carrier. Despite this, we still do not have a full understanding of the release mechanisms and ways in which drug release can be modified. This is probably due to the complexity of drug release from PLG-based DDSs. This work has focused on release mechanisms, and the different factors that influence drug release, and has contributed with pieces of the puzzle of drug release.

9.1 Method development

A novel method for measuring the diffusion of proteins and other substances through PLG films was developed. A procedure for spraying thin films of PLG onto PVDF filters was developed. The method was thoroughly evaluated with regard to: (i) the effect of stirring, (ii) the number of samples necessary to obtain a reliable effective diffusion coefficient, (iii) the effect on diffusion of a filter protecting the PLG film from erosion, (iv) the dependence of diffusion on the concentrations of the solutes, and (v) adsorption of the solutes to the films and filters.

The diffusion of hGH was measured, and the reproducibility was considered to be sufficient to study the diffusive properties of PLG

films. The diffusion of hGH was measured after $ZnCl_2$ had been used to increase the porosity of the PLG films, and the results indicated a considerable time lag before a continuous network of pores of sufficient pore-size had been formed. A size-exclusion effect was observed on diffusion through the PLG films when studying simultaneous diffusion of hGH and glucose.

9.2 Divalent cations

Divalent cations in the *in vitro* buffer increased the rate of pore formation in PLG films. Zinc cations had a greater effect than magnesium and calcium cations.

Encapsulated zinc acetate increased the effective diffusion coefficient of lysozyme through PLG films after 14 to 18 days of degradation. PLG films with zinc cations also became porous, while PLG films without zinc cations only developed cavities on the surface.

The pore forming effect of divalent cations was probably due to Lewis-acid-catalyzed hydrolysis of PLG. The pore forming effect could not be explained in terms of solubility, as the solubility of the water-soluble fraction of PLG decreased in the presence of divalent cations in buffers. Polymer erosion was significantly faster when zinc cations were present in the buffer, and the pH inside the PLG films was lower. PLG films with zinc cations became thinner rapidly after the same lag period seen in diffusion measurements. These results support the theory that zinc cations catalyze PLG degradation.

Salts of divalent cations are sometimes co-encapsulated in PLG formulations in order to stabilize proteins, and the pore forming effect should be borne in mind, although a basic anion may counteract the effect. The effect probably also depends on the polymer M_w and the number of interactions between the PLG and divalent cations. Salts of divalent cations, especially zinc salts, could be used as drug release modifiers, in addition to stabilizers.

The results also show that a buffer containing the same salts as those in plasma, among those magnesium and calcium cations, and in the same concentrations, affected the formation of pores differently than PBS.

This is one possible explanation, among others, of the problems associated with *in vitro–in vivo* correlations.

9.3 Pore formation and pore closure

Pore closure was increased in a release medium with low pH, with a low-M_w PLG with a relatively low degree of hydrophobicity, or at high temperature.

At pH 3.0, the PLG samples contracted, absorbed hardly any water, became more hydrophobic, and T_g decreased significantly. This could be explained by the less dissociated carboxyl acids at low pH, which made the polymer chains less charged and more hydrophobic. At pH 7.4, the samples instead swelled and absorbed a large amount of water. Pore closure may have been caused by at least two different physico-chemical processes in these two cases: (i) polymer–polymer interactions driven by the hydrophobic effect, causing separation from water and rearrangement of the polymer chains, and (ii) polymer–water interactions that led to a more homogeneously swollen polymer mass, instead of distinct regions of polymer and pores. In both cases, polymer mobility is an important factor.

Pore closure was not detected in a high-M_w PLG, which demonstrates the importance of initial porosity in such systems. Pores form slowly and do no close in high-M_w PLGs.

The pH affects pore formation and pore closure in different ways. The highest porosity was found at pH 5–6, probably due to a combined effect of rapid pore closure below and above these values and relatively rapid hydrolysis in this slightly acidic environment.

Pore closure probably affects the rate of drug release, as the encapsulated drug is often released through water-filled pores. The effect of pH on both pore formation and pore closure may be important, as the pH may decrease significantly from the physiological value (7.4) due to inflammatory reactions *in vivo* and acidic polymer degradation products. The effect of pH should thus be borne in mind when evaluating experimental results. This effect may also cause heterogeneity in the porous structure of PLG matrices, as the microclimate may vary in

position. Pores were found not to develop homogeneously throughout the polymer, but instead to form close to the surface initially, and then further towards the center with time, while the closure of pores was observed at the surface.

9.4 Transport resistance

No diffusion of lysozyme, hGH, or glucose though thin PLG films that had absorbed a large amount of water was detectable. An estimate of the average effective diffusion coefficient from the release of hGH encapsulated in PLG-coated microspheres suggested that the diffusion of hGH was slowed down about one million times through the polymer compared to that in pure water. As diffusion was slow even for the small, hydrophilic, non-charged non-interacting glucose molecules, the reason for slow diffusion seems to lie in the transport properties of the PLG.

The diffusion of lysozyme through heavily degraded PLG films (35 days) increased with increased surface porosity, which indicated that the major transport resistance was located at the surfaces.

Using fluorescent probes attached to dextran, and confocal microscopy, large non-porous regions were found inside the PLG films after 21 days of degradation.

The results of the diffusion studies, and the non-porous regions found using confocal microscopy and SEM suggest that considerable transport resistance can be found inside the polymer matrix during the first stage of degradation, and that the most significant transport resistance is found at the surfaces at a later state of degradation. Pores of insufficient size may also cause considerable transport resistance.

9.5 Release mechanisms

The term release mechanism has been used in the literature with two different meanings: as a description of the way in which release takes place, and as a description of the physico-chemical process controlling the release rate. In this thesis, the former is called a *true release*

mechanism, and the latter a *rate-controlling release mechanism*. True release mechanisms are: diffusion through water-filled pores, diffusion through the polymer, osmotic pumping and erosion (i.e. no transport). Examples of rate-controlling release mechanisms are: water absorption, erosion (i.e. increased drug transport), pore closure and polymer–drug interactions. Rate-controlling release mechanisms are important, as they give information of how drug release can be modified.

Diffusion through water-filled pores is the most commonly dominating true release mechanism, as the encapsulated drug is usually a protein or a peptide, which are too large to diffuse through the polymer. Osmotic pumping rarely occurs, as the low-M_w PLGs often used swell, and osmotic pressure is likely to be equalized. Drug release due to erosion without drug transport is also rare, as drug transport is often faster than polymer degradation/erosion.

All these true release mechanisms have been found to determine the *way* in which the drug was released, and several rate-controlling release mechanisms have been found to control the *rate* of release. This is probably because of the complex interactions of all the different factors that influence drug release from PLG-based DDS. Drug release is often preceded by a chain of processes, and the dominant process may change with time and space, or when a parameter is altered. This complexity provides many ways of solving a particular problem during pharmaceutical development, and many ways of modifying drug release. General and mechanistic research contributes pieces of the puzzle of drug release, which is useful in pharmaceutical development.

9.6 Suggestions for future work

9.6.1 Diffusion studies

It would be interesting to study the diffusion of ions, especially divalent cations, through PLG matrices. This could perhaps be done by measuring the conductivity. Ions with an atomic number higher than 12 are detectable using x-ray energy dispersive spectroscopy, and their location inside PLG matrices could theoretically be mapped.

Water absorption in the early stage of polymer–water interactions could be investigated using nuclear magnetic resonance (NMR). The rate of

water transport and whether water diffuses homogeneously or preferably in growing pores (after their formation) could be investigated.

9.6.2 Divalent cations

The effect of zinc and other divalent cations, and the effect of concentration, on higher-M_w PLGs should be studied, and degradation constants calculated using gel permeation chromatography. Deeper insights into the interactions between zinc and PLG could perhaps be obtained using NMR or Fourier transform infrared imaging. It would also be interesting to study the effect of divalent cations, encapsulated or in *in vitro* buffers, on the release of encapsulated drugs.

9.6.3 Pores

Questions remain to be answered regarding the formation and closure of pores. Some questions are partly answered but deeper investigations could be done. For example, which characteristics promote pore formation at one point, rather than another? Is pH different at such points? Why exactly do pores form close to the surface initially and are developed further into the film with time? Different methods of analysis would be required to answer these questions. Perhaps the development of a pore could be studied "live" in an aqueous environment using atomic force microscopy with a fluid cantilever holder. Confocal microscopy could also be used to study diffusion of polymer chains coupled to fluorescent probes.

It would also be interesting to study pore formation and pore closure using environmental scanning electron microscopy, as the samples do not have to be dried.

PLG chains of different molecular weights may not be homogeneously distributed in a matrix after manufacturing. These polymer chains differ in hydrophobicity, and may be distributed differently in emulsion-based preparation techniques. If the system is homogeneous initially, will it remain so during degradation? Could islets of PLGs with specific molecular weights be formed, and would such islets become pores? It would be interesting to attach fluorescent probes to polymer chains with a specific M_w and track their location in a matrix of PLGs with different molecular weights using confocal microscopy.

9.6.4 PLG and environmental characteristics

It would be interesting to use multivariate analysis or principal component analysis to obtain mathematical models or to find correlations between the properties of PLG and its physico-chemical behavior.

The effects of the characteristics of the *in vivo* environment that can not be mimicked *in vitro* need to be further investigated. It would be useful if pH maps of the region surrounding administered DDSs could be obtained. It would be interesting to study the impact of different *in vivo* characteristics, for example, lipids, enzymes and if possible macrophages, separately *in vitro*.

9.6.5 DDSs and applications

Nanoparticles have interesting applications such as target delivery and oral delivery. These are relatively new areas of research where many approaches to obtain the desired release characteristics remain to be investigated. Surface modification or blending with different materials can be used to achieve a specific function, for example, the ability to target specific cells.

Oral delivery is a considerable advantage for patients, and various strategies are available to improve bioavailability. For example, particles could be coated with Eudragit®, which is resistant to acids, in order to aid uptake in the intestinal tract. The effect of particle size and blends of PLG with other materials can be studied in order to identify the optimal characteristics for drug delivery via Peyer's patches and the lymphoid system. The targeting of regions in the intestine with a thin mucus layer is another interesting field of investigation.

It would be interesting to design new DDSs, for example, different PLG layers on particles or implants. Differences in PLGs, drug loading, and additives could be used to tailor the release profile.

It would also be interesting to study how PLG particles with different properties could be used to control the release profile. High-M_w PLGs with controlled porosity are attractive, as they degrade slowly and

produce a low amount of acids. However, the release rate probably decreases with time as a result of the decreasing concentration gradient. Small non-porous particles of lower-M_w PLGs might counteract the decrease in drug release by increasing the rate of release as pores are formed. Adding zinc salt to the polymer might further increase the release rate after a lag phase, similar to the result described in Paper III. The particles should preferably be small to avoid large acid gradients. The blend of two different formulations in different proportions may be useful when tailoring the release profile.

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Measurement of Protein Diffusion Through Poly(D,L-Lactide-Co-Glycolide)

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A novel method was developed for studying the diffusion of proteins through poly(D,L-lactide-co-glycolide) (PLG), using a diffusion cell. To develop improved formulations for the controlled release of encapsulated drugs it is important to understand the underlying release mechanisms. When using low-molecular-weight PLG as the release-controlling polymer, diffusion through the pores is often proposed as the main release mechanism. The experimental set-up and method of determining the diffusion coefficient were thoroughly evaluated with regard to the reliability and the influence of the stirring rate. A procedure for spraying thin films of PLG onto a filter, which could be placed in the diffusion cell, was optimized. The method was then applied to the determination of the diffusion coefficient of human growth hormone (hGH) through a PLG film. The results show that the method enables measurements of the diffusion coefficient through the polymer film. Neither the stirring rate nor the concentration of hGH influenced the diffusion coefficient. The diffusion coefficient of hGH through degraded PLG films was $5.0 \cdot 10^{-13}$ m^2/s, which is in the range that could be expected, i.e., several orders of magnitude smaller than its the diffusivity in pure water. The reproducibility was good, considering the dynamic properties of PLG, i.e., the difference in diffusion coefficients, at, for example, different stages of degradation and for different compositions of PLG, is expected to be much higher. The variation is probably also present in PLG films used for controlled-release formulations. Although the PLG film contains a large amount of water, a considerable time elapsed before pores of sufficient size formed and diffusion through the film started. In two-component diffusion experiments, the difference in diffusion rate did not correspond to the difference in molecular weight of the solutes, indicating a size exclusion effect. This method can be used to study the effect of changes in the formulation specification. By studying the change in the diffusion coefficient through the degradation process of PLG, or similar polymers, a better understanding of diffusion and, thus, also release mechanisms can be obtained.

Keywords diffusion, poly(D,L-lactide-co-glycolide), protein, hGH, controlled release

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INTRODUCTION

During the past decade there has been great interest in controlled release of drugs due to the benefits of using proteins therapeutically.[1] Proteins can not be given orally and are usually injected.[2] Controlled-release formulations decrease the frequency of such injections, thus increasing patient compliance.

Poly(D,L-Lactide-Co-Glycolide) (PLG) is often used to control the drug release as it is biodegradable and nontoxic,[3–5] and the duration of the release can be controlled over a wide range.[6–8] The polymer porosity changes due to the swelling and degradation, and thus the diffusivity changes continuously.[9]

Diffusion of the drug through water-filled pores is often proposed as the main release mechanism in PLG formulations. To gain knowledge about the release mechanisms it is, therefore, important to study diffusion through

PLG. Diaphragm cells have been shown to be economical and accurate for measurements of diffusion.[10–14] The diaphragm cell has been used for measurements on polymer gels, membranes,[10–16] and in the field of topical and transdermal drug delivery.[17,18] However, many reports on diffusion measurements lack a thorough evaluation of the conditions under which the measurements were made, and there are many factors that may contribute to an erroneous result (e.g., stirring rate and dependence of the diffusion coefficient on concentration).[19,20]

Measurements based on the diffusion cell technique must be relatively short, because the diffusion coefficient changes due to swelling and degradation of PLG. Because of this the membrane has to be thin, which results in problems of mechanical stability and measurement of the thickness of the membrane. Some researchers use release data to calculate diffusion coefficients,[21,22] but in these cases diffusion may not be the only mechanism involved, which results in an ''apparent'' diffusion coefficient lumping the different phenomena together, and the change in diffusion coefficient is not seen.

The problems concerning PLG and diaphragm cells are addressed and a novel method for measuring the diffusion of proteins and small substances through PLG is presented in this article. This method can be used to obtain better understanding of diffusion and release mechanisms by studying the change in the diffusion coefficient at different stages of the release process. Examples of other applications are comparison of the diffusivity through different PLG compositions, studies of the effects of additives, and comparison of the diffusivity of different drugs, which helps determination of optimal formulation specifications. This method also has the advantage that it can be used for other polymers similar to PLG.

DIFFUSION CELL—THEORETICAL DESCRIPTION

This method was developed for measuring the diffusion of water-soluble proteins through a membrane of PLG. Diffusion takes place in the water absorbed by the polymer, and the polymer structure is not accessible to the protein.

To calculate the diffusion coefficient Fick's law is used.[23]

$$j = -D_e \frac{dC}{dz} \qquad [1]$$

The mass flux through the film, j [g/(m^{2}s)], is expressed in terms of the effective diffusion coefficient D_e. This is, of course, lower than the diffusivity in pure water, due to the porosity of the film and the tortuous pathway of the solute through the film.

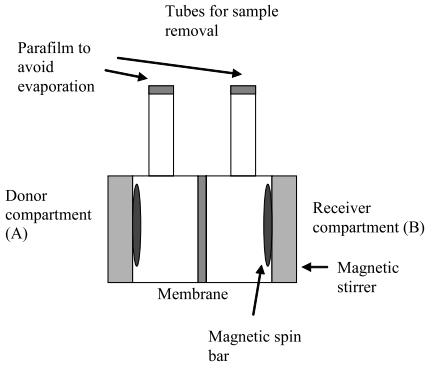

Figure 1. Schematic illustration of the diffusion cell.

When using a diaphragm cell (Figure 1) pseudo steady state diffusion, a condition close to steady state, is often applicable.[10] The pseudo steady state assumption has been investigated and is fully justified if the ratio of the volume of the membrane to the volume of the compartments is less than 0.1.[10]

The solution of Eq. 1 together with a mass balance over the two compartments is:[10]

$$K = \frac{1}{A\left(\frac{1}{V_A} + \frac{1}{V_B}\right)} \times \frac{\ln\left(\frac{C_{A1} - C_{B1}}{C_{A2} - C_{B2}}\right)}{t_2 - t_1} \qquad [2]$$

The suffixes A and B represent the donor and receiver compartment, respectively. Suffixes 1 and 2 are sample numbers. K is a mass transfer coefficient, A is the diffusion area, and t denotes time. V_A and V_B are the volumes of the compartments A and B, and C is the concentration.

Eq. 2 can be applied to measurements on several samples. The logarithmic values of the difference in the concentrations are plotted against time, resulting in a slope that equals the second part of the right-hand side of Eq. 2, but with a negative sign. The slope can be calculated with linear regression, and K can then be calculated knowing the constants A, V_A, and V_B.

Eq. 2 is not valid during the initial lag phase when the fluxes into and out of the membrane are not equal, and this time should be excluded from the evaluation. After the lag phase, the mass balance over the membrane should

be checked to avoid discrepancies that might be due to unequal fluxes, for example experimental failures.

When the polymer film is made by spraying the polymer onto a filter, the total diffusion resistance consists not only of the resistance through the polymer film but also of the resistance through the filter. The total mass transfer resistance is thus the sum of the mass transfer resistances according to Eq. 3.

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{D_{e\,filter}} \quad [3]$$

K is the mass transfer coefficient calculated from Eq. 2. Thickness and effective diffusivity through the film and filter are denoted l and D_e, respectively. By rearranging Eq. 3 the effective diffusion coefficient for the PLG film can be obtained by simply subtracting the filter resistance ($l_{filter}/D_{e\,filter}$), which can be determined in initial experiments.

A number of factors should be taken into consideration when calculating D_e.

- The stirring rate—If the stirring rate is too low, stagnant layers at the membrane surface will lead to an additional mass transfer resistance. Too high a stirring rate may cause forced convection through the membrane. Appropriate stirring rates are those that lie on the plateau when D_e is plotted as a function of stirring rate.[20]
- Adsorption—Adsorption of the protein onto the polymer results in an increased lag time.
- Dependence of D_e on concentration–The concentration dependence is influenced by the properties of the protein, especially the isoelectric point, pI. Therefore, the choice of ionic strength and pH of the buffer is important.[24,25]
- Temperature dependency–Liquid diffusion coefficients are very temperature dependent.

MATERIALS AND METHODS

Materials

The PLG (RG502H and RG756) was obtained from Boehringer Ingelheim Pharma KG (Germany), polysorbate 80 from Uniqema (Belgium), PVDF-filters and nylon filters from Millipore AB (Sweden), dialysis membranes (Spectra®Por, MWCO 3500) from Spectrum Laboratories, Inc. (USA and Canada), glucose from BHD Laboratory Supplies (England), and human growth hormone (hGH) from Novo Nordisk A/S (Denmark).

Methods

All diffusion experiments were carried out at 37°C. The temperature was controlled by placing the diffusion cell in a water bath. Duplicate samples were withdrawn from each compartment and when necessary replaced by the same volume and concentration to avoid a decrease in the volumes. Glucose was analyzed using a reagent kit (Randox) and ultraviolet (UV) absorbance, and human growth hormone (hGH) were analyzed using high pressure liquid chromatography (HPLC) with UV detection (ambient temperature, TSK2000 SW column, Tosoh Corporation, Japan). To avoid duplication of the experimental error of the concentration analysis, the concentration in the donor compartment was calculated by a mass balance.

Evaluation of Stirring Rate, Reproducibility, and Number of Samples Necessary

Ten measurements of the diffusion coefficient of glucose through a dialysis membrane were carried out at stirring rates ranging from 150 to 820 rpm. The medium used in the diffusion experiment, PBS (phosphate buffer saline), pH 7.4, consisted of 30 mM sodium phosphate, 82 mM NaCl, 0.5 mM $CaCl_2$, 3 mM NaN_3 and 0.1% poloxamer 188 NF. The surface water was carefully removed from the membrane before placing it in the cell. The thickness of the membrane was measured before and after the experiment using a calliper. An equal volume of buffer, 7–8 mL, was added to the compartments and the exact volumes were determined by weighing. The buffer added to the donor compartment was supplemented with the solute. In these experiments the solute was glucose and the concentration 5 mg/mL. To evaluate the number of withdrawn samples needed for an accurate result, the diffusion coefficient was calculated using all samples. This was then compared with the diffusion coefficient obtained by using other numbers of samples.

Spraying PLG Films

Films of PLG a few μm thick, which contained 2% polysorbate 80, were made by spraying a solution onto a filter of polyvinylidene fluoride (PVDF) using a Hüttlin spray nozzle. The filter, which had a pore size of 0.65 μm, was mounted on a rotating wheel, which made the filter pass through the spray at determined intervals. The PLG and polysorbate 80 were dissolved in ethyl acetate. Spraying parameters were optimized by evaluating the sprayed film using scanning electron microscopy (SEM) (Philips XL30 microscope, Philips, the Netherlands). The

optimal values of the parameters were found to be 5 cm spray distance, 1.5 bar atomizing pressure, 10 mL/min spray feed, 12 minutes of spraying, 60 rpm rotational speed, and a 1% PLG solution. To obtain a film of as uniform thickness as possible, the film was sprayed in many spray sequences during which the spraying nozzle was directed towards three different places on the filter, and the filter turned 90°. Twelve films were made simultaneously.

Evaluation of Nylon Filter

To protect the PLG film from erosion caused by stirring, a nylon filter was clamped against the PLG film around the edges in the diffusion cell. The influence of the nylon filter on diffusion was investigated by conducting diffusion measurements of glucose in the same way as explained earlier, but with the nylon filter clamped onto the dialysis membrane without any PLG film in the diffusion cell. The diffusion experiments were also carried out with only the nylon filter in the diffusion cell.

Concentration Dependence of the Diffusion of hGH

The diffusion coefficient of hGH through the PVDF filter was measured at concentrations of 5, 20, and 30 mg/mL of hGH in PBS, pH 7.4. The measurements were conducted at least in duplicate. The measurements were carried out as described earlier but with a stirring rate of 150 rpm.

Thickness Measurements

The thickness of the sprayed PLG films on filters was measured using an Olympus BHT microscope, an Olympus DP10 camera (both Olympus, Japan) and the software Image Pro Plus, version 4.1 (Media Cybernetics Inc., USA). Small slices of the film and filter were cut and clamped at the ends by two pieces of glass and placed in a holder that kept the slices vertical. The film slices, placed in this equipment, were incubated and treated in the same way as the PLG films used for diffusion measurements. Three PLG films from each batch of 12 films sprayed simultaneously were used to estimate the thickness of all 12 films. An average thickness was calculated based on a total of 450 measurements, prior to and after a diffusion experiment. The thickness of the PVDF filters was measured in the same way, and this thickness was subtracted from the total thickness of the film and filter.

Measurement of Diffusion Coefficient of hGH through PLG

The PLG film, which was sprayed onto a PVDF filter, was incubated for 14 days in Hepes buffer with 1 mM $ZnCl_2$, pH 7.4, at 37°C. The composition of Hepes buffer was 75 mM Hepes, 115 mM NaCl, 15.4 mM NaN_3, and 0.1% poloxamer 188 NF. The surface water was carefully removed from the filter and film before placing it in the cell with the nylon filter. The buffer in the diffusion cell was Hepes buffer without $ZnCl_2$. The initial concentration of hGH in the donor compartment was 30 mg/mL. The change in the concentration in the compartments due to sample withdrawal and replacement was insignificant. The temperature, stirring rate, and volumes of the compartments were as described previously. The experiment was repeated three times. Diffusion measurements of hGH through a PLG film incubated for 5 days in PBS and one PLG film incubated for 8 days in a buffer denoted ''plasma buffer,'' pH 7.4, were also carried out. The composition of the plasma buffer was 60 mM Hepes buffer, salts similar to those in plasma,[26,27] 3 mM NaN_3, and 0.1% poloxamer 188 NF.

Simultaneous Measurement of the Diffusion Coefficient of Glucose and hGH through PLG

The PLG film (sprayed onto a PVDF-filter) was incubated for 10 days in Hepes buffer with 1 mM $ZnCl_2$, at 37°C. The buffer in the diffusion cell was Hepes buffer without $ZnCl_2$. Both glucose and hGH were added to the donor compartment, 5 mg/mL and 30 mg/mL, respectively.

RESULTS AND DISCUSSION

Evaluation of Stirring Rate, Reproducibility, Number of Samples Necessary, the Influence of the Nylon Filter, and Adsorption

The stirring rate did not affect the diffusion coefficient (Figure 2). The stirring rate was sufficient to prevent stagnant layers from forming at the dialysis membrane; and stagnant layers are, therefore, not expected to form at the PLG films or at PVDF filters. The stirring rate was not high enough to promote forced convection through the dialysis membrane, since the mass transfer did not increase with increasing stirring rate. The lowest stirring rate tested, 150 rpm, was chosen to reduce the risk of erosion of the PLG film.

The standard deviation of the 10 measurements of the diffusion coefficient of glucose through dialysis mem-

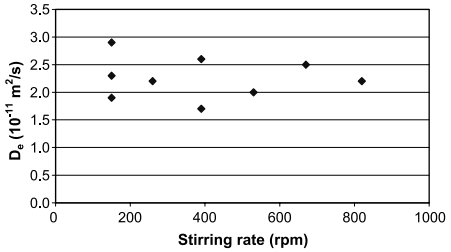

Figure 2. The diffusion of glucose through dialysis membrane: dependence of the diffusion coefficient on the stirring rate.

brane was 15%. Although standard deviations of other diffusion cells have been lower,[10] 15% is sufficient for measurements using a dynamic polymer such as PLG. Since the polymer swells and is degraded, the effective diffusion coefficient will be very different at different stages of degradation. This variation must be considered when evaluating the release rate from formulations using PLG as the rate-controlling polymer.

To further protect the PLG film, a nylon filter was clamped close to the PLG surface in the cell. When repeating one of the 10 diffusion measurements using a nylon filter, the diffusion coefficient was the same, indicating that the nylon filter did not affect the diffusion. Also, in the diffusion experiment with only the nylon filter in the cell, the mass transfer was too fast to be measured. Since no diffusion resistance of this filter was detectable, the nylon filter did not decrease the diffusion surface at all.

The least number of withdrawn samples necessary for an accurate result was 5. An example of a plot of the diffusion coefficient against the number of samples used for calculation is shown in Figure 3. The slope that equals

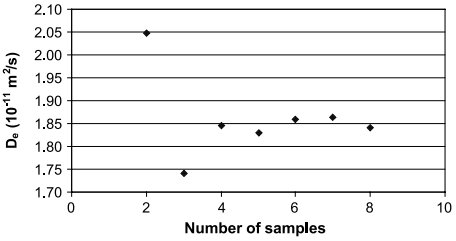

Figure 3. The diffusion coefficient of glucose through dialysis membrane when different numbers of withdrawn samples are used in the evaluation. Stirring rate 150 rpm. The reliability of the diffusion coefficient depends on the number of withdrawn samples used for calculation.

the right hand side of Eq. 2, built up by the withdrawn samples, and which the diffusion coefficient is based on, becomes more reliable with many samples. The diffusion coefficient is fairly constant when more than four samples are used for evaluation.

Adsorption studies were also carried out. Neither glucose nor hGH was adsorbed onto any of the films, membranes, or filters used in contact with the solutes.

Influence of Concentration and Determination of Diffusion Coefficient Through the Filter

Diffusion coefficients of hGH through the PVDF filter at different concentrations are shown in Figure 4. The decrease in the diffusion coefficient with higher concentration was within the standard deviation of the reproducibility and is, therefore, considered to be negligible. A minor decrease, or no decrease at all, was expected since a buffer of quite high ionic strength was used. Similar results indicating a lack of or weak concentration dependence have been reported for many proteins at high ionic strength. For example, the diffusion coefficient of lysozyme only decreased marginally with the concentration in a buffer with an ionic strength of 0.20 M.[25] Diffusion was not dependent on the concentration of BSA, at least up to 20 mg/mL, in a buffer with an ionic strength of 0.1 M and pH above the isoelectric point.[24] In the diffusion measurements using PLG films Hepes buffer was used, while PBS was used in the experiments to investigate concentration dependency using only PVDF filters. Since the ionic strength of Hepes buffer is not far from that of PBS, the concentration dependence of the diffusion coefficient should not be stronger in Hepes buffer ($I_{PBS} \approx 0.15$ M, $I_{Hepes} \approx 0.15$ M). These values are in the same range as 0.1 M and 0.20 M, as referred to earlier, which was high enough for concentration dependence to be negligible. Based at the results of these experiments it was decided to use

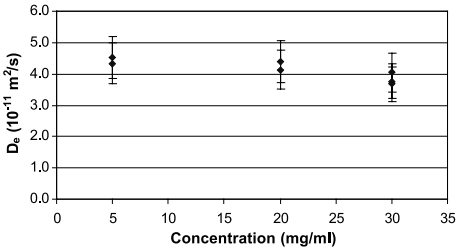

Figure 4. Influence of concentration of hGH on the diffusion coefficient. Diffusion of hGH through PVDF filters.

Table 1
Diffusion of hGH through PLG films, incubated for 14 days in 75 mM Hepes buffer with 0.1% poloxamer 188 NF and 1 mM $ZnCl_2$, at 37°C data

Expt. no.	$l_{film}/D_{e\ film}$ (10^{-7} s/m)	D_e (10^{13} m^2/s)
1	1.6	7.1
2	1.8	6.0
3	4.2	2.6
4	2.6	4.3
Average		5.0

$l_{film}/D_{e\ film}$ is the mass transfer resistance in the PLG film, l is the thickness and D_e is the effective diffusion coefficient.

a concentration of hGH of 30 mg/mL in the PLG film experiments. Thus, the average diffusion coefficient at 30 mg/mL ($3.8 \cdot 10^{-11}$ m^2/s) was used in the calculation of the diffusion coefficient in the PLG film [Eq. 3]. This high concentration was chosen to reduce the experimental time and, thus, allow the diffusion measurements to be carried out under as constant conditions as possible.

Measurement of Diffusion Coefficient of hGH Through PLG

The effective diffusion coefficient (D_e) of hGH through a film of PLG was calculated according to the procedure described earlier. The results are listed in Table 1.

The thicknesses of the swelled and degraded PLG films averaged 11 μm. Since all the films were sprayed simultaneously, three films were used for thickness measurements and the average applied to all the films. The standard deviation of the thickness of films sprayed simultaneously was 1.2 μm. The concentration in the donor compartment was calculated using mass balance based on the concentration in the receiver compartment and the thoroughly analyzed initial concentration in the donor compartment. The analyzed concentration in the donor compartment varied considerably, probably due to the high dilution factor required for analysis. An indication of this is that duplicate samples taken from the donor compartment differed more than duplicate samples taken from the receiver compartment. This made the mass balance difficult to check, but since no indication of increasing or decreasing total amount of hGH in the cell throughout the experiment was observed, the mass balance was considered valid.

The average diffusion coefficient is in the range that could be expected, i.e., several orders of magnitude smaller than the diffusivity in pure water (see Table 2). The diffusion coefficient of proteins through PLG is expected to be very low because of the high molecular weight of proteins and the high transport resistance in PLG. Calculations on diffusion of very large proteins or macromolecules through PLG or poly(lactide) (PLA) based on release experiments show diffusion coefficients in the range of 10^{-17} to 10^{-15} m^2/s.[22,28,29] However, the diffusion coefficient is highly dependent on the degree of swelling and degradation. For example, the diffusion coefficient for hGH in PLG films degraded for 14 days (Table 1) were 10 times higher than in PLG films degraded for 10 days (Table 2). The reproducibility is good, considering the dynamic properties of PLG, e.g., the swelling, degradation, and the fact that the sprayed films may not be identical. The standard deviation of the method was 15%. The variation when using degraded PLG films was higher (see Table 1). The difference in diffusion coefficient at different stages of degradation as well as for different proteins and different compositions of PLG, are expected to be much higher than the variation in repeated measurements. The variation is probably also present in PLG films used for controlled-release formulations, which results in a variation in the controlled-release formulation. Similar performance has been

Table 2
Diffusivities of glucose and hGH in water (D_{aq}), through the filter and the PLG film

	D_{aq} (10^{10} m^2/s)	$D_{e\ filter}$ (10^{11} m^2/s)	$D_{e\ film}$ (10^{14} m^2/s)	$D_{e\ filter}/D_{aq}$	$D_{e\ film}/D_{aq}$
Glucose	8.2.[33,34]	22	67	0.24	0.00074
hGH	1.3 [32]	3.8	4.9	0.29	0.00038
Ratio glucose:hGH	6.3	5.8	14	0.80	1.9

The ratio of the diffusivity for glucose and hGH shows that PLG film had a size exclusion effect. $D_{e\ film}$ is the average of two experiments in which the PLG films had been incubated for 10 days in Hepes buffer with 1 mM $ZnCl_2$. The D_{aq} of hGH has been calculated using the relation between diffusivity and molecular weight for proteins proposed by young.[32] The D_{aq} of glucose at 37°C has been calculated using the value at 30°C[33] and the fact that diffusivity is proportional to the temperature (in Kelvin) and inversely proportional to the dynamic viscosity.(From Ref. [34].)

observed for polymer films used for single units making up a multi-pellet system.[30,31]

The diffusion cell technique is a method of determining the influence of polymer film mass transfer resistance. The measured diffusion coefficient is not influenced by other release mechanisms, which might be the case when calculating the diffusion coefficient from release experiments. This method can be used to estimate effects of changes in the formulation and to better determine optimal formulation specifications. It can also be used to obtain better understanding of diffusion and release mechanisms by studying the change in diffusion coefficient in different phases of the release process, assuming the morphology of the PLG film and drug delivery system is not too different.

Dependence of Diffusivity on Pore Formation

The diffusivity changes over a long period of time in the case of PLG, due to swelling, degradation, and erosion, and these changes may have a considerable effect on the release rate. In a parallel study it was shown that pores are formed due to swelling and degradation of the polymer, and these processes are among other factors affected by buffer salts.[9] In the present work, it was shown that pores had to be formed before hGH was able to diffuse through the PLG film. The diffusion rate was measured for different incubation times of the film before the diffusion experiments. Although the PLG film contained about 3 times as much water as polymer after 5 days of incubation in PBS, the pores were too small for hGH to diffuse through the film (Figure 5). Likewise, 8 days of incubation in Hepes buffer with salts similar to those in plasma was not long enough for sufficient pore formation. After 14 days of incubation in Hepes buffer with 1 mM $ZnCl_2$ the diffusivity was measurable. Analysis of the surface of the film with SEM after the experiments showed that it was free of pores in the experiments when the diffusivity was not measurable, but contained pores when hGH was seen to diffuse through the film (Figure 6).

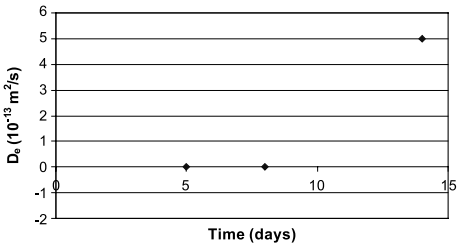

Figure 5. Pores of sufficient size have to be formed before proteins can diffuse through PLG. One PLG film was incubated for 5 days in PBS, one was incubated for 8 days in Hepes buffer with salts similar to those in plasma and four PLG films were incubated for 14 days in Hepes buffer with 1 mM $ZnCl_2$.

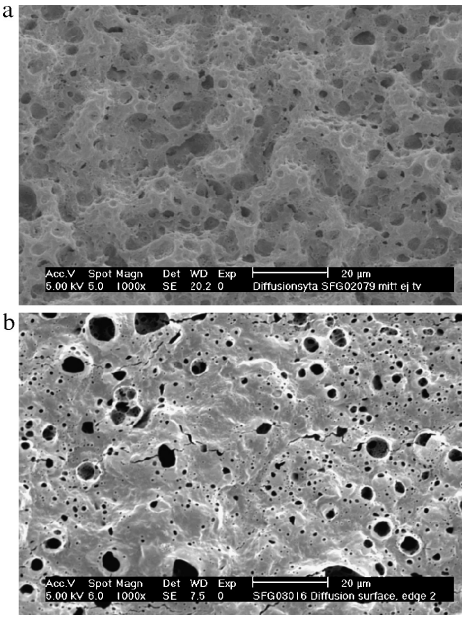

Figure 6. PLG films after diffusion experiments in which the film had been incubated: a) for 8 days in Hepes buffer with salts similar to those in plasma and b) for 14 days in Hepes buffer with 1 mM $ZnCl_2$. Cavities can be seen in a) but no pores.

These results show that the formation of pores of sufficient size for protein permeation is a prerequisite for the release of encapsulated proteins, assuming the release mechanism is diffusion through pores of water. Water absorption alone does not necessarily form pores of sufficient size.

Simultaneous Diffusion of hGH and Glucose

Measurements of the simultaneous diffusion of glucose and hGH through PLG were performed, and the results presented in Table 2. The ratio of $D_{e\ glucose}$ to $D_{e\ hGH}$ was higher in PLG (14) than in the filter (5.8) used as support, showing that the diffusivity of hGH in PLG was reduced more than the diffusivity of glucose. The same

ratio for diffusion in water, $D_{aq\ glucose}$ to $D_{aq\ hGH}$ is 6.3, which indicates that the filter had no size exclusion effect. However, since the ratio was 14 in PLG, the polymer has a significant size exclusion effect.

The size exclusion effect of PLG is dependent on how far the process of pore formation has proceeded. At some point the pores will reach a sufficient size for glucose, but not hGH, to diffuse through, and at a later point the pores will be large enough for both solutes to pass through.

CONCLUSIONS

A novel method for measuring the diffusion of proteins through a PLG film, in which the diffusivity changes continuously, was developed and evaluated. A diffusion cell, similar to an Ussing cell, was used and the reproducibility of the results was shown to be sufficiently good to study the diffusive properties of a PLG film. The diffusion coefficient of hGH was shown to be independent of the concentration. A procedure for spraying thin films of PLG onto a PVDF filter was developed.

The diffusion coefficient of hGH through a PLG film was determined using Fick's law for pseudo-steady state diffusion. The diffusion coefficient was in the expected range, i.e., several orders of magnitude smaller than the diffusivity in pure water. The reproducibility was good considering the dynamic properties of PLG, e.g., swelling and degradation. The difference in diffusion coefficient at different stages of degradation as well as for different PLG compositions, is expected to be much higher than the variation in repeated measurements.

Using this method it was shown that although the PLG film contained several times more water than polymer, as dry substance, the protein did not diffuse through the film until the pores were large enough. It was also shown that PLG has a size exclusion effect, since the diffusivity of hGH decreased much more than did that of glucose.

The presented diffusion cell technique makes it possible to study diffusion alone, without the influence of other release mechanisms present during release experiments. Determination of the diffusion coefficient in different phases of the release process, comparison of diffusivity through different PLG compositions, studies of the effects of additives and comparison of the diffusivity of different drugs are examples of applications.

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Paper II

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Effect of Divalent Cations on Pore Formation and Degradation of Poly(D,L-lactide-co-glycolide)

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Poly(D,L-lactide-co-glycolide) (PLG) is probably the biodegradable polymer most often used for polymeric controlled-release formulations. Different salts have been shown to affect the swelling and degradation of PLG, which, in turn, affect the release of encapsulated drugs. In this investigation the effect of divalent cations was especially investigated. Films of PLG were incubated in phosphate buffer saline (PBS), a buffer containing salts similar to plasma, Hepes buffer, and Hepes buffer with $ZnCl_2$, $CaCl_2$, $MgCl_2$, or Na_2CO_3 added. Pore formation at the surface and inside the film was analyzed by scanning electron microscopy. The samples were also analyzed gravimetrically at predetermined intervals to determine the mass loss, and for some samples the pH within the PLG films was determined by confocal microscopy. Pores were formed faster in the presence of all divalent cations, and the results indicated a greater degradation rate in the presence of Zn^{2+}. The catalyzing effect of the divalent cations on degradation was attributed to their ability to act as Lewis acids. Pores were formed more slowly in PBS than in a buffer containing salts similar to plasma, which should be considered when choosing the in vitro release medium.

Keywords controlled release, poly(D,L-lactide-co-glycolide), degradation, pore formation, divalent cations, diffusion, in vitro–in vivo correlation

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INTRODUCTION

The use of poly(D,L-lactide-co-glycolide) (PLG) in parenteral controlled-release formulations has been investigated for two decades, and PLG is one of the polymers most often used for this application. The interest in PLG is due to the fact that it is biodegradable and nontoxic, and availability of different kinds of PLG make a wide range of release rates possible.[1] As well as microparticles consisting of PLG, other systems use encapsulation of drugs in a hydrophilic matrix, which is subsequently coated with PLG[2] where the PLG coating layer acts a diffusion-hindering membrane.

To control the release, it is important to know the underlying release mechanisms. This is especially true because during release PLG is subjected to water absorption and swelling, hydrolysis, and erosion.[3] The encapsulated drugs can be released in three main ways: diffusion through the polymer network, diffusion through water-filled pores, or by erosion of the polymer. Diffusion through water-filled pores is often proposed as the main release mechanism when the encapsulated drug is a water-soluble protein and when low-molecular-weight PLG is used. Thus, pore formation is a very important step. Pores are formed either by swelling,[4] dissolution of the polymer,[5] or a combination of the two.

When developing a new sustained-release formulation it is important to obtain an in vitro–in vivo correlation. Thus, it is important to consider the influence of buffer salts in the in vitro medium on PLG degradation when choosing the release medium. Phosphate buffer saline (PBS) is often used in in vitro experiments to mimic

tissue fluids, without considering the different ionic species in body fluids. Different cation salts may be present in protein-delivery systems for the following reasons:

- As a buffer substance to prevent a fall in pH in PLG formulations during degradation[6–8]
- As a protein and peptide stabilizer[9–15]
- As a precipitant or complexing agent[16,17]

Various salts have been shown to affect the degradation of PLG and the release of encapsulated drugs. There have been many reports on the effects of encapsulated salts, such as NaCl, $ZnCO_3$, $MgCO_3$, $CaCO_3$, and $Mg(OH)_2$ on water absorption, hydrolysis rate, mass loss, pH within the film, and pore formation.[4,6,7,8,18,19] However, an encapsulated salt may not affect PLG in the same way as a salt in the surrounding buffer. For example, the encapsulated salt may dissolve and diffuse out, resulting in increased porosity. There have been few reports on the effects of buffer salts in the release medium, and the results have sometimes been contradictory. For example, low water absorption due to salt in the release medium has been reported.[20] On the other hand, the same initial water absorption in deionized water and PBS, followed by faster water absorption in PBS, has also been reported.[21] Cations are able to form complexes with PLG. It has been suggested that this leads to changes in the plasticizing effect of degradation products and of the autocatalytic effect on hydrolysis.[22] In particular, Zn has been reported to form a complex with PLG.[18,20,23] In most reports on the effect of a salt, the anion but not the cation is discussed. Although some of the effects of salt on the degradation of PLG have been established, others are not understood.

This article describes an investigation into the influence of the composition of the release medium, especially concerning divalent cations, on the degradation of PLG. Pore formation was investigated, as well as mass loss and pH inside the PLG films incubated in buffers containing different salts. This will contribute to the understanding of the release mechanism and improve formulation design to obtain a predetermined release rate and release duration.

MATERIALS AND METHODS

Materials

PLG (RG502H, 50:50 lactide/glycolide, with an approximate molecular weight of 12 000 g/mol and polydispersity of 1.2) was obtained from Boehringer Ingelheim Pharma KG (Germany), polysorbate 80 from Uniqema (Belgium) and fluorescent probes [tetramethylrhodamine (TMR) and Oregon green dextran conjugates, 70 kDa] from Molecular Probes Europe BV (The Netherlands). Hepes acid and Na-Hepes salt were obtained from ICN Biomedicals Inc. (USA). NaCl, $MgCl_2 \cdot 6H_2O$, Na_2CO_3, KH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, and $NaH_2PO_4 \cdot H_2O$ were obtained from Merck (Germany). $CaCl_2 \cdot 2H_2O$ and $MgSO_4$ were obtained from Sigma Co. (USA), $ZnCl_2$ from Fluka Chemie AG (Switzerland), NaN_3 from Acros Organics (USA), and KCl from Janssen Chimica (Belgium). All salts were of analytical grade.

Methods

Preparation of Films

PLG and polysorbate 80 were dissolved in ethyl acetate, and PLG films containing 2% polysorbate were prepared by casting. Films without any detergent, such as polysorbate 80, have been shown to differ in wettability from microparticles, as some of the detergent used in microparticle preparation remains on the microparticles.[2,24,25,26] The PLG films contained about 5% (w/w) ethyl acetate after preparation, as measured by gas chromatography. The residual solvent diffuses into the buffer immediately at incubation and does not affect the porosity as shown in initial experiments. The buffer alone, which is absorbed by the polymer immediately, plasticizes the polymer and shifts the glass transition temperature (Tg) below the incubation temperature. To analyze the pH inside the film, fluorescent pH-sensitive probes were encapsulated in films using the same casting procedure, but in three steps. First, the bottom half of the film was cast. Then the fluorescent probes were suspended in a small portion of the remaining PLG-polysorbate solution and cast onto the bottom layer. The probes sedimented through this solution. Finally, the rest of the PLG-polysorbate solution was cast on top, resulting in the fluorescent probes being entrapped in the middle. Only films used for pH analysis contained fluorescent probes.

Buffers

The ionic species investigated were Zn^{2+}, Mg^{2+}, Ca^{2+}, and CO_3^{2-}, and the buffers used are described in Table 1. The osmolality of the buffers was analyzed by using a vapor pressure osmometer (Vapro 5520, Vesco Inc., USA). Hepes buffer was used as a reference and was compared with Hepes buffer with the addition of 1 mM of the chloride or sodium salt of the ions studied. Because PBS is often used in in vitro experiments, this buffer was also investigated. To investigate the in vitro–in vivo correlation, a buffer containing salt, similar to plasma,[27] denoted

Table 1
Buffers used in the experiments

Buffer	Composition	Osmolality (mmol/kg)
Hepes buffer	37.5 mM Hepes acid, 37.5 mM Na-Hepes, 115 mM NaCl, 15.4 mM NaN_3	345
Hepes buffer + $ZnCl_2$	Hepes buffer, 1 mM $ZnCl_2$	344
Hepes buffer + $MgCl_2$	Hepes buffer, 1 mM $MgCl_2$	342
Hepes buffer + $CaCl_2$	Hepes buffer, 1 mM $CaCl_2$	353
Hepes buffer + Na_2CO_3	Hepes buffer, 1 mM Na_2CO_3	371
PBS	12 mM $NaH_2PO_4 \cdot H_2O$, 18 mM $Na_2HPO_4 \cdot 2H_2O$, 82 mM NaCl, 0.5 mM $CaCl_2$, 3 mM NaN_3	224
"Plasma buffer"	137 mM Na^+, 4.35 mM K^+, 2.1 mM Ca^{2+}, 1.8 mM Mg^{2+}, 0.8 mM SO_4^{2-}, 114 mM Cl^-, 0.7 mM PO_4^{3-}, 3 mM NaN_3, 60 mM Hepes	300

All buffers were adjusted to pH 7.4.

"plasma buffer," was also included. In plasma the pH-regulating system is CO_3^{2-}, but this had to be replaced by Hepes because of problems due to precipitation.

Incubation

Pieces of the films (about 130 mg, about 150 μm thick, with a diameter of 20 mm) were cut out by using a punch and incubated at 37°C in buffer with a volume of 0.24 mL buffer per mg PLG. Samples were collected and analyzed with regard to mass loss and pore formation at the surface and inside the film on days 1, 2, (3), 4, 7, 9, 11, and 14. The pH inside the film was also analyzed by using pieces incubated in Hepes buffer with and without $ZnCl_2$. These pieces (about 150 μm thick, with a diameter of 12 or 14 mm) were cut out from films containing fluorescent probes. The buffer was replaced by fresh buffer on each sampling occasion (except day 3). To facilitate the transfer of the PLG samples for weighing, they were placed on metal nets that could be lifted. Each experiment was run in duplicate.

Analysis

Mass loss was calculated by weighing the sample prior to incubation and after washing and drying the sample in a vacuum chamber. The formation of pores was investigated by using scanning electron microscopy, SEM (Philips XL30 microscope, Philips, The Netherlands). To investigate the cross section, pieces were sliced and mounted so that the cross section faced up. The samples were sputtered with gold (108 Auto Sputter Coater, Cressington Scientific Instruments Ltd, UK). The pH inside the films was analyzed by measuring the fluorescence of pH-sensitive probes using an MRC-1024 confocal system (Bio-Rad Laboratories, UK) attached to a Nikon Eclipse microscope (Nikon, Japan) and the software Laser Sharp (Bio-Rad Laboratories, UK). Similar pH measurements or pH mapping based on confocal microscopy and pH-sensitive fluorescent probes have been carried out by others.[7,28] In the present investigation two kinds of probes were used: Oregon green and TMR, both conjugated to 70 kDa dextran to limit diffusion. Laser wavelengths of 488 nm and 568 nm were used simultaneously to excite Oregon green and TMR, respectively. The intensity of the fluorescence emission of Oregon green (wavelength 522 nm) depends on the pH, whereas the fluorescence of TMR (wavelength 598 nm) is independent of the pH. The intensity ratio of green to red emission is thus related to pH. A calibration curve relating pH to intensity ratio was obtained by measuring the fluorescence emission of pH standards. Probes dissolved in the pH-adjusted buffer were used as standards. Hepes buffer with and without $ZnCl_2$ was used to obtain separate standard curves for the samples degraded in these two buffers. There was not any difference in these standard curves, which proves that Zn^{2+} did not interfere with the fluorescent emission. The lower limit of pH measurement was 3.7, because the intensity ratio of the emission was close to zero at this pH. Oregon green is pH-sensitive above this limit, which makes it possible to measure the pH above 3.7.

Solubility of Oligomers

The purpose of this analysis was to investigate whether the divalent cations increased the soluble fraction, which could explain the increased rate of pore formation. Here, the term solubility applies to the fraction of the PLG compound that has sufficiently low molecular weight (i.e., oligomers) and is thus sufficiently hydrophilic to be water soluble. Because the mean molecular weight of this particular PLG was low (12 000 g/mol) the water-soluble fraction was present from the beginning and increased as the molecular weight decreased due to hydrolysis. Buffers may affect the hydrophilicity of PLG, by ionic interaction or complex formation and thus also the water-soluble fraction.[18,20,23] The solubility of PLG oligomers in the different buffers was studied by using PLG powder. Enough PLG to ensure that the amount of oligomers dissolved was not limited by the amount added was incubated for 10 min under stirring at 37°C. The same amount of PLG and volume of buffer were used for all samples. The samples were centrifuged and undissolved PLG was removed. Dissolved PLG oligomers were degraded to their monomers in sodium hydroxide under ultrasonication for 1 hr. After adding HCl to obtain neutral pH, the concentration of L-lactate was analyzed by using a Sire® Biosensor P100 and a Sire® L-Lactate Kit (Chemel AB, Sweden). Because the proportions of L-lactate, D-lactate and glycolide were known, the concentration of PLG could be calculated. The experiment was run in duplicate.

RESULTS AND DISCUSSION

Effect of Divalent Cations on Pore Formation and Degradation

Pore Formation

Pore formation was investigated at the surface and inside the films. The time, elapsed before pores appeared is given in Table 2. Pores were formed at the surface on day 1 in all buffers with divalent cations (Figure 1), but not until several days later without these cations. PBS also contained Ca^{2+}, at a concentration of 0.5 mM, but showed slower pore formation, probably due to Ca^{2+} binding to phosphate. This is an indication of faster degradation in the presence of divalent cations, which in turn implies faster diffusion of drugs through the pores formed. Zn^{2+} is known to act as a Lewis acid and may thereby catalyze the degradation. The other divalent cations should also be able to act as Lewis acids, and thus these results could be expected. The surface of all films appeared completely smooth prior to incubation when examined with SEM (Figure 1). It should be noted that the films sometimes varied in appearance within a sample. The images shown in Figures 1 and 2 can be considered to be representative of the samples. The times presented in Table 2 are similarly typical for the films.

Faster pore formation in the presence of divalent cations was also seen when studying cross sections of the samples (Table 2). Pores were formed close to the surface on day 1, and the porous region grew toward the center of the film, as shown in Figure 2 for Hepes buffer with $ZnCl_2$ added. The time at which the whole cross section was porous is also given in Table 2. It is obvious that the porous structure, which formed faster when divalent cations were added, will lead to a faster diffusion rate, and subsequently the faster release of an encapsulated drug.

The pores observed in the cross sections grew larger with time and seemed to coalesce to form very large pores. This coalescence of pores occurred earlier when divalent cations were present, which again indicates faster degradation. The growth and coalescence of very small pores into larger pores, and the release of encapsulated drug when the pores were large enough and the structure porous enough, have been reported by others.[5] In a previous study[29] we found a size-exclusion effect. The difference in diffusion rate of high-molecular-weight drugs and low-molecular-weight substances in the partly degraded PLG did not correspond to the difference in molecular weight.

Adding Na_2CO_3 to HEPES buffer seemed to slow down the formation of pores, both at the surface and inside the sample (Table 2). It is possible that this was due to the buffering effect of CO_3^{2-}, although the concentration of CO_3^{2-} was small. The effect of a divalent cation might be counteracted by the CO_3^{2-} anion, if such a salt is used.

Table 2
Time required for pores to appear at the surface and for pores to be formed throughout the whole film (days) for the different buffers

Buffer	Hepes	Hepes + $ZnCl_2$	Hepes + $MgCl_2$	Hepes + $CaCl_2$	Hepes + Na_2CO_3	Plasma buffer	PBS
Pores at surface (Figure 1)	7	1	1	1	9	1	4–9
Pores throughout film (Figure 2)	9–14	7	7	4–7	11–>14	4–7	7–11

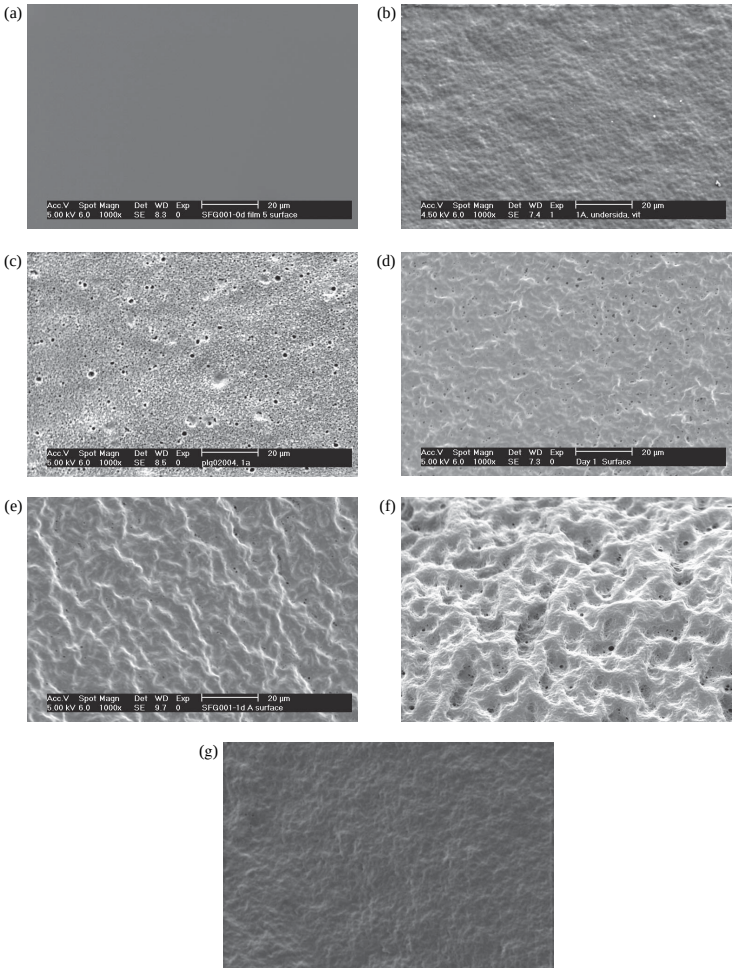

Figure 1. The surface on day 0 (a). The surface on day 1 in (b) Hepes buffer, (c) Hepes buffer with $ZnCl_2$, (d) Hepes buffer with $MgCl_2$, (e) Hepes buffer with $CaCl_2$, (f) plasma buffer, and (g) PBS. Magnification 1000 ×.

Divalent cations are sometimes used in controlled-release formulations, and the results of this investigation show that these salts affect the formation of pores in the polymer controlling drug release. Basic salts of Mg, Ca, and Zn are used to neutralize the acid degradation products.[6,7,19] There are many reports on the use of Zn to stabilize the protein.[9,11–15] However, the effect of Zn on PLG is not discussed. Faster release when Zn was used in the formulation can be seen from the results in reports, although this was not the objective of the investigations.[13,14] It is surprising that a slower release of a Zn-conjugated drug has also been reported.[30] However, different drugs were compared, and the different release rates were explained by the indicated different distributions of the different drugs in the microparticles. The results of this investigation also show that PBS affects the formation of pores differently from a buffer containing ions similar to plasma. Because pore formation is usually the step governing

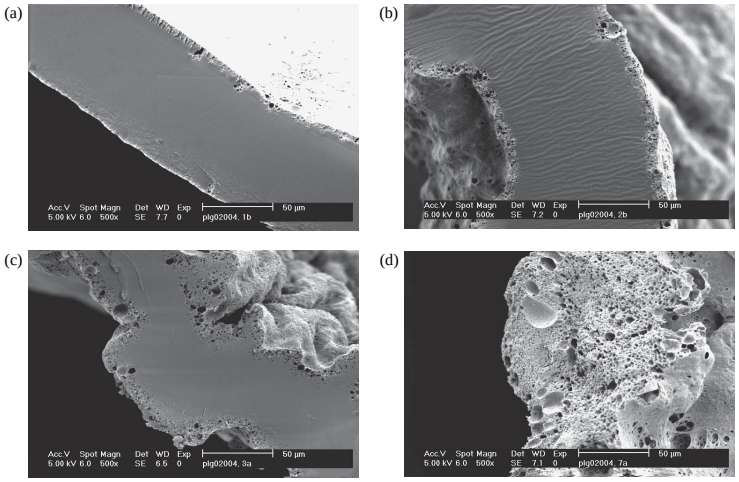

Figure 2. Cross sections of samples showing degradation in Hepes buffer with 1 mM $ZnCl_2$ (a) on day 1, (b) on day 2, (c) on day 3, and (d) on day 7. Magnification 500 ×.

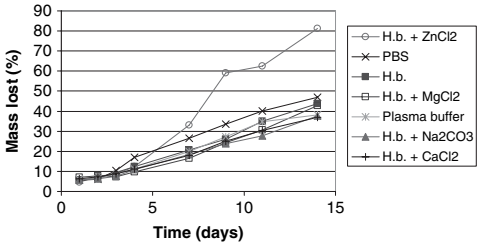

Figure 3. Mass loss of PLG in the different buffers. H.b. denotes Hepes buffer. The deviation of duplicate samples from the average was about 5%, which means that the experimental error is insignificant.

in the release rate, the choice of buffer is important, especially because PBS is often used for in vitro experiments.

Mass Loss

The mass loss of the PLG films is presented in Figure 3. The mass loss was similar in all buffers except in Hepes buffer containing $ZnCl_2$, in which it was higher after a lag time, which indicates that Zn^{2+} has a catalyzing effect on degradation. The deviation of duplicate samples from the average was only about 5%, which means that the experimental error is insignificant. The effect of Zn^{2+} was in agreement with the analyses of pore formation.

The samples became porous throughout the whole thickness of the film between days 4 and 7, which corresponds to the time when mass loss increased and the degradation products could diffuse out faster. Mg^{2+} and Ca^{2+} should also be able to act as Lewis acids, but no such effect was observed in this investigation. Zn^{2+} might be a better Lewis acid, and the concentration of Mg^{2+} and Ca^{2+} may have been too low to have an effect. Lewis acids are known to act to varying degrees with different Lewis bases, depending on the frontier orbitals and the energy of the electrons participating in the bonding.[31] Zn, which belongs to group IIB of the periodic table, differs in its electron structure from Mg and Ca, which belong to group IIA. Similar results have been reported on the encapsulation of water-soluble salts such as $ZnSO_4$ and $MgSO_4$, or sparingly soluble salts such as $ZnCO_3$, $MgCO_3$, and $CaCO_3$ in PLG.[18] Pores were formed faster, but the degradation rate was slower in the case of the CO_3^{2-} salts and was not affected by the SO_4^{2-} salts. Zhang et al attributed the faster pore formation to faster water absorption. However, in the present investigation water absorption was equally fast during the first 4 days of incubation in the different buffers (data not shown). A possible explanation of the results in these investigations is that the degradation rate was higher locally where very small pores started to form.[5] However, the overall degradation was slower due to the neutralizing effect of CO_3^{2-} or was too low to be detected in the cases of SO_4^{2-}, $MgCl_2$, and $CaCl_2$.

This kind of mass loss should affect the release rate, and it should be considered when using zinc salts in controlled-release PLG formulations.

pH Within the PLG Film

The results of pore formation and mass loss analyses indicate a higher degradation rate when $ZnCl_2$ is added to Hepes buffer. Another indication of a higher degradation rate is a low pH within the film. The initial low pH is due to dissolution of water-soluble oligomers. Figure 4 shows the pH inside the film during incubation in Hepes buffer and Hepes with $ZnCl_2$ added. The initial low pH increased due to absorption of the buffer. The pH increased most in Hepes buffer without Zn^{2+}. In the Hepes buffer containing $ZnCl_2$, the pH increase was instead very small. This further indicates that Zn^{2+} acts as a catalyst for hydrolysis and forms acidic degradation products at a higher rate than without Zn^{2+}. The pH was probably lower than 3.7 on day 1, but, as mentioned in Materials and Methods, this value is the limit of this particular pH-sensitive prob. A pH as low as 1.8 has been reported in PLG.[7] On day 2, and during the rest of the experiment, the pH values were above the limit in the present investigation. The variation in pH within a PLG film was small.

It is well known that hydrolysis produces acidic degradation products that autocatalyze the reaction. At sufficiently low molecular weight, the oligomers dissolve and, depending on the pore network, the dissolved oligomers either diffuse out of the film or remain inside. The increase in hydrolysis rate in the presence of Zn^{2+} resulted in a lower pH inside the film due to the accumulation of degradation products which, apart from their autocatalyzing effect, are known to increase degradation by plasticizing the polymer. The hydrolysis rate is often investigated by monitoring the decrease in molecular weight of PLG using gel permeation chromatography (GPC). An attempt was made to measure the molecular weight of the samples in this investigation using GPC and light scattering. However, it was impossible to obtain reliable data because a significant part of the samples had a molecular weight low enough to be soluble in water soon after the start of degradation, and the samples were not soluble in tetrahydrofuran, which was used in the analysis. Different solvents and mixtures of solvents and water were tested, but none of them were better than tetrahydrofuran.

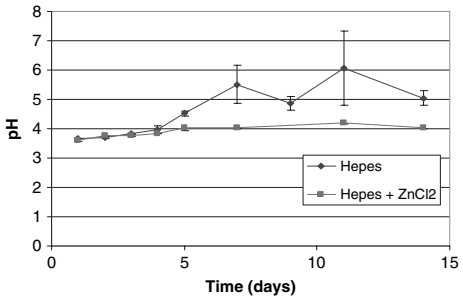

Figure 4. pH inside the PLG film during incubation in Hepes buffer with and without $ZnCl_2$. The error bars show the difference between duplicate experiments, which was too small to be seen in Hepes buffer with $ZnCl_2$. The pH on day 9 within the films incubated in the presence of $ZnCl_2$ could not be determined because very large differences in pH were obtained within the samples on this day.

Solubility of Oligomers

As mentioned above, the solubility of PLG applies to the fraction of the compound that has sufficiently low molecular weight (i.e., oligomers) and is thus sufficiently hydrophilic to be water soluble. The purpose of this analysis was to investigate whether the divalent cations increased the soluble fraction, which could explain the increased rate of pore formation. Because the duration of this experiment (maximum 1 hr before removing undissolved PLG) should only cause an insignificant decrease in molecular weight and because duplicate samples incubated for shorter times did not show lower solubility, the measured solubility should not be significantly affected by hydrolysis. However, this solubility should only be used for comparison, and not as an absolute solubility, because the soluble fraction increases with longer incubation times due to degradation. As shown in Figure 5, the solubility of PLG oligomers decreased with the addition of each of the divalent cations, particularly in $ZnCl_2$ and in plasma buffer. PBS also contained Ca^{2+}, but no effect was seen probably due to Ca^{2+} binding to phosphate and the lower concentration. This means that the faster formation of

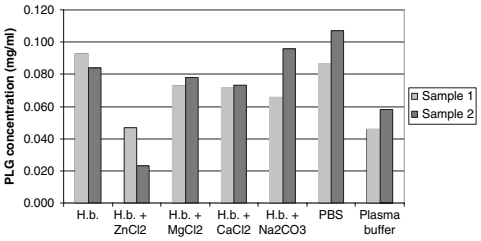

Figure 5. Comparison of the solubility of PLG in the investigated buffers. The term solubility applies to the water-soluble fraction of the polymer. Samples 1 and 2 are duplicates.

pores in the presence of divalent cations is due to increased degradation and not to higher solubility of oligomers.

The decreased solubility of oligomers may be a result of complex formation between PLG and divalent cations, resulting in less hydrophilic oligomers with lower solubility. Complex formation of Zn^{2+} with carbonyl oxygen at the ester sites, and by Zn^{2+} substituting for hydrogen at the terminal carboxylic acid, has been reported previously.[20,23] Mg^{2+} and Ca^{2+} have also been reported to form complex or interact with PLG.[18,20]

Observations Regarding Pore Formation

The porosity at the surface was lower than that inside the film in all samples, except for a short period at the beginning of the experiments. The higher porosity inside the film was the result of the faster hydrolysis due to the presence of acidic degradation products. This confirms results reported by others.[32–34] Unlike some other reports,[5] the present investigation shows that the pores did not develop homogeneously throughout the polymer film. Pores were first formed close to the surface, and the porous region then grew with time toward the center. This may be explained as follows. Pore formation is probably a combination of degradation, which is catalyzed by divalent cations, and swelling due to water absorption. Pores are initially formed at the surface, and these grow faster in the presence of divalent cations. Water penetrates PLG rapidly (data not shown), as has also been reported by others,[5,35] and thus water is probably also present in the nonporous region. Because more water is present close to the surface than in the middle of the film, the polymer swells and becomes porous. At the border between porous and nonporous regions, greater amounts of degradation products probably dissolve than in the nonporous region due to the greater area of water–polymer contact. This results in the formation of pores and in the movement of the border between the porous and nonporous regions toward the center. As can be seen from Table 2 this movement was faster in the presence of divalent cations catalyzing the hydrolysis. The results show that the diffusion resistance does not develop homogeneously throughout the film, which could be important when designing models for simulating release.

In all buffers, except Hepes containing Na_2CO_3, the porosity at the surface started to decrease or completely disappeared at some point in time. This would result in a slowing down of the diffusion of encapsulated drugs. This annealing effect could be due to the decrease in the glass transition temperature (Tg), and the critical softening temperature reaching 37°C. It is well known that Tg decreases with time due to degradation and the plasticizing effect of absorbed water.[36] Annealing of pores when Tg decreased 10–15°C below the incubation temperature has been reported by others.[37] They called this temperature (10–15°C above Tg) the critical softening temperature. Other examples of a decrease in the porosity of the structure as a result of an incubation temperature higher than Tg have been reported.[32,38] The reason why annealing was not seen in the presence of Na_2CO_3 may be the slow degradation, resulting in a critical softening temperature higher than 37°C throughout the whole experiment.

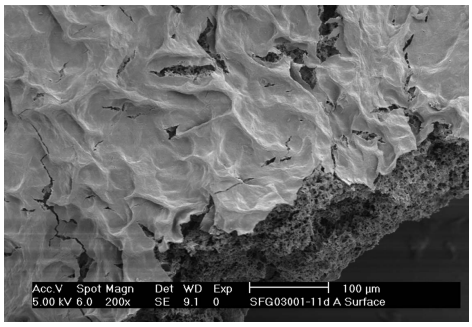

Figure 6. The "skin" formed on the PLG film due to faster degradation inside the film, after 11 days of incubation in Hepes buffer with 1 mM $CaCl_2$.

The increasing porosity inside the film and annealing of pores at the surface resulted in the formation of a "skin" on the surface of the PLG film, as shown in Figure 6, for Hepes buffer with $CaCl_2$, which was reported previously.[36,39] Thus, both annealing due to lower critical softening temperature, as well as pore formation, are results of degradation. The reason that the interior of the film was not annealed was probably that it was too porous for annealing to take place. After the skin has formed, this will probably be the barrier of greatest transport resistance.

CONCLUSIONS

Faster degradation in the presence of Zn^{2+} was indicated by faster pore formation, faster mass loss after a lag phase, and lower pH inside the film. In all buffers with divalent cations (except PBS, probably due to Ca^{2+} binding to phosphate and the lower concentration), pores were formed at the surface much faster than in other buffers, and this was explained by the divalent cations acting as Lewis acids catalyzing hydrolysis. The divalent

cations made the water-soluble fraction of the polymer (i.e., oligomers) less soluble, probably by complex formation. Thus, the increase in mass loss in the presence of Zn^{2+} and the increase in pore formation were not due to increased solubility, but to an increase in the hydrolysis rate.

Although degradation was heterogeneous (i.e., faster inside the film than at the surface due to acid degradation products), the formation of pores within the film followed a different pattern. The region close to the surface became porous faster than the center. A skin was formed on the surface, as pores annealed after different incubation times in different buffers. This was probably due to the critical softening temperature having decreased to the incubation temperature. This complex pattern of pore formation and annealing of pores results in a change in the diffusion resistance in time and space, which should be kept in mind when simulating release.

Faster pore formation, due to faster degradation, is an important factor that influences release patterns, although there is probably a lag phase before complete networks of pores of sufficient size have been formed. This investigation shows that PBS affects pore formation differently from a buffer containing ions similar to those in plasma.

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Paper III

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Encapsulated zinc salt increases the diffusion of protein through PLG films

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ABSTRACT

The use of microspheres and nanospheres of poly(D,L-lactide-co-glycolide) (PLG) as a controlled-release drug delivery system has been the subject of great interest for at least two decades within the field of pharmaceuticals. Salts of zinc and other divalent cations are sometimes co-encapsulated in PLG particles to control the pH or to stabilize encapsulated proteins or peptides. Zinc salts are known to affect pore formation and other processes that may lead to the release of an encapsulated drug. In this study the effect of encapsulated zinc acetate on protein diffusion through PLG films was investigated. PLG films, with and without encapsulated zinc acetate, were degraded in Hepes buffer for different periods of time. The films were subsequently subjected to various kinds of analyses: diffusion properties (using a diffusion cell), porosity (using scanning electron microscopy) and thickness (using light microscopy and an image-analysis program). Encapsulated zinc acetate had a considerable effect and increased the diffusion coefficient of lysozyme through PLG films degraded for 18 days or longer. Films containing zinc acetate became porous, while those without zinc acetate only developed cavities on the surface. Zinc salts may thus be used as release-modifying agents. This effect should be considered when using zinc salts as protein stabilizers or pH neutralizers.

1. Introduction

Microspheres and nanospheres of poly(D,L-lactide-co-glycolide) (PLG) have been studied during the last two decades to determine their usefulness as controlled-release drug delivery systems. The number of protein pharmaceuticals that have to be injected, due to low bioavailability, has increased greatly in recent years (Walsh, 2003). One of the advantages of controlled-release formulations is the reduction in the number of injections required. Another obvious advantage is that a constant drug concentration can be maintained in the blood.

PLG is the biodegradable polymer most commonly used for this application. Some of the reasons for the extensive use of PLG are its biocompatibility, its approval by regulatory authorities, and the possibility of controlling the duration of drug release, ranging from days (Liu et al., 2003) to many months (Lagarce et al., 2005). The microspheres may consist of a matrix in which the drug is dispersed, or the drug may be encapsulated in a hydrophilic core which is subsequently coated with a layer of PLG acting as a diffusion-controlling membrane (Reslow et al., 2002).

To be able to control the release, it is important to have the knowledge of the factors affecting the process. The main release mechanism is diffusion through the pores formed by swelling (Webber et al., 1998; Liu et al., 2005) and degradation/erosion (Batycky et al., 1997; Kim and Park, 2004; Berkland et al., 2007). These processes are affected by a number of factors (Fredenberg, 2004). In a previous paper we showed that the rate of pore formation increased in the presence of divalent cations, especially by zinc ions (Fredenberg et al., 2007).

Salts of zinc and other divalent cations are sometimes co-encapsulated in PLG particles to control the pH (Shenderova et al., 1999; Tracy et al., 1999; Zhu and Schwendeman, 2000). Zinc salts are also used to stabilize encapsulated proteins or peptides (Johnson et al., 1997; Carino et al., 2000; Lam et al., 2000; Takada et al., 2003; Takenaga et al., 2004; Bilati et al., 2005). There have been numerous reports on the effects of zinc salts on water absorption, hydrolysis rate, mass loss, pore formation, release rate and pH inside the particle (Zhang et al., 1997; Tracy et al., 1999; Zhu and Schwendeman, 2000; Li and Schwendeman, 2005; Houchin et al., 2007). These results have mainly been attributed to the anion, and not to the zinc cation. Higher porosity has been found when salts of zinc or other divalent cations are encapsulated, and this has been attributed to faster water absorption due to higher osmotic pressure (Zhang et al., 1997; Zhu and Schwendeman, 2000; Kang and Schwendeman, 2007). This may be true, but the catalysing effect of divalent cations, reported in our previous work, probably also increased the porosity. There have also been reports of changes in the drug release rate in the presence of zinc ions, although this

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was not always the objective of the investigation (Cleland et al., 1997; Lam et al., 2000; Zhu and Schwendeman, 2000; Takada et al., 2003; Ishihara et al., 2005; Kang and Schwendeman, 2007). In some studies, the release rate was found to increase, while in others it decreased. However, as it will be discussed later, the decrease in release rate can be explained by other factors.

This paper describes the effect of encapsulated zinc acetate on protein diffusion through PLG films. Furthermore, the influence on pore formation and the porosity of the PLG films was studied. The method employed for diffusion measurements was based on a diffusion cell technique, which has been described previously (Fredenberg et al., 2004). Lysozyme was chosen as a model protein.

2. Materials and methods

2.1. Materials

PLG (RG502H, 50:50 lactide/glycolide, with an approximate molecular weight of 12,000 g/mol and a polydispersity of 1.2) was obtained from Boehringer Ingelheim Pharma KG (Germany). Polysorbate 80, lysozyme (14,100 g/mol) and sodium Hepes salt were obtained from Sigma–Aldrich Inc. (USA). Hepes acid was obtained from Research Organics (USA) and NaCl, zinc acetate and ethyl acetate were obtained from Merck KGaA (Germany). NaN_3 was obtained from VWR International Ltd. (UK) and PVDF filters and nylon filters from Millipore AB (Sweden). All salts were of analytical grade.

2.2. Film preparation

The method of spraying films has been described in detail before (Fredenberg et al., 2004). Briefly, films of PLG containing 2% polysorbate 80 and also some 5% zinc acetate were made by spraying a solution onto a polyvinylidene fluoride (PVDF) filter using a Hüttlin spray nozzle. The filter, which had a pore size of 0.65 μm, was mounted on a rotating wheel, and the filter thus passed through the spray at determined intervals, in order to mimic a normal coating process. The PLG, polysorbate 80 and zinc acetate (when used) were dissolved in ethyl acetate. Twelve films were made simultaneously to ensure reproducibility.

2.3. Incubation

The PLG films, with and without zinc acetate, were degraded in 75 mM Hepes buffer containing 115 mM NaCl and 5 mM NaN_3, pH 7.4, at 37 °C. Triplicate samples were degraded for 7, 14, 18, 21 and 35 days before performing diffusion measurements. For thickness measurements five strips from three of the films were degraded under the same conditions, for the same periods of time.

2.4. Diffusion measurements

The method used for diffusion measurements and the method of evaluation have been described previously (Fredenberg et al., 2004). A diffusion cell was used. The PLG film, sprayed onto a filter, formed the barrier between the stirred donor and receiver compartments, and a fibre optic probe measuring the UV-absorption ("Dip probe accessory", Varian Inc., USA) was placed in the receiver compartment, see Fig. 1. The openings were covered with parafilm to avoid evaporation and the diffusion cell was placed in a water bath at a temperature of 37 °C. A coarse nylon filter, pore size 60 μm, was mounted together with the PLG film to protect the film from possible erosion caused by the stirring. This filter does not influence the mass transport rate, which was thoroughly evaluated (Fredenberg et al., 2004).

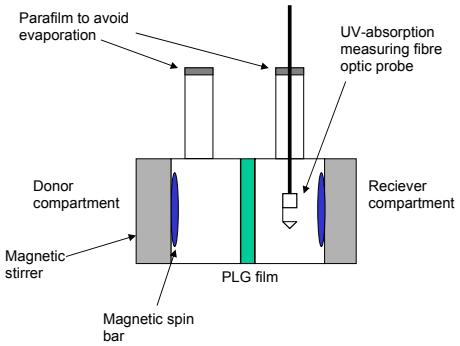

Fig. 1. The diffusion cell and the UV-absorption measuring fibre optic probe.

The UV-absorption measuring fibre optic probe together with a Cary 50 Bio spectrophotometer (Varian Inc., USA) was used to measure the concentration of lysozyme in the receiver compartment during the diffusion experiments. A sample of known concentration was measured each day to ensure the accuracy of the measurements. The baseline was checked by measuring the UV absorbance in pure Hepes buffer, used for dissolution, and any deviation from zero was subtracted when calculating the concentrations. The initial concentration in the donor compartment was measured before addition. The concentration in the donor compartment was then calculated from the concentration in the receiver compartment using a mass balance. The accuracy of this mass balance was checked at the end of the experiments by measuring the concentration in the donor compartment.

2.5. Initial experiments necessary for determining the diffusion coefficient

Initial experiments were performed to confirm that the diffusion coefficient was independent of concentration, by measuring the diffusion through the PVDF filter with 5 and 10 mg/ml lysozyme in Hepes buffer. The experiments were run in triplicate.

Possible adsorption of lysozyme onto PLG films, with and without zinc acetate, and the PVDF filter was also evaluated in initial experiments. Films degraded for 10 days in Hepes buffer at 37 °C were incubated for 1 day in Hepes buffer containing 53 μg/ml or 5 mg/ml lysozyme, which are representative of the concentrations in the two compartments at the beginning of the diffusion process. The concentration of lysozyme was measured before and after incubation. Triplicate samples were used.

The diffusion coefficient of lysozyme through the PVDF filter was also determined in initial measurements. The measurements were conducted in the same way as the subsequent diffusion measurements, except that only the PVDF filter was mounted in the diffusion cell. Measurements were made in triplicate.

2.6. Calculation of the effective diffusion coefficient

The theory of diffusion measurements using this kind of diffusion cell, and the calculation of the diffusion coefficient have been described in our previous work (Fredenberg et al., 2004). Briefly, the calculation is based on Fick's law:

$$j = -D_e \frac{dC}{dz} \quad (1)$$

The mass flux through the film, j [g/(m^2 s)], is expressed in terms of the effective diffusion coefficient D_e. When using a diaphragm cell, pseudo-steady-state diffusion, a condition attained after a short time lag, is often applicable. The solution of Eq. (1) together with a mass balance over the two compartments results in (Westrin, 1991):

$$K = \frac{1}{S(1/V_A + 1/V_B)} \cdot \frac{\ln(C_{A1} - C_{B1}/C_{A2} - C_{B2})}{t_2 - t_1} \quad (2)$$

The subscripts A and B denote the donor and receiver compartment, respectively. Subscripts 1 and 2 denote sample numbers. K is a mass transfer coefficient, S is the diffusion area and t denotes time. V is the volume of the compartments A and B, and C is the concentration. To increase the accuracy, many measurements should be made at different times. When the logarithmic concentration ratio in Eq. (2) is plotted against time, the value of K can be determined from the slope of the line.

When a polymer film is made by spraying the polymer onto a filter, the total diffusion resistance consists not only of the resistance through the polymer film but also that through the filter. The total mass transfer resistance is thus the sum of the mass transfer resistances according to Eq. (3):

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{D_{e\,filter}} \quad (3)$$

where l is the thickness of the film or filter. By rearranging Eq. (3) the effective diffusion coefficient for the PLG film can be obtained by simply subtracting the filter resistance ($l_{filter}/D_{e\,filter}$), which was determined in the initial experiments.

2.7. Thickness measurement

Strips of PLG films degraded in Hepes buffer, as described above, were mounted so that the edge faced upwards in holders made specially for this application. Photographs were taken of the edge using a BX50F4 microscope from the Olympus Optical Co. Ltd. (Japan) and a SSC-DC38P digital camera from Sony Co. (USA). The thickness was determined using the software Image Pro Plus, version 4.1 (Media Cybernetics Inc., USA). The PLG films used for thickness measurements were made simultaneously with those used for diffusion experiments. Three different films were used and five strips were cut from each film. Three pictures were taken of each strip and ten measurements were made on each picture, giving a total of 450 measurements for each determination of the thickness.

2.8. Porosity

The surfaces of the diffusion area of all the PLG films were examined to study the pore formation, using a JSM-6700F field emission scanning electron microscope from Jeol Ltd. (Japan). The samples were sputtered with gold prior to inspection.

3. Results and discussion

3.1. Film preparation

The PLG films obtained by spraying had thicknesses of 7.5–9.5 μm, calculated from their weight and a density of 1.3 g/cm^3. This density was determined gravimetrically by us (data not shown) and has also been reported by others (Duvvuri et al., 2005). The standard deviation of the film thickness within each batch of 12 films made simultaneously was 0.2 μm. The PLG films were non-porous and completely smooth (Fig. 2). The very low standard deviation obtained in this kind of spraying process is probably much lower than that obtained when coating microspheres (Borgquist et al., 2004).

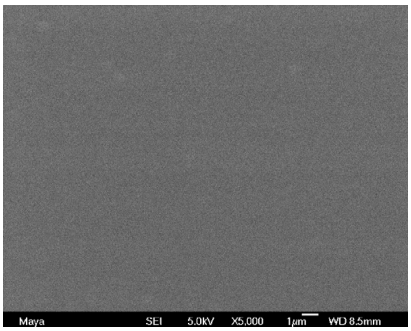

Fig. 2. The surfaces of the sprayed PLG films were smooth and non-porous before incubation in buffer.

3.2. Initial experiments

The diffusion coefficient of lysozyme did not depend on the concentration, as can be seen in Fig. 3. This is consistent with reports in the literature showing that the diffusion coefficient of lysozyme in water is independent of the concentration up to 8 mg/ml (Ross Colvin, 1952). A concentration of 5 mg/ml of lysozyme in Hepes buffer was chosen as the initial concentration in the donor compartment.

The possible adsorption of lysozyme onto the PLG films, with and without zinc acetate, after 10 days of degradation and at different concentrations of lysozyme was investigated. A very small amount of lysozyme was found to be adsorbed. However, the amount was considered not to influence the calculation of the diffusion coefficient because: (1) the amount was small; (2) mathematical correction of the lysozyme concentration due to adsorption resulted in an insignificant change in the diffusion coefficient; (3) after an initial lag phase, during which adsorption takes place, the adsorption does not influence the flux and this lag phase was excluded from the calculations. The adsorption can be minimized by shielding, i.e. neutralizing the surface charges of the lysozyme molecule by the addition of salt. It has been shown that an ionic strength of 0.1 M is sufficient for this (Mattisson, 1999). In the present study the ionic strength of the Hepes buffer was 0.16 M, which means that the adsorption due to charge effects was minimized.

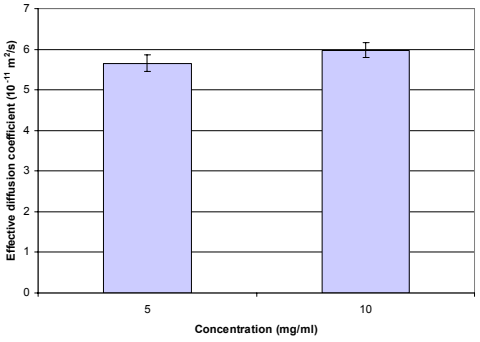

Fig. 3. The effective diffusion coefficient of lysozyme did not depend on the concentration.

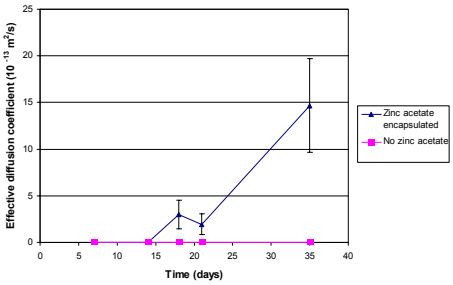

Fig. 4. Encapsulated zinc acetate increased the effective diffusion coefficient of lysozyme after 14 days of degradation. There was no measurable diffusion at 7 and 14 days of degradation. The error-bars show the standard deviation.

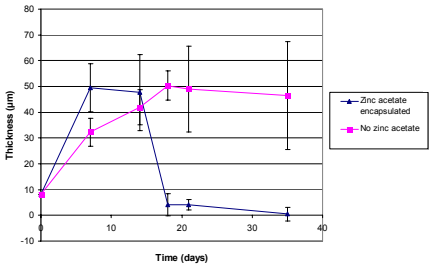

Fig. 5. The thickness of the PLG films with and without zinc acetate.

The effective diffusion coefficient of lysozyme through the PVDF filter was determined to be 5.7×10^{-11} m^2/s. This is close to the diffusion coefficient in pure water, which means that the PVDF filter contributes very little to the total mass transfer resistance. However, this was taken into consideration to ensure that the correct value of the pure mass transfer coefficient in the PLG film was obtained, according to Eq. (3).

3.3. The effect of encapsulated zinc on diffusion

The diffusion coefficient of lysozyme through PLG film, with and without zinc acetate, was measured after 7, 14, 18, 21 and 35 days of degradation. There was no detectable diffusion of lysozyme through the films not containing zinc acetate after any of the degradation periods. Encapsulated zinc acetate had a considerable effect on the effective diffusion coefficient, as can bee seen in Fig. 4. After 14 days of degradation there was a detectable, but very slow, diffusion of lysozyme through the PLG film in one of three replicates, but it was too small to be calculated. Fourteen days of degradation was thus probably the time required for pores sufficiently large for diffusion to form a connected network.

This effect of zinc cations on the effective diffusion coefficient was expected, based on a previous study, in which zinc cations were found to increase the porosity and erosion of PLG films (Fredenberg et al., 2007). We suggest that zinc cations act as a Lewis acid, thereby catalysing the hydrolysis of the polymer, which induces erosion and pore formation. Surprisingly, the diffusion coefficient was lower after 21 days' incubation than after 18. However, the difference was small and within the errors that can be expected from the reproducibility of the mass transport resistance of degraded PLG films. The interesting phenomenon of pore closure, and the formation of a less porous skin, which could cause a reduction in effective diffusion, has been reported previously (Park, 1995; Fredenberg et al., 2007; Kang and Schwendeman, 2007). After 35 days' incubation, the films containing zinc cations were almost completely degraded. However, there were no pores on the surface of films without zinc acetate.

The thickness of the PLG films, with and without zinc, is shown in Fig. 5. As could be expected from the effective diffusion coefficient measurements, the PLG films containing zinc acetate were considerably eroded between 14 and 18 days of degradation. The thickness measurements also confirmed that very little of the zinc-containing PLG films remained after 35 days. The PLG films without zinc absorbed water and were eroded slowly.

The porosity of the surface of PLG films after diffusion measurements was, as concluded from SEM analyses, in agreement with the effective diffusion coefficient. The PLG films with zinc acetate were porous from day 18, as illustrated in Fig. 6. The PLG films without zinc acetate contained cavities from day 18, but no continuous pores. It is of course possible that pores, too small to be seen in this investigation and too small for diffusion, were present. It can be expected that water completely fills the pores, and it is thus often assumed that the amount of water absorbed is a measure of the porosity. However, the pores must be continuous from one side of the film to the other, and sufficiently large for diffusion to take place. The facts that the surface of PLG films without zinc had no pores, and thus no diffusion was detectable despite the long degradation period, and that a great amount of water had been absorbed in the films, support the theory of skin formation. This will be investigated in a future study.

Zinc is used as a protein stabilizer, and salts of zinc and a base anion may be used as pH neutralizers. This study shows that the encapsulated zinc cation affects the mass transport resistance of the polymer, and this should be taken into consideration when using such salts. Zinc salts may have many effects:

1. As mentioned above, zinc cations have a pore forming and degrading effect, which facilitates drug release. We suggest that a base anion may more or less counteract this effect as it neutralizes acid catalysis of the hydrolysis.
2. Our previous study (Fredenberg et al., 2007) showed that the interaction between zinc cations and PLG made the polymer more hydrophobic, which may initially lead to slower water absorption. This is supported by the thickness measurements shown in Fig. 5, and by the differences in morphology (Fig. 6a–d) showing the degree of swelling of the films. This increase in hydrophobicity has also been speculated upon by others (Pratt et al., 1993). However, water absorption is rarely the process that controls the release rate in (relatively) hydrophilic PLG with low molecular weight, which is often used for the controlled release of encapsulated drugs.
3. The zinc–protein interaction may affect the solubility of the protein and the stability, which in turn will influence the release rate (Takenaga et al., 2004).

There have been some reports on the controlled release of drugs co-encapsulated with zinc salts. An increase in the rate of release of protein in the presence of zinc or other divalent cations has been reported, although it was not the objective of some investigations (Lam et al., 2000; Zhu and Schwendeman, 2000; Takada et al., 2003; Kang and Schwendeman, 2007). Surprisingly, a slower release rate has also been reported (Ishihara et al., 2005). However, as the authors noted, this may be due to the different drugs used, which were probably distributed differently in the particles. There has also been one report of zinc

carbonate causing an increase in the release rate at some concentrations but a decrease at others (Cleland et al., 1997). However, the decrease may be explained by the low concentration of zinc carbonate, which might just have been sufficient to keep the protein in an undissolved state. Some investigations indicate greater release, resulting in a higher area under curve (AUC) in vivo, as a result of stabilization of the protein (Johnson et al., 1996; Takada et al., 2003).

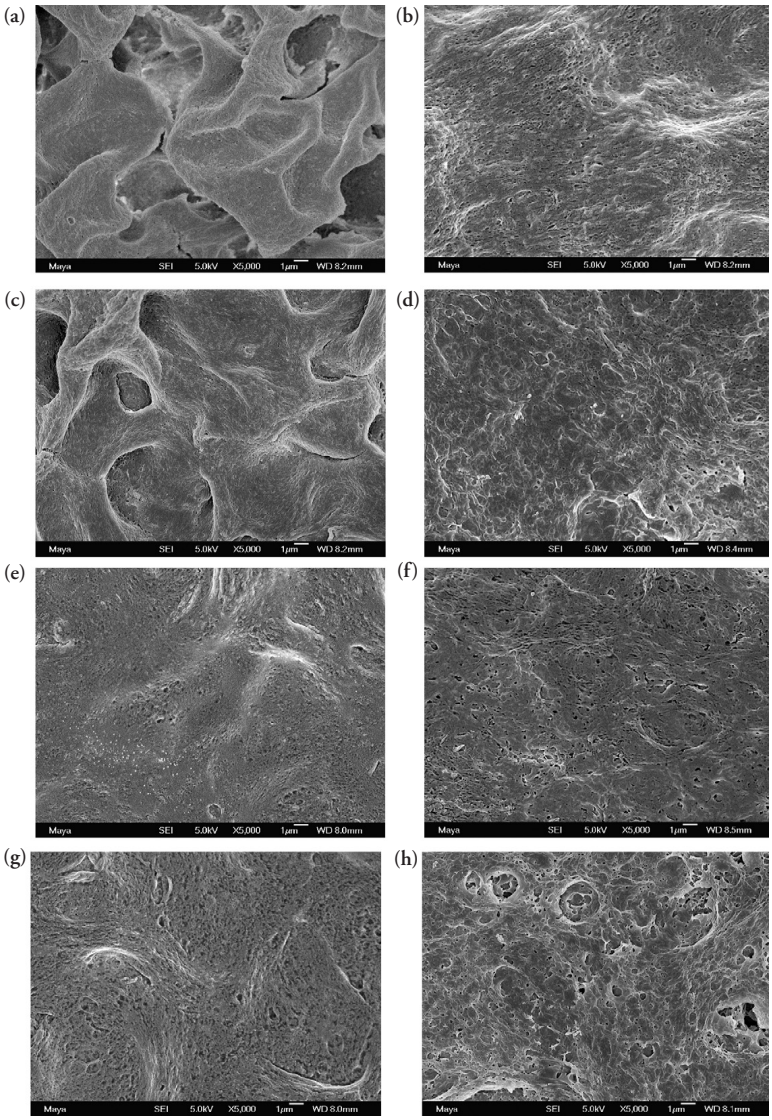

Fig. 6. The porosity of the surface of PLG films after diffusion measurements. The left column: no zinc acetate. The right column: zinc acetate encapsulated. (a and b) 7 days, (c and d) 14 days, (e and f) 18 days, (g and h) 21 days and (i and j) 35 days. PLG films without zinc acetate contained cavities after 18, 21 and 35 days' degradation. However, the PLG films with zinc acetate contained continuous pores after these time periods. Magnification 5000×.

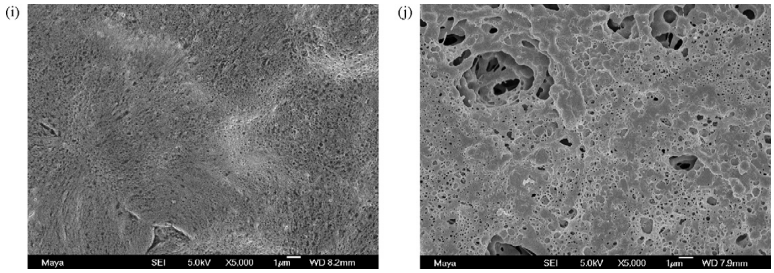

Fig. 6. (*Continued*).

The present study shows that zinc salts may be used as release-modifying agents in addition to protein stabilizers or pH neutralizers.

4. Conclusions

Encapsulated zinc acetate increased the diffusion coefficient of lysozyme through PLG films after 18 days' or more degradation. The PLG films containing zinc acetate were degraded and became thinner more rapidly than those without zinc. Films containing zinc acetate also became porous, while PLG films without zinc only developed cavities on the surface. The pore forming effects and the increase in release rate should be considered when using such salts as protein stabilizers or pH neutralizers. Although the effect of zinc could be counteracted by the presence of base anions, due to decreased acid catalysis of the hydrolysis, zinc salts may be used as release-modifying agents, as well as protein stabilizers or pH neutralizers.

Acknowledgement

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Paper IV

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Pore formation and pore closure in poly(D,L-lactide-co-glycolide) films

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ABSTRACT

Pore formation and pore closure in poly(D,L-lactide-co-glycolide)-based drug delivery systems are two important processes as they control the release of the encapsulated drug. The phenomenon pore closure was investigated by studying the effects of the pH and the temperature of the release medium, and the properties of the polymer. Poly(D,L-lactide-co-glycolide) (PLG) films were subjected to a pore forming pre-treatment, and then pore closure was observed simultaneously with changes in glass transition temperature, wettability (contact angle), water absorption and mass remaining. To further understand the effect of pH, combined pore formation and pore closure were studied at different pH values. Pore closure was increased in a release medium with low pH, with a low-molecular-weight PLG of relatively low degree of hydrophobicity, or at high temperature. Pore closure occurred by two different mechanisms, one based on polymer–polymer interactions and one on polymer–water interactions. The mobility of the PLG chains also played an important role. The surface of the PLG films were more porous at pH 5–6 than at lower or higher pH, as pore formation was relatively fast and pore closure were less pronounced in this pH range. The pH had a significant impact on the porous structure, which should be kept in mind when evaluating experimental results, as the pH may be significantly decreased *in vitro*, *in vivo* and *in situ*. The results also show that the initial porosity is very important when using a high-molecular-weight PLG.

1. Introduction

Poly(D,L-lactide-co-glycolide) (PLG) is a biocompatible polymer that has been used extensively in various areas, such as the controlled release of encapsulated drugs [1], tissue engineering [2], healing of bone defects [3], cancer treatment [4] and vaccines [5]. PLG is the most frequently used biodegradable polymer in the controlled release of encapsulated proteins or peptides. The reasons for its success are its biodegradability, its biocompatibility and the fact that it has been approved for parenteral use by the regulatory authorities around the world. Furthermore, its physico-chemical behavior, and thus the drug release profile, can be tailored by selecting PLGs with the appropriate properties, for example, molecular weight and the lactide:glycolide ratio [6–8]. Blending or co-polymerizing PLG with other materials further extends the possibility of controlling its physico-chemical behavior [9–11].

Encapsulated proteins or peptides diffuse through water-filled pores [12–15]. The release rate is thus very dependent on the porosity of the polymer, and it is important to understand both pore *formation* and pore *closure* when tailoring release from PLG-based formulations. The phenomenon pore closure and also the development of a surface layer less porous than the interior due to heterogeneous degradation, have been observed previously [16–22], but are unfortunately not often mentioned when discussing release mechanisms. Temperature has been shown to affect pore closure [23], and the structural collapse of the polymer has been suggested as one reason for pore closure [20]. Less deep pores have been reported on the surface of microspheres stored at high humidity when a plasticizing agent was added to the polymer [24], and the authors suggested that the polymer chains were rearranged due to the increased mobility of the polymer. However, the phenomenon is far from well understood.

Pore formation has been more discussed in the literature. It has been shown that pores are formed both by water absorption and by degradation/erosion of the polymer [25–27]. These processes, in turn, are influenced by a great number of factors, for example the presence and the concentration of salts, plasticizing excipients used and the properties of the polymer [6–8,21,28,29]. Another such factor is pH. As hydrolysis is acid-catalyzed, the common opinion is that pores are formed faster at lower pH [22,26,30]. However, the pH also affects the degree of polymer terminal carboxyl acid dissociation, which determines the charge of polymer chains. This may be important in the arrangement of polymer chains and thus possibly in pore closure. Both pore formation and pore closure probably take place simultaneously and constantly, and the porosity of the polymer is likely to be affected by both. The domination of these processes may vary with pH, which may decrease significantly from the normal physiological

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value (7.4) during polymer degradation. Inflammatory reactions and formation of a fibrous capsule surrounding PLG microspheres may decrease the local pH *in vivo* [31,32], while acid degradation products from PLG may decrease the pH inside PLG drug delivery systems and of the release medium *in vitro* [33–35].

The purpose of this study was to identify the mechanisms governing the phenomenon of pore closure. The effects of the pH and the temperature of the release medium, and the properties of the polymer were investigated. To our knowledge, no studies have been carried out regarding the effect of the properties of the polymer, such as its molecular weight or hydrophobicity, on pore closure. This study is complementary to previous studies on the effect of the pH and the temperature of the release medium on pore closure [16,23]. Pore closure was studied simultaneously with the changes of the glass transition temperature (Tg), wettability (contact angle), water absorption and mass remaining. To further understand the effect of pH of the release medium, combined pore formation and pore closure were studied at different pH values.

2. Materials and methods

2.1. Materials

Three different PLGs were obtained from Boehringer Ingelheim Pharma KG (Germany), namely RG502H (50:50 lactide:glycolide, with an approximate molecular weight (MW) of 12 kDa), RG504H (50:50 lactide:glycolide, approximate MW 45 kDa) and RG756 (75:25 lactide:glycolide, approximate MW 80 kDa). Polysorbate 80 and sodium Hepes salt were obtained from Sigma-Aldrich Inc. (USA), and Hepes acid from Research Organics (USA). NaCl, $ZnCl_2$ and ethyl acetate were obtained from Merck KGaA (Germany). NaN_3 was obtained from VWR International Ltd (UK) and polyvinylidene fluoride filters (pore size 0.65 μm) were purchased from Millipore AB (Sweden). All salts were of analytical grade.

2.2. Film preparation and sample pre-treatment

Polymer films (about 150 μm thick), were cast on glass dishes from solutions in ethyl acetate (67 mg/ml). Polysorbate 80 was co-dissolved in this solution (1.3 mg/ml) and encapsulated in the PLG film order to mimic a relevant pharmaceutical system utilizing PLG films coated onto microparticles [36]. Solutions of the PLG denoted RG756 also contained 10% (w/w) NaCl particles in relation to the weight of PLG. A polyvinylidene fluoride filter (105 μm thick and pore size 0.65 μm) was encapsulated in all films intended for analysis of pore formation or wettability to provide mechanical support. The filters were placed on the glass dishes and the polymer solutions were poured onto the filters. The filters were completely encapsulated in the PLG films and did not interfere with the analyses. After drying at ambient conditions for 10 days and vacuum drying at room temperature for 7 days, circular samples with a diameter of 1 cm were cut from the film. Pores were created in samples intended for studies on pore closure (see below), while those intended for a study on combined pore formation and pore closure at different pH were not subject to any pore-forming pre-treatment, and were thus smooth and non-porous. Pores were created in samples of PLG denoted RG502H by incubation in Hepes buffer (see Section 2.3) with 1 mM $ZnCl_2$, pH 7.4, for two days at 37 °C. $ZnCl_2$ has been found to increase the rate of pore formation, probably by acting as a Lewis acid and thereby catalyzing degradation [17,28]. Pores formed due to the presence of $ZnCl_2$ during this short period of time were located at the surfaces [17]. Samples of PLG denoted RG504H were incubated for four days in the same way to create pores. The molecular weight of the PLG denoted RG756 was too high for pore forming pre-treatment with $ZnCl_2$. In order for pore formation to occur due to (catalyzed) hydrolysis and erosion within the first few days, a part of the polymer chains must be sufficiently short to reach the molecular weight necessary for dissolution within this time. The pore forming pre-treatment was instead based on a porogen. The encapsulated NaCl particles in samples denoted RG756 were released within four days of incubation in Hepes buffer, as analyzed by scanning electron microscopy (SEM) (see Section 2.4). These pores were located on one of the surfaces of the samples, as the NaCl particles settled to the bottom during drying. $ZnCl_2$ was added to the Hepes buffer during these four days, with the purpose of avoiding unknown effects of $ZnCl_2$ at comparison of the different PLGs. After the pore forming pre-treatment, which was two days for PLG denoted RG502H and four days for the other PLGs, the samples were incubated in Hepes buffer without $ZnCl_2$, and the analysis of pore closure started.

Table 1
Experimental design for the investigation of pore closure.

PLG	MW (kDa)	Relative degree of hydrophobicity	Temperature (°C)	pH of the release medium
RG502H	12	Low	37	7.4
RG502H	12	Low	37	3.0
RG502H	12	Low	9	7.4
RG502H	12	Low	45	7.4
RG504H	45	Average	37	7.4
RG756	80	High	37	7.4

2.3. Incubation

All the samples (those containing pores and those not pre-treated) were incubated in 75 mM Hepes buffer containing 115 mM NaCl and 5 mM NaN_3, pH 7.4, at 37 °C. Samples of different PLGs were incubated in release medium with a pH of 3.0 or pH 7.4, at temperatures of 9 °C, 37 °C or 45 °C. Table 1 presents the experimental design.

Samples intended for studies on the effect of release medium pH on both pore formation and closure were made of PLG denoted RG502H, and were incubated in Hepes buffer with the pH adjusted to 3.0, 5.0, or 6.0 using HCl, or pH 7.4 (no adjustment). The release medium was refreshed continuously to keep the pH constant. At predetermined intervals the samples were investigated with regard to porosity, water absorption and mass loss, and in the study of pore closure the glass transition temperature (Tg) and wettability were also investigated. All the analyses were performed on triplicate samples.

2.4. Scanning electron microscopy

The samples were washed and vacuum dried. The effect of the drying method, i.e. freeze drying or vacuum drying, on the porosity was investigated in an initial experiment. The drying method did not have any effect on the result (data not shown). The porosity was studied using a JSM-6700F field emission scanning electron microscope from Jeol Ltd (Japan). The samples were sputtered with gold prior to inspection. Triplicate samples were analyzed.

2.5. Wettability

The wettability was measured using the captive bubble method to determine the contact angle. PLG samples were mounted in a device allowing the sample to be submerged in water. An air bubble was placed on the downward facing surface of the PLG sample using a hypodermic needle. The equipment was thoroughly cleaned using acids (1:1 HCL:HNO_3) or ethanol in an ultrasonic bath to ensure that contaminants did not interfere with the measurements. The contact angle was measured using a Melles Griot Invaritar P/N 59 LGF 410 camera and the software program Windrop for windows XP. Triplicate samples were analyzed.

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2.6. Differential scanning calorimetry

The glass transition temperature (Tg) was analyzed using a DSC 6200 calorimeter (Seiko Instruments Inc., Japan). The samples were washed, vacuum dried and placed in aluminum pans (TA Instruments, USA, ref no. 900790.901). The pans were hermetically sealed and an empty pan was used as a reference. The samples were scanned at a rate of 10 °C/min with a temperature sweep up to 100 °C, starting at −20 °C. The Tg calculated from the second heating cycle using the soft ware program Exstar 6000 (Scientific & Medical Products Ltd. UK). Triplicate samples were analyzed.

2.7. Water absorption and mass loss

Water absorption and mass were determined by weighing the samples in wet state (W_{wet}) and after drying in a vacuum chamber to constant weight (W_{dry}). W_0 denotes the initial weight. Triplicate samples were analyzed.

$$\text{Water absorption} = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100(\%)$$

$$\text{Mass loss} = \frac{W_0 - W_{dry}}{W_0} \times 100(\%)$$

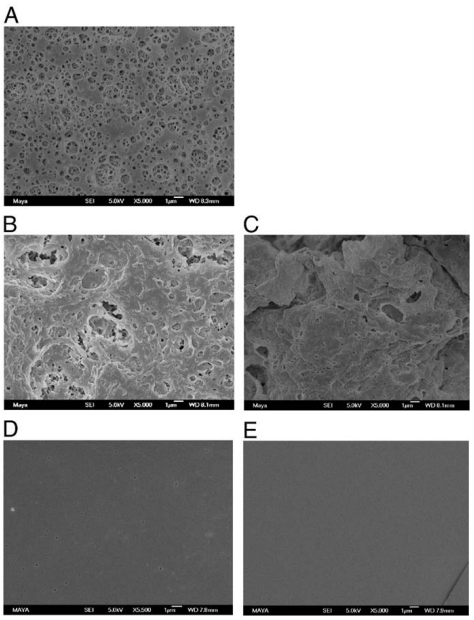

Fig. 1. Porosity directly after pre-treatment (A), after 2 more days of incubation (B and D) and after 12 days (C and E). PLG MW 12 kDa, 37 °C and pH 7.4 (B and C) and pH 3.0 (D and E). Magnification 5000×.

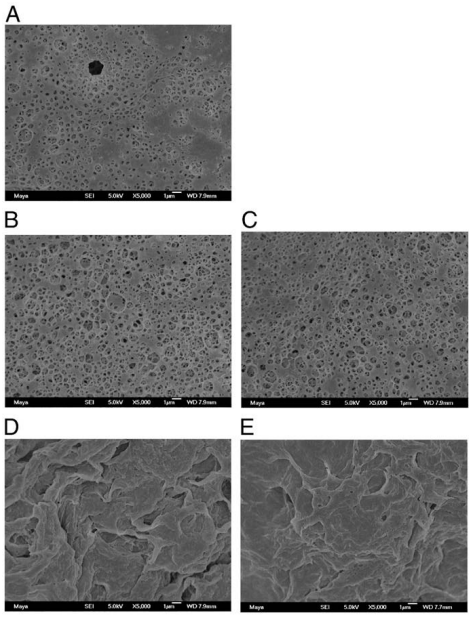

Fig. 2. Porosity directly after pre-treatment (A), after 2 more days of incubation (B and D) and after 12 days (C and E). PLG MW 12 kDa, pH 7.4 and 9 °C (B and C) and 45 °C (D and E). Magnification 5000×. See Fig. 1 for the comparable experiment carried out at 37 °C.

3. Results and discussion

3.1. Pore closure

All the factors investigated, i.e. the pH and the temperature of the release medium, and the properties of the polymer, influenced pore closure, as observed by scanning electron microscopy (Fig. 1–3). Pore closure occurred rapidly at low pH, high temperature and when using a PLG of low molecular weight and a relatively low degree of hydrophobicity. The effect of each factor will be discussed separately below.

3.1.1. Effects of the pH of the release medium

Pore closure was faster at pH 3.0 than at pH 7.4 during the 26 days of observation, although pore closure began within two days at both pH values (Fig. 1). The pores were completely closed at pH 3.0, but not in pH 7.4. Pore formation is commonly believed to be enhanced at low pH due to the well-known acid-catalyzed hydrolysis of PLG [22,26,30]. These results show that pH may affect the polymer in more than one way. In this case, pore closure probably occurred more rapidly than pore formation. Water absorption was slower at the lower pH (Fig. 4), which was somewhat unexpected, as the acid-catalyzed hydrolysis reduces the molecular weight of PLG, which in turn makes the polymer chains more hydrophilic [37]. The samples degraded at pH 7.4 became highly swollen, degraded and sometimes fell apart during the last period of the analyses, which caused the fluctuations seen in Fig. 4A. The results from the pore closure and water absorption analyses can be explained by the lack of dissociation of the terminal carboxyl acids of the polymer chains at pH 3.0, which makes the polymer less charged and more hydrophobic. Measures of wettability confirmed this (Table 2). It is likely that polymer–polymer

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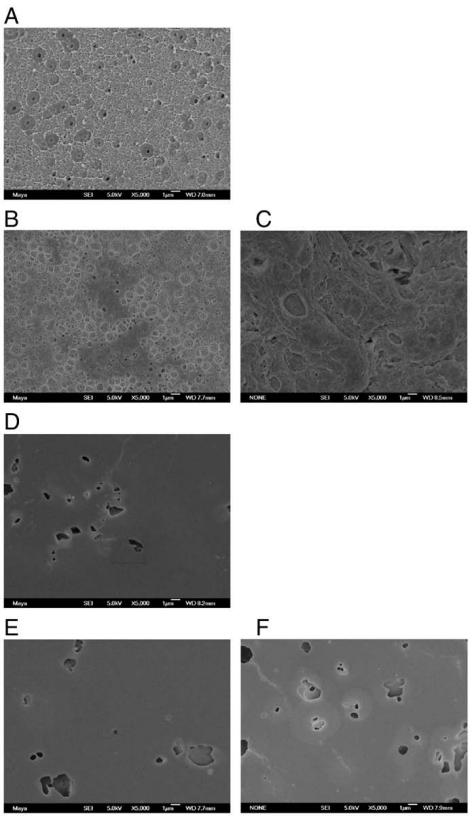

Fig. 3. Porosity directly after pre-treatment (A and D), after 2 more days of incubation (B and E) and after 12 days (C and F). pH 7.4, 37 °C and PLG MW 45 kDa (A-C) and 80 kDa (D-F). Magnification 5000×. See Fig. 1 for the comparable experiment with PLG MW 12 kDa.

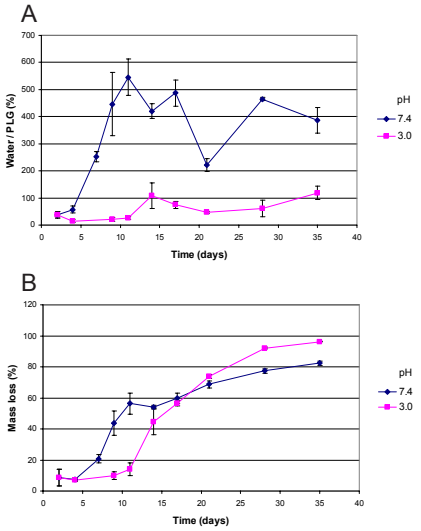

Fig. 4. Water absorption (A) and mass loss (B) at different pH values. The error bars show the standard deviation. The polymer samples were subjected to a pore forming pre-treatment in presence of $ZnCl_2$ at pH 7.4 and 37 °C during the first two days.

interactions resulting from the more hydrophobic nature of the polymer constituted the driving force for pore closure. The higher interfacial tension between water and polymer encourages as small contact area between the two phases as possible, and release of surface bound water from two hydrophobic polymer areas attracting each other is a drive for contraction. This would also explain why the water uptake by the polymer was low. Visually, there was an obvious difference between the samples: those in medium of pH 3.0 contracted into a lump, while those in medium of pH 7.4 swelled and spread out.

Another important factor contributing to the faster pore closure at pH 3.0 was the considerable increase in polymer chain mobility, evidenced by a significant decrease in Tg (Fig. 5). There have also been reports of constant or even increased Tg in acidic environments, however, those PLG matrices contained drugs and, according to our results, the period of degradation was too short for an effect to be seen, [30,38] (we observed no effect until after 4 days of degradation, see Fig. 5). Acid catalysis of the hydrolysis of PLG is well known, as mentioned above, and a lowering of the molecular weight of the polymer chains results in a lower Tg and higher mobility [19,39]. This mobility is important as a rigid polymer will not contract, or will only contract slowly, even if the interfacial tension is strong. This is further discussed in the next section. Rearrangement of polymer chains due to increased mobility has also been suggested as the explanation of the observation that pores on the surface of microspheres stored at high relative humidity became less deep when a plasticizing agent was added [24]. As shown in Fig. 5, the Tg remained constant at pH 7.4, although a slight decrease of Tg was expected. This was probably due to a faster loss of plasticizing substances such as polysorbate 80 and PLG degradation products, which counteracted the decrease in the Tg. At the beginning of the experiment, mass loss was slower at pH 3.0, but by the end of the experiment, mass loss was greater at pH 3.0 (Fig. 4). Mass loss is influenced by both the rate of hydrolysis and the rate of transport of water-soluble degradation products out of the samples, as PLG degradation products become soluble in water when they have been hydrolyzed down to approximately 1100 g/mol [37]. As the porosity was lower at pH 3.0, the transport rate was slower and counteracted the faster hydrolysis.

This rapid and complete pore closure at low pH may play an import role during drug release, as pH may be low *in vivo*, *in vitro* and *in situ*. As mentioned in Section 1, inflammatory reactions and formation of a fibrous capsule surrounding PLG microspheres may decrease the local pH *in vivo* [31,32], while acid degradation products from PLG decrease

Table 2
Wettability, expressed as the contact angle, at the two pH values studied. The standard deviation is given in parentheses. High wettability and low hydrophobicity result in a small contact angle between the air bubble in water and the surface. The polymer samples were subjected to a pore forming pre-treatment in presence of $ZnCl_2$ at pH 7.4 and 37 °C during the first two days.

pH	Day 2 (°)	Day 4 (°)	Day 9 (°)
3.0	36.7 (7.1)	54.7 (3.1)	53.4 (3.1)
7.4	36.7 (7.1)	28.2 (0.44)	34.4 (3.5)

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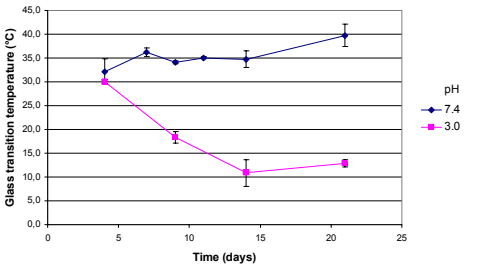

Fig. 5. The change of the glass transition temperature over time at the two pH values studied. The error bars show the standard deviation.

the pH of the release medium *in vitro* [33]. The release medium is therefore usually replaced continuously in the studies described in the literature. However, the actual decrease in pH is seldom reported. The pH *in situ*, i.e. inside the PLG particles, has been found to be as low as 1.8, also due to acid degradation products, and the probability the pH being about 3 has been shown to be high [34,35]. The common belief regarding the effect of pH on release rate is that degradation, and thus drug release, is faster at low pH. Both faster [21,40] and slower release have been reported [41]. The effect of pH on drug release is further complicated by the fact that polymer–drug interactions and the rate of drug dissolution, which influence drug release, may depend on the pH. The results of this work show that pH 3.0 may have a retarding effect on drug release due to pore closure.

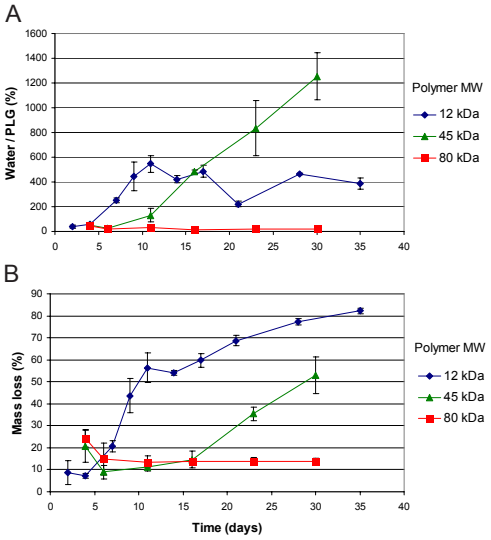

Fig. 6. Water absorption (A) and mass loss (B) for the different PLGs. The error bars show the standard deviation. The polymer samples were subjected to a pore forming pre-treatment in presence of $ZnCl_2$ at pH 7.4 and 37 °C during the first two (12 kDa) or four days (45 and 80 kDa).

3.1.2. Properties of the polymer

Pore closure was faster with a low molecular-weight polymer with a relatively low degree of hydrophobicity Fig. 2. Pores began to close within two days in the 12 kDa polymer with the lowest hydrophobicity among the three chosen PLGs. In the 45 kDa polymer with an average hydrophobicity, pores began to close within 7 days, although not clear until 19 days. Pores in the 80 kDa polymer with the highest hydrophobicity were not closed at all. The hydrophobicity depends on the molecular weight, but also the lactide:glycolide ratio and if the polymer chains are end-capped. As expected, a low molecular-weight and less hydrophobic polymer absorbed more water, and the rate of the polymer mass loss was faster (Fig. 6). Thus, a difference in the Tg between the different polymers was expected. The Tg differed initially (Table 3), but after incubation there were no significant differences (data not shown). Plasticizing substances such as polysorbate 80 and PLG degradation products were probably lost at different rates, which could compensate for the effects of the molecular weight and hydrophobicity. The relative degree of hydrophobicity affected the wettability at the beginning of the incubation period (Table 3). Later, the films were unfortunately too degraded and too swollen for reliable measurements.

The more pronounced pore closure associated with low molecular weight and relatively low degree of hydrophobicity can be explained by the mobility and flexibility of the polymer chains and their ability to mix with water. Polymer chains that diffuse easily are more likely to spread and cover pores. Instead of distinct pores, a more swollen and homogeneous polymer structure was formed. Pores were not closed in the high-molecular-weight and highly hydrophobic PLG, although the interfacial tension between water and the hydrophobic polymer would be a driving force for contraction of the polymer, similar to the case of low pH discussed above. The difference was the mobility of the polymer chains, which enabled pore closure in the low-molecular-weight polymer with decreased Tg, but not in the rigid high-molecular-weight polymer.

These results show that the initial porosity of a drug delivery system is very important when using high-molecular-weight PLG of relatively high hydrophobicity. At low initial porosity, the release would be very slow due to slow water absorption and degradation. However, if the initial porosity is high, the pores will not close, and the drug release may be faster than when using a low-molecular-weight PLG. This should be considered when choosing the properties of the polymer.

3.1.3. Temperature

Pores were closed faster as the temperature increased Fig. 3. Pores were not closed at all at 9 °C. Pores began to close within two days at both 37 and 45 °C. However, pores were closed faster during the next 13 days at 45 °C. After that, the samples at 45 °C were too degraded to be analyzed. The faster pore closure at higher temperature was expected and in agreement with a previous report [23]. Increasing the temperature increases the mobility of the polymer, and the polymer chains can diffuse and cover pores more easily, forming a more homogeneous surface layer. A higher temperature also increases the ability of the polymer to mix with water, which results in faster water absorption (Fig. 7). The pores were, however, not completely closed, because of the counteracting process of pore *formation* which also was

Table 3
Initial glass transition temperature (Tg) and wettability (expressed as contact angle) after four days of incubation. High wettability and relatively low hydrophobicity result in a small contact angle. The standard deviation is given in parentheses.

Properties of the PLG	Initial Tg (°C)	Contact angle after four days of degradation (°)
MW: 12 kDa, low hydrophobicity (RG502H)	42 (0.46)	28 (0.44)
MW: 45 kDa, average hydrophobicity (RG504H)	46 (0.46)	38 (0.46)
MW: 80 kDa, high hydrophobicity (RG756)	50 (0.66)	49 (4.1)

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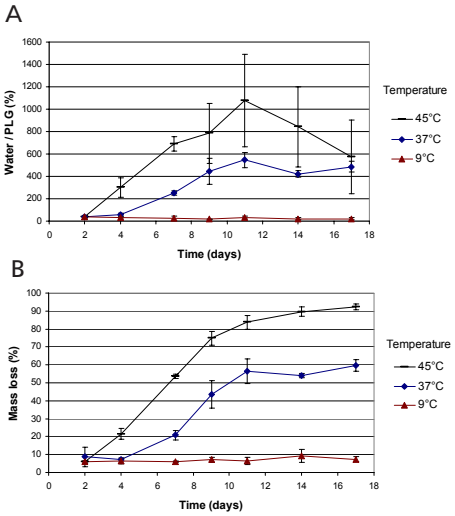

Fig. 7. Water absorption (A) and mass loss (B) at different temperatures. The error bars show the standard deviation. The polymer samples were subjected to a pore forming pre-treatment in presence of $ZnCl_2$ at pH 7.4 and 37 °C during the first two days.

faster. Increasing the temperature increased the rate of hydrolysis and the mass loss (Fig. 7). The highly degraded polymer lost its structure and the surface of the samples could not be completely annealed. The effect of different incubation temperatures could not be seen in measurements of Tg and wettability, probably for the same reasons as described in Section 3.1.2.

3.1.4. The mechanism of pore closure

Pores closure may be caused by at least two different physical events, a polymer–polymer interaction, where polymer–polymer attraction is mainly driven by the hydrophobic effect, and a polymer–water interaction that leads to a more homogenously swollen polymer gel. In both cases polymer mobility will be an important factor.

The results suggest that pore closure at pH 3.0 was driven by the polymer–polymer interaction. Attraction of two hydrophobic polymer areas separated by a water-filled pore releases the surface-bound water and increases the entropy, resulting in a more energetically stable system. In addition, the decrease in the surface tension by separation of water and hydrophobic polymer is more energetically favorable. It was clearly observed visually that the films contracted during the experiments at pH 3.0. The molecular weight of the polymer was low, which promotes polymer chain mobility, allowing separation from water. The high-molecular-weight and highly hydrophobic PLG denoted RG756, would probably also gain considerably in terms of energy by separating the polymer mass from the water-filled pores by contracting, but the polymer chains were too long and rigid.

Pore closure in a highly mobile polymer at pH 7.4 that absorbed water was instead probably driven by swelling of the polymer network. At this pH, where the polymer was charged and thus much more hydrophilic, it had a higher tendency to take up water, as can be seen in Fig. 6. It is thus likely that the polymer chains diffused easily and created a more homogenously swollen polymer network that no longer contained distinct pores. In contrast to the contracting samples at low pH, these samples visually swelled, as evidenced by an increase in thickness and diameter. However, pore closure did not occur when the temperature was lowered to 9 °C, at which the polymer was more rigid. High temperature on the other hand increased the mobility, and thus also the rate of pore closure.

3.2. Pore formation and pore closure at different pH values

The results discussed above show that pore closure occurred at both pH 3.0 and at pH 7.4, but at different rates and as a result of different mechanisms. The porous structure of a PLG matrix will be determined by the combined effects of pore formation and pore closure, which are taking place simultaneously. To understand these processes better, pore formation and pore closure was investigated not only at pH 3.0 and 7.4, as above, but also at pH 5.0 and 6.0. The purpose was to investigate whether there was an optimal pH for pore formation. The SEM analysis showed that pores did not form at pH 3.0, and samples incubated at pH 5–6 had the most porous surfaces (Fig. 8). Pore closure could be seen at all pH values after different periods of time.

Water absorption was faster at high pH and slower at low pH (Fig. 9). The explanation, as mentioned in Section 3.1.1, lies in the degree of dissociation of the polymer terminal carboxyl acids. The polymer chains are more charged and hydrophilic at higher pH. The degree of water absorption and swelling explained the very distinct and different appearances of SEM images of samples at different pH values (Fig. 8). Samples at pH 3 and 5 showed smooth surfaces, while those incubated at pH 6, and even more at pH 7.4, were creased.

The processes of pore formation and pore closure were taking place continuously at all the pH values studied. However, at pH 3.0, pore closure was so dominant that pores were not seen. The polymer–polymer interaction, caused by the lack of dissociated terminal carboxyl groups of the polymer chains, and driving pore closure at pH 3.0, should become much less strong with increasing pH. Pore closure was also rapid at pH 7.4, as shown in Section 3.1.2, due to the diffusion of the highly mobile and hydrophilic polymer chains. This polymer–water interaction should become less strong with decreasing pH. Between pH 3.0 and 7.4, pore closure should thus be less strong, while hydrolysis, which leads to pore formation, is relatively fast due to acid catalysis. This explains the optimal pore formation at pH 5–6, seen in Fig. 8. It should be noted that the effect of the pH of the

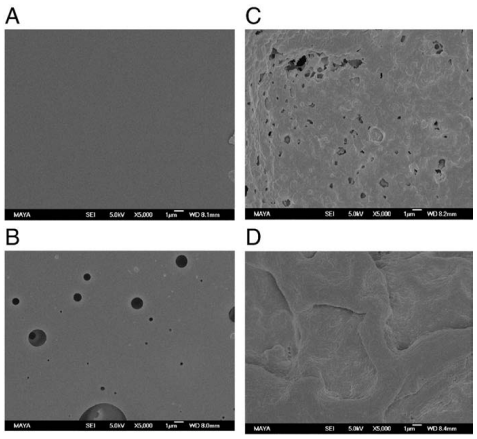

Fig. 8. Pore formation after 10 days of incubation at pH 3.0 (A), pH 5.0 (B), pH 6.0 (C) and pH 7.4 (D).

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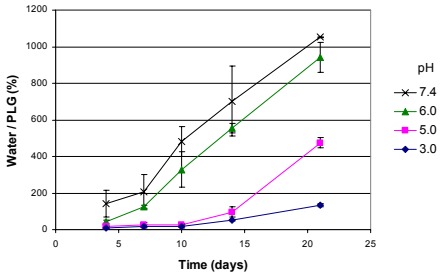

Fig. 9. Water absorption at differentpHvalues. The error bars showthe standard deviation.

release medium on pore formation and pore closure is probably dependent on the molecular weight and the mobility of the polymer chains.

The pH microclimate within a particle or film of PLG can be quite heterogeneous, which means that the porous structure could differ throughout the polymer mass. The formation and closure of pores may be explained by such local pH differences. As mentioned above, it is important to know the pH changes *in vitro*, *in vivo* and *in situ* (e.g. due to dissolved acid polymer degradation products and to inflammatory reactions in the body) as the rate of drug release may depend on this.

4. Conclusions

Pore closure was increased in a release medium with low pH, with a low-molecular-weight PLG of relatively low degree of hydrophobicity, or at high temperature. Pore closure occurred by two different mechanisms, depending on the pH and the degree of dissociated terminal carboxylic acids, which governed the hydrophobicity of the polymer. The results of this study suggest that pore closure at pH 3.0 was driven by polymer–polymer interactions, in which the attraction of two relatively highly hydrophobic areas, separated by a water-interactions caused the release of surface-bound water, increasing the entropy of the system. The surface tension also made the separation of water and hydrophobic polymer more energetically favorable. At pH 7.4, on the other hand, the results suggest polymer–water interactions. The pores in the low-molecular-weight polymer with highly mobile polymer chains and low hydrophobicity, seemed to be closed by diffusion of polymer chains that covered the pores, forming a more swollen and homogeneous polymer structure. This was facilitated at high temperature.

The initial porosity of a drug delivery system is very important when using a high-molecular weight-PLG with a relatively high degree of hydrophobicity, as pores are formed slowly due to slow degradation and water absorption. Pore will not close, and if the porosity is high, the release may be faster than when using a low-molecular-weight PLG.

The highest porosity of the surfaces of the PLG films was seen at pH 5–6. At these pH values, degradation/erosion, and thus pore formation, was relatively fast, while pore closure was less pronounced than at lower and higher pH.

The effect of pH on pore formation and pore closure should be kept in mind, as a significant decrease in pH may occur *in vitro*, *in vivo* and *in situ* due to acid degradation products of the polymer and inflammatory reactions *in vivo*. The pH microclimate within a particle or film of PLG can be quite heterogeneous, which means that the porous structure also may differ throughout the polymer mass. Unexplained formation and closure of pores reported in other studies may be due to local differences in pH. The results of the present study show that conclusions regarding drug release and release mechanisms must be drawn with pH in mind.

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Paper V

Development of Mass Transport Resistance in Poly(Lactide-co-Glycolide) Films and Particles – a Mechanistic Study

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Abstract

Poly(D,L-lactide-co-glycolide) (PLG) is the most frequently used biodegradable polymer in the controlled release of an encapsulated drug. The purpose of this work was to explain the surprisingly slow diffusion through this polymer, and locate the major source of transport resistance. Diffusion of human growth hormone (hGH) and glucose through PLG films was undetectable (using a diffusion cell), although the degraded polymer contained several times more water than polymer mass. *In vitro* release of hGH from PLG-coated particles also showed a surprisingly slow rate of release. Non-porous regions inside the PLG films were detected after three weeks of degradation using dextran-coupled fluorescent probes and confocal microscopy. The findings were supported by scanning electron microscopy. Diffusion through PLG films degraded for five weeks was significantly increased when the porosity of both surfaces was increased due to the presence of $ZnCl_2$ in the buffer the last three days of the degradation period. The results

indicated high transport resistance inside the films after three weeks of degradation, and at the surfaces after five weeks of degradation. These results should also be applicable to microparticles of different sizes. Knowledge on the reason for transport resistance is important in the development of pharmaceuticals and when modifying the rate of drug release.

Keywords: Diffusion, Transport resistance, Pore closure, Poly(D,L-lactide-co-glycolide), Release mechanism, Degradation

1. Introduction

The use of biopharmaceuticals, for example, peptides and proteins, and hydrophobic drugs with low oral bioavailability, is growing (Närhi and Nordström, 2005; Pisal et al., 2010; Wiscke and Schwendeman, 2008). The oral bioavailability of these groups of pharmaceuticals is low, and administration by injection is almost always necessary, which leads to discomfort for the patient. The frequency of injections can be decreased by the use of controlled release of encapsulated drugs, which is beneficial for patients who require daily and/or long-term treatment. Poly(D,L-lactide-co-glycolide) (PLG) has been the subject of intense research for this purpose for two decades (Houchin and Topp, 2008), largely due its biodegradability, biocompatibility and the fact that it has been approved for parental use by the regulatory authorities. Furthermore, the physico-chemical properties and thus the release profile, can be tailored by selecting PLGs with appropriate properties, such as the molecular weight and the lactide:glycolide ratio (Tracy et al., 1999; Kranz, 2000; Ravivarapu et al., 2000; Zolnik and Burgess, 2008). Other applications of PLG-based formulations are single-shot vaccines (Feng, 2006; Jiang, 2005; Shi, 2002), local drug delivery (e.g. cancer treatment or antibiotics) (Sastre et al., 2007; Weinberg et al., 2008; Xu and Czernuszka, 2008), targeted drug delivery (e.g. molecules with affinity for the target attached to PLG nanoparticles) (Chittasupho et al., 2009; Cruz et al., 2010) and tissue engineering (Wei et al., 2006).

Knowing the release mechanisms and the factors that influence the rate of release is vital. Two release mechanisms are mainly discussed in the literature: diffusion and degradation/erosion, and the release rate is often said to be diffusion-controlled initially and degradation/erosion-

controlled during the final part the release period (Alexis, 2005; D'Souza et al., 2005; Johnson et al., 1997; Lam et al., 2000; Mollo and Corrigan, 2003; Zolnik et al., 2006). However, there are many processes that influence the rate of drug diffusion and the degradation kinetics, for example polymer–drug interactions (Blanco and Alonso, 1997), drug–drug interactions (Kang et al., 2008), heterogeneous degradation leading to a less permeable surface layer (Park, 1995), water absorption (Liu et al., 2005) and pore closure (Kang and Schwendeman, 2007; Wang et al., 2002). Knowledge regarding these more detailed processes or events is necessary if we are to understand the release mechanisms in detail and be able to control the release rate.

An encapsulated drug may be released in three ways:

1. drug transport through the polymer phase,
2. drug transport through water-filled pores,
3. due to dissolution of the polymer encapsulating the drug (which does not require drug transport).

Drug transport through water-filled pores is the most common way, as the encapsulated drug is usually a large hydrophilic molecule, and drug release usually starts before the onset of any significant polymer erosion. The transport takes place either by diffusion (driven by the concentration gradient) or convection (driven by a force such as osmotic pressure) (Cussler, 1997). Although the latter has been reported (Jonnalagadda and Robinson, 2000; Ryu et al., 2007), osmotic pressure is usually compensated by swelling of the polymer, and diffusion is therefore the most common process (Guse et al., 2006; Kim et al., 2006; Sansdrap and Moës, 1997; Webber et al., 1998). Pore formation and pore closure are thus two very important processes. Pore formation is influenced by the rate of water absorption and the rate of degradation/erosion (Matsumoto et al., 2005; Mochizuki et al., 2008; Siepmann et al., 2002). Due to the auto-catalytic nature of degradation, the rate of pore formation, and thus drug diffusion, may not be homogeneous throughout the polymer matrix. Similarly, the rate of the other process, pore closure, may be heterogeneous throughout the polymer matrix. Kang and Schwendeman (2007) used confocal microscopy to follow the diffusion of fluorescent probes and found that the pores were closed at the surface. At physiological pH, this is probably due to the polymer–water interaction. However, at pH 3.0, pore closure may be caused by the hydrophobic effect of non-dissociated

carboxyl acids (Fredenberg et al., 2011). A local microclimate of pH 3.0 is not unlikely at some places inside PLG particles or films due to the acid gradient and auto-catalytic nature of degradation (Ding and Schwendeman, 2008; Shenderova et al., 1999.). Therefore, it is possible that pore closure can occur locally within the polymer matrix as well as on the surface. The processes leading to the release of an encapsulated drug are complex; for example, low pH may increase both the rate of pore formation and the rate of pore closure. It is important to know the location of the highest transport resistance within a PLG drug delivery system (DDS) in order to modify the release rate. This has not been thoroughly investigated.

In a previous study, we found the diffusion of lysozyme through thin PLG films to be undetectable, despite the fact that the films had lost approximately 40% of their polymer mass, and the amount of water absorbed was several times greater than the polymer mass (Fredenberg et al., 2009). Others have also reported slow diffusion or surprisingly slow drug release from PLG microspheres (Batycky et al., 1997; Berkland et al., 2007.) In this work, we studied the diffusion of the human growth hormone (hGH) (with the opposite charge to that of lysozyme) and glucose (a small un-charged, non-interacting molecule) to confirm that the previously observed slow diffusion of lysozyme was not a result of protein–polymer interactions or protein–protein interactions. We also investigated the reasons of the slow diffusion using scanning electron microscopy (SEM), confocal microscopy and fluorescent probes. A diffusion cell was used for diffusion measurements, and *in vitro* release of hGH from PLG-coated microspheres (StratoSphere HL™) was studied. The diffusion of proteins encapsulated in particles, and the influence of thick or thin PLG layers are discussed. In this paper, we extend the discussion regarding simple diffusion-controlled or degradation/erosion-controlled release in PLG films and PLG particles by showing the occurrence of regions with surprisingly high mass transfer resistances.

2. Materials and methods

2.1. Materials

PLG (RG502H, 50:50 lactide:glycolide, with an approximate molecular weight of 12 kDa) was obtained from Boehringer Ingelheim Pharma KG

(Germany). Polysorbate 80, lysozyme (14100 g/mol), sodium HEPES salt and starch from rice were obtained from Sigma-Aldrich Inc. (USA), and HEPES acid from Research Organics (USA). NaCl, $ZnCl_2$, Na_2HPO_4, NaH_2PO_4, glucose, mannitol, zinc acetate and ethyl acetate were obtained from Merck KGaA (Germany), and NaN_3 from VWR International Ltd (UK). Polyvinylidene fluoride filters (pore size 0.65 μm) were purchased from Millipore AB (Sweden). Dextran-coupled fluorescent probes, tetramethylrhodamine-dextran (TMR-dextran), were obtained from Invitrogen AB (Sweden). The hGH used for diffusion experiments was a kind gift from Novo Nordisk A/S (Denmark). The sodium hyaluronic acid (1.56 mDA) was a kind gift from Hyaltech Ltd (Edinburgh Scotland). All salts were of analytical grade.

2.2. Film preparation

PLG films (about 150 μm thick), containing 2% (w/w) polysorbate 80, were cast on glass dishes from solutions in ethyl acetate. These films are henceforth denoted "thick films". After drying at ambient conditions for 10 days and in a vacuum chamber for 7 days, circular samples with a diameter of 1 cm were cut from the films for measurements of water absorption and mass loss, or for confocal microscopy of absorbed fluorescent probes. A polyvinylidene filter was encapsulated in PLG films intended for diffusion measurements for mechanical support, and samples with a diameter of 3 cm were cut to fit the diffusion cell. Thick films containing 3% zinc acetate (w/w) were made the same way, but zinc acetate was also dissolved in the solution of ethyl acetate.

PLG films (7.0 ± 1.0 μm thick), also containing 2% (w/w) polysorbate 80, were sprayed from solutions in ethyl acetate onto polyvinylidene filters. These films are henceforth denoted "thin films". This process has been described previously (Fredenberg et al., 2004). Briefly, the polymer solution (containing 1% (w/w) polymer dissolved in ethyl acetate together with polysorbate 80) was sprayed using a Hüttlin (Germany) spray nozzle. The filter, which had a pore size of 0.65 μm, was mounted on a rotating wheel, and the filter thus passed through the spray at determined intervals, in order to mimic a normal coating process. Twelve films were made simultaneously to ensure reproducibility. The films were dried at ambient conditions for 1 day and in a vacuum chamber for 5 days.

2.3. Preparation of StratoSphere high load microparticles

Hyaluronic acid was dissolved in 50mM Na-phosphate pH 6.4 (1% w/w). Rice starch and hGH were dispersed in the hyaluronic acid solution, and the dispersion was mixed with a spatula to a homogeneous thick mixture. The cores of the microparticles were prepared using a proprietary spray freezing technology (WO 2008/ 128 992 A1). Briefly, liquid nitrogen was filled into a stainless steel vessel. A spray nozzle capable of providing two gas flows: one atomizing the fluid to be sprayed, and one surrounding the atomizing gas flow, was used (Hüttlin). Carbon dioxide was used for both gas flows. PLG (RG502H) was dissolved in ethyl acetate, which was emulsified in water using polysorbate 80 as emulsifier. The cores were coated with PLG, by spraying the emulsion, using a Hüttlin Kugelcoater HKC005. During the final stage of this coating process, the microparticles were coated with a thin layer of mannitol, to inhibit particle aggregation during storage. The microparticles were dried in a vacuum chamber.

2.4. Thickness of the films

The thickness of PLG films was measured using a BX50F4 microscope from the Olympus Optical Co. Ltd (Japan) and an SSC-DC38P digital camera from Sony Co. (USA). Strips of PLG films were mounted so that the edge faced upwards in holders made specially for this purpose. The PLG films, from which the strips were cut, were made simultaneously with those used for diffusion experiments, and the strips were degraded under the same conditions as the samples used for diffusion measurements. The software Image J, version 1.37, (U.S. National Institutes of Health, available on the internet at http://rsb.info.nih.gov/ij) was used to calculate the thickness of the films. Three different films were used to determine the thickness of the thin sprayed films. Measurements were made on five strips cut from each film. Three images were taken of each strip, and five measurements were made on each image, giving a total of 225 measurements for each determination of the thickness. To determine the thickness of the thick cast films, five strips were cut from one film, three images were taken of each strip, and five measurements were made on each image (75 measurements).

2.5. Water absorption and mass loss

Water absorption and polymer mass loss were measured gravimetrically. Samples were weighed in the wet state (W_{wet}) and after drying in a vacuum chamber to constant weight (W_{dry}). W_0 denotes the initial weight. Triplicate samples were analyzed.

$$\text{Water absorption} = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100 \quad (\%) \qquad \text{Eq. 1}$$

$$\text{Mass loss} = \frac{W_0 - W_{dry}}{W_0} \times 100 \quad (\%) \qquad \text{Eq. 2}$$

2.6. Scanning electron microscopy

The porosity of the surface and inside PLG films was analyzed using a JSM-6700F field emission scanning electron microscope from Jeol Ltd (Japan). The samples were washed and dried. The effect of the drying method, i.e. freeze drying or vacuum drying, on the porosity was investigated in an initial experiment. The drying method did not have any effect on the result (data not shown) and vacuum drying was employed. The samples were sputtered with gold prior to inspection. Triplicate samples were analyzed.

2.7. Diffusion measurements of hGH and glucose through thin PLG films

Thin PLG films were degraded in 75 mM HEPES buffer containing 115 mM NaCl and 5 mM NaN_3, pH 7.4, at 37°C for 21 days. The buffer was changed regularly to keep the pH constant. After this period of degradation, the films were placed in the diffusion cell and measurements of simultaneous diffusion of hGH and glucose were carried out. This procedure has been described previously (Fredenberg et al., 2004). Briefly, the thin PLG film, sprayed onto a filter, formed the barrier between the stirred donor and receiver compartments. The openings were covered with Parafilm to avoid evaporation, and the diffusion cell was placed in a water bath at a temperature of 37°C. A coarse nylon filter, pore size 60 μm, was mounted together with the PLG film to protect the film from possible erosion caused by stirring. This

filter does not influence the mass transport rate, which was thoroughly evaluated (Fredenberg et al., 2004). Samples were withdrawn from the receiver compartment and replaced with fresh buffer. The experiment was terminated after 24 hours, and the PLG films were prepared for SEM analysis. The initial concentrations in the donor compartment (approximately 4.3 mg/ml hGH and 1.3 mg/ml glucose) were measured. The concentrations in the donor compartment were then calculated from the concentrations in the receiver compartment using a mass balance. The accuracy of this mass balance was checked at the end of the experiments by measuring the concentration in the donor compartment. Diffusion was measured through three PLG films.

The concentration of hGH was analyzed using SEC-HPLC with UV detection and a TSK2000 SW column (Tosoh Corporation, Japan). The concentration of glucose was analyzed using high-pH, anion-exchange chromatography, coupled with pulsed amperometric detection (HPAEC-PAD), with an ED40 electrochemical detector and a Carbo Pac PA10 guard and analytical column (DIONEX, USA).

2.8. Diffusion measurements of lysozyme through thick PLG films and increase in the surface porosity

Thick PLG films were degraded in HEPES buffer at 37°C for 35 days. The buffer was changed regularly to keep the pH constant. In order to determine if the main mass transfer resistance was located at the surfaces, the surfaces of some of the thick films were made more porous by adding 1 mM $ZnCl_2$ to the HEPES buffer during the last three days of the 35-day degradation period. Three days of this pore-forming treatment with $ZnCl_2$ results only in pores close to the surface (Fredenberg et al., 2007).

The diffusion of lysozyme through the films, with and without the pore-forming treatment, was measured as described above, except that no samples were withdrawn from the receiver department. A fiber-optic probe (Dip probe accessory, Varian Inc. USA) was inserted into the receiver compartment. This probe was used together with a Cary 50 Bio spectrophotometer from Varian Inc. (USA) to measure the change in the concentration of lysozyme with time by measuring the UV absorbance at 280 nm. The experiments were terminated after 4 days, and the PLG films were prepared for SEM analysis. Three films with increased surface

porosity and three untreated films were subjected to diffusion measurements and SEM analysis.

2.9. Calculation of the diffusion coefficient

The method of calculating the diffusion coefficient is based on Fick's law. The mass transfer coefficient is calculated from the change in the concentrations in the two compartments with time (Westrin, 1991):

$$K = \frac{1}{S\left(\frac{1}{V_A} + \frac{1}{V_B}\right)} \times \frac{\ln\left(\frac{C_{A1} - C_{B1}}{C_{A2} - C_{B2}}\right)}{t_2 - t_1} \qquad \text{Eq. 3}$$

The subscripts *A* and *B* denote the donor and receiver compartment, respectively. Subscripts 1 and 2 denote sample numbers. *K* is the mass transfer coefficient, *S* is the diffusion area, and *t* denotes time. *V* is the volume of each compartment, and *C* is the concentration. The total mass transfer resistance (*1/K*) is the sum of the mass transfer resistances of each layer, i.e. the PLG film and the filter for mechanical support. In the case of the thin PLG films sprayed onto the filter, the pores of the filter were filled with HEPES buffer. The total mass transfer resistance can be described as:

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{\frac{\varepsilon}{\tau} \times D_{water}} \qquad \text{Eq. 4}$$

where *l* is the thickness of the film or filter. The right-hand term in Equation 4 is the transfer resistance of the filter, and is determined by the thickness of the filter, the porosity (ε) and the tortuosity (τ) of the filter, and the diffusion coefficient in the buffer D_{water}. By rearranging Equation 4 the effective diffusion coefficient for the PLG film ($D_{e\,film}$) can be obtained by simply subtracting the filter resistance, which has been determined previously for hGH, glucose and lysozyme (Fredenberg et al., 2004; Fredenberg et al., 2009).

Thick PLG films were cast together with a filter for mechanical support, which meant that the pores of the filter were filled with PLG. The total mass transfer resistance can be described as:

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{\frac{\varepsilon}{\tau} \times D_{e\,film}} \qquad \text{Eq. 5}$$

The parameter ε/τ can be calculated by comparing the effective diffusion coefficient in the filter to the diffusion coefficient in water. All other parameters in Equation 5 could be measured.

2.10. Visualization of fluorescent probes in PLG films using confocal microscopy

Thick PLG films were degraded in HEPES buffer with 1 mM $ZnCl_2$ at 37°C for two days (to decrease any possible mass transfer resistance at the surface). The samples were then degraded in HEPES buffer with 1 mg/ml TMR-dextran, and the fluorescent probes were allowed to diffuse into the samples for 19 days. The buffer containing the dissolved fluorescent probes was changed continuously to maintain constant pH. The location of TMR-dextran inside the PLG films was analyzed with an LSM510 META inverted confocal microscope. The excitation wavelength was 543 nm, and an LP560 long-pass filter was used to detect the emission, i.e. detection of wavelengths above 560 nm. Fluorescence was detected with pinhole settings corresponding to 1 Airy unit, and the samples were scanned from one side to the other in steps of 1.41 μm. The gain was set to 643, which was below the detection of background fluorescence of the polymer. The stability of the pH-independent TMR-dextran was investigated by measuring the fluorescence after incubation in HEPES buffer at 37°C for 21 days. The samples were scanned three times at different locations.

2.11. In vitro release of hGH from PLG-coated microparticles

Microspheres (10 mg) were suspended in 1.5 ml HEPES buffer and placed on a tilting board at 37°C. At predetermined time intervals, the supernatant was collected after centrifugation to determine the amount of hGH released, using HPLC with UV detection, as described in Section 2.7. Triplicate samples were prepared for analysis at each point in

time. The pH of the HEPES buffer was checked regularly, and the buffer in samples intended for analysis after 21 days' was changed after 21 days, at which the pH had decreased from 7.4 to 6.8.

3. Results and discussion

3.1. Diffusion of hGH and glucose through thin PLG films

In order to confirm that the lack of detectable diffusion of lysozyme through thin degraded PLG films in our previous work was not the result of the properties of the protein chosen, such as charge or hydrophobicity, additional diffusion measurements were carried out with hGH and glucose as solutes. Human growth hormone is a protein of approximately the same size as lysozyme (22 kDa compared to 14 kDa) but with the opposite charge at physiological pH. Glucose is a small (180 Da), uncharged, and under these conditions, inert molecule, which means that degradation, aggregation and absorption to the polymer can be ruled out. The PLG films were degraded for 21 days in HEPES buffer at 37°C before diffusion measurements.

The diffusion experiments did not show any diffusion of either hGH or glucose. No pores were visible at the surfaces of the thin films after the experiments (Figure 1), according to SEM. The limit for the detection of diffusion of hGH with this method was 960 times slower than the rate of diffusion in water at 37°C, and for glucose it was 3100 times slower (Table 1). Compared to the rate of diffusion through the filters used for mechanical support of the thin PLG films, the limit of detection was 280 slower for hGH and 850 times slower for glucose (Table 1). This means that the rate of diffusion, if there was any, was slowed down at least several hundred times by the polymer. This is surprising, considering that after 21 days of degradation, the PLG films contained about 10 times more water than PLG mass due to water absorption (swelling measured using microscopy, data not shown) and they had lost approximately 27% of their polymer mass (Figure 2). The rate of diffusion of glucose and proteins through a hydrogel containing this amount of water is usually in the range of 10^{-10} to 10^{-11} m^2/s (Andersson et al., 1997; Axelsson et al., 1991; Brandl et al., 2010), which is at least 100 – 1000 times faster than through the PLG films. The filter used for mechanical support has an approximate porosity of 70%, and the factor of diffusion retardation in

this filter was 3.5. If the 27% polymer mass lost were regarded as porosity, the reduction in diffusion rate based on the porosity would be about 10 (70/27 x 3.5). However, these values were, as mentioned above, much greater (a factor of more than 280 for hGH and 850 for glucose). Importantly, these diffusion measurements also show that the undetectable diffusion of lysozyme was not an artifact. To investigate this slow diffusion, additional diffusion experiments were performed on thicker films using lysozyme as the solute (see Section 3.3).

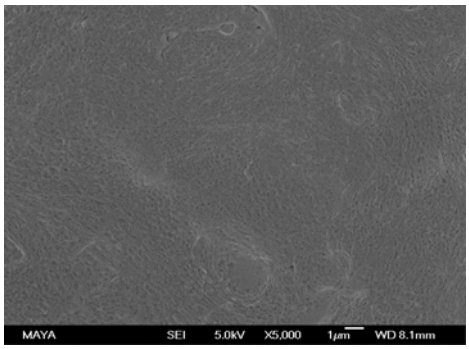


Figure 1. SEM image of the surface of a thin film after measurements of the diffusion of hGH and glucose. It can be seen that the surface was not porous.

Table 1. Diffusion through thin PLG films compared to diffusion in water and through the filter used for mechanical support.

Substance	**Effective diffusion coefficient in PLG films (10^{-13} m^2/s)**	***Diffusion coefficient in water (37°C) (10^{-13} m^2/s)**	**Factor of retardation, compared to water**	**Effective diffusion coefficient in the filter (10^{-13} m^2/s)**	**Factor of retardation, compared to the filter**
hGH (n=3)	Less than 1,4	1300	More than 960	380	More than 280
Glucose (n=3)	Less than 2,6	8200	More than 3100	2200	More than 850

*(He and Niemeyer, 2003; Landolt and Börnstein, 1969)

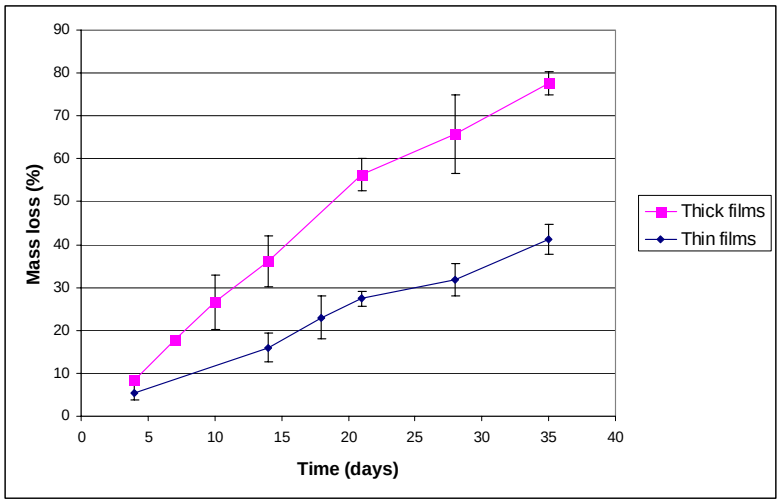


Figure 2. Mass loss from thick and thin PLG films. The error bars show the standard deviation. (n=3).

3.2. In vitro *release of hGH encapsulated in PLG-coated particles*

In order to compare the results from the diffusion measurements with a more relevant pharmaceutical system, the *in vitro* release of hGH encapsulated in particles coated with the same PLG as used for the PLG films in the previous section was investigated, see Figure 3. The conventional manufacturing method used by StratoSphere Pharma AB was modified in order to be able to compare the results. The modifications were: (i) hGH is generally used in the form of small particles in the preparation of the microparticles but was used in dissolved state, (ii) zinc ions, generally used for maintaining stability and low solubility of hGH, were not used, and (iii) only a single PLG copolymer was used in the coating whereas a mixture of two copolymers is generally used. The *in vitro* release had a high initial release (burst release), presumably related to these changes, followed by a fairly linear release. Minimal burst release is normally important in pharmaceutical development, however, it was not an issue in these experiments. The control of release kinetics by application of a PLG coating on microparticles by air suspension technology is generally capable of providing a low initial release of hGH *in vitro* and *in vivo*, including man (Jostel et al., 2005; Reslow et al., 2002).

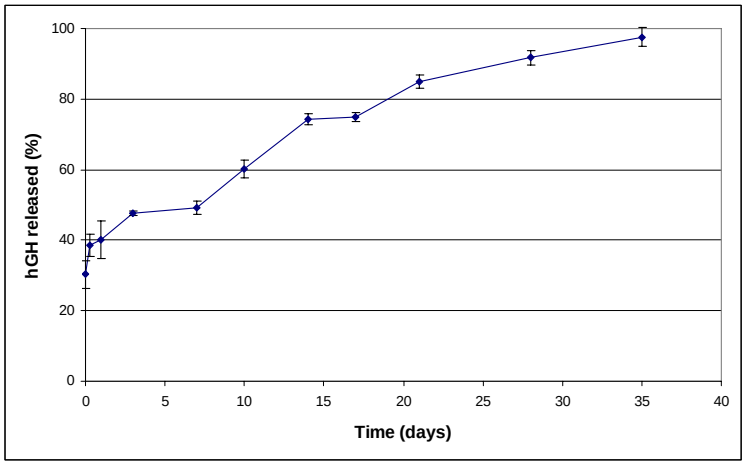


Figure 3. In vitro *release of hGH encapsulated in PLG-coated particles. The error bars show the standard deviation. (n=3). The manufacturing method used by StratoSphere Pharma AB was modified in order to be able to compare this result to other measurements (see the discussion above).*

As can be seen in Figure 3, the release was relatively linear up to 21 days, at least after the burst release during the first day ($R^2 = 0.97$). The release from large PLG particles is often more sigmoidal (Berkland et al., 2007; Sansdrap and Moës, 1997). This is a result of the greater pH gradient and more pronounced auto-catalytic hydrolysis in large PLG matrices, which explains the faster mass loss from the thicker films, shown in Figure 2. As zero-order release often is preferred, formulations utilizing thin PLG coatings may be a better choice than large PLG particles. The slow and relatively linear release is promising for the development of pharmaceuticals. For example, patients requiring daily injections of hGH would benefit greatly from a once-a-month formulation.

The duration of the release, 35 days, is long considering the rate of water absorption and degradation/erosion of the polymer. The thickness of the polymer coating on the particles (approximately 15 μm) was similar to the thickness of the "thin films" (7 μm), and the data presented in Figure 2 could therefore be used to estimate the approximate amount of water absorbed and the amount of polymer mass lost. After 35 days of incubation, the PLG coating probably contained about 10 times more water than polymer mass and had lost approximately 40% of its polymer mass. Diffusion through such a system should be relatively fast, as discussed in Section 3.1.

The effective diffusion coefficient through the PLG coating can be determined from Equation 6 (Cussler, 1997).

$$M = \frac{D_e}{l} c_{sat} A t \qquad \text{Eq. 6}$$

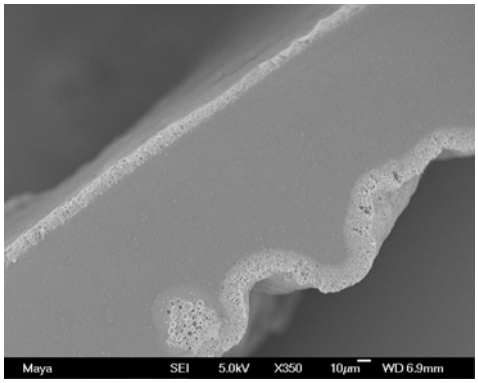
M denotes the mass released, D_e the effective diffusion coefficient and l the thickness of the PLG coating. A is the surface area, c_{sat} is the concentration within the particles and t denotes time. Based on the particle size (approximately 100 μm), the initial thickness and swelling of the PLG coating, and an estimated concentration of hGH of 10 mg/ml within the particles, the average effective diffusion coefficient was calculated to be on the order of 10^{-16} m^2/s. As some of the parameters change with time, including the diffusion coefficient, this is only a rough estimate. However, it is clear that the diffusion is much slower than in water (1.3×10^{-10} m^2/s): about one million times slower. The enormous total surface area of the microparticles compensates for the slow diffusion. Others have also reported low diffusion coefficients for drug transport thorough PLG, ranging from 10^{-13} to 10^{-16} m^2/s for small drugs (Alexis et al., 2004; Hsu et al., 1996; Klose et al., 2008; Siepmann et al., 2002) and from 10^{-17} to 10^{-19} m^2/s for proteins (Batycky et al., 1997; Berkland et al., 2007). The rate of diffusion depends on many factors, such as the molecular weight and the physico-chemical properties of the drug and the PLG used, the additives, the initial porosity of the DDS, and the *in vitro* conditions. Diffusion coefficients can therefore rarely be directly compared. However, it is evident that the retarding power of PLG is high. Berkland et al., (2007) also reported a surprisingly and unexplainably slow drug release from particles constituting of a PLG similar to the relatively hydrophilic, low-molecular-weight PLG used in this study, which should result in relatively rapid drug release. The possible reasons for the slow diffusion observed in this study will be addressed in the next section.

3.3. Possible explanations of the slow diffusion

Three possible explanations of the slow diffusion are: (i) pore closure at the surface, (ii) non-porous areas within the PLG film and (iii) insufficient pore size. These are discussed in detail below.

3.3.1. Pore closure at the surface

Pore closure at the surface of PLG films has been demonstrated previously, as mentioned in Section 1. Thus, it is possible that a significant part of the total transport resistance is located at the surface. In order to test this hypothesis, diffusion measurements were carried out using thick films with and without the surfaces having been exposed to $ZnCl_2$, which increases the rate of pore formation (Fredenberg et al., 2007). The PLG films in this study were less porous at the surface than inside the films (data not shown). Three days of exposure to $ZnCl_2$ was chosen to assure that only the regions close to the surfaces were affected. Figure 4 shows that pores were formed close to the surface after four days in the presence of $ZnCl_2$ in the buffer, while pores were formed inside the film after four days when zinc acetate was encapsulated inside the film.



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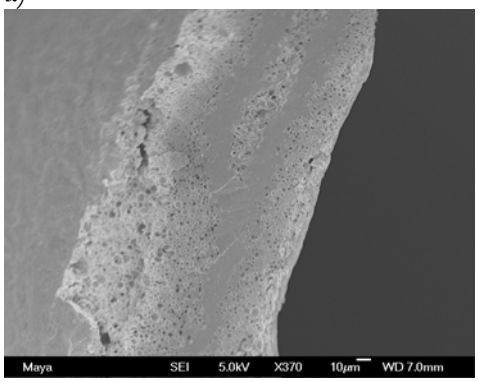


b)

Figure 4. Pore formation after 4 days of degradation. a) $ZnCl_2$ dissolved in the buffer resulted in pore formation close to the surfaces. b) Zinc acetate encapsulated inside the PLG film resulted in pore formation inside the film.

The diffusion of lysozyme was significantly faster through the PLG films that had been treated with $ZnCl_2$ to increase the surface porosity (Figure 5). These films had lost almost 80% of their polymer mass, according to the mass loss profile in Figure 2, and contained about 6 times more water than polymer mass, according to water absorption measurement (data not shown). However, diffusion was retarded by more than 100 times due to the transport resistance of the polymer. The increased porosity at the surfaces increased the diffusion coefficient eight times (Figure 5). This indicates that there was considerable transport resistance at the surfaces. Any change in the conditions of the surface of DDSs, for example, the formation of cracks or the detachment of small pieces of microparticles, may thus significantly influence the release rate. The difference in porosity between the interior and the surface, where a piece of the surface has become detached, can be seen in Figure 6. It was difficult to measure the thickness of the $ZnCl_2$-treated films precisely. However, the inaccuracy in these measurements would not account for the difference in the diffusion coefficients.

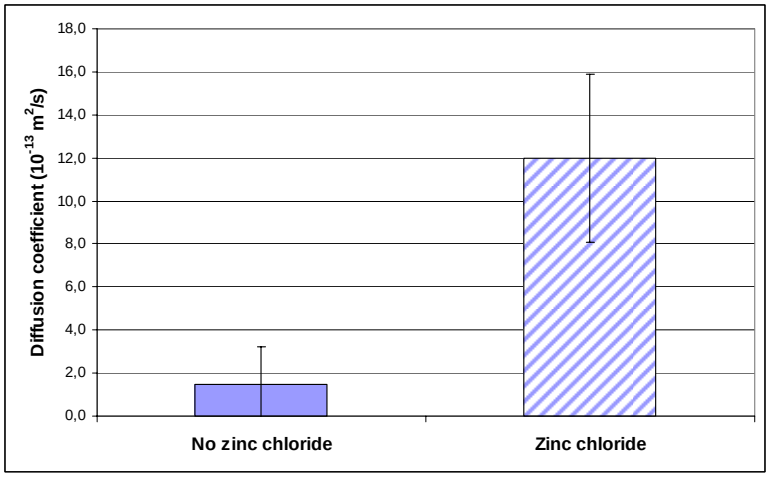


Figure 5. A significant increase in diffusion coefficient was observed when the surfaces of the PLG films were made more porous by the presence of $ZnCl_2$ in the buffer during the last three days of the 35-day degradation period. The error bars show the standard deviation. (n=3).

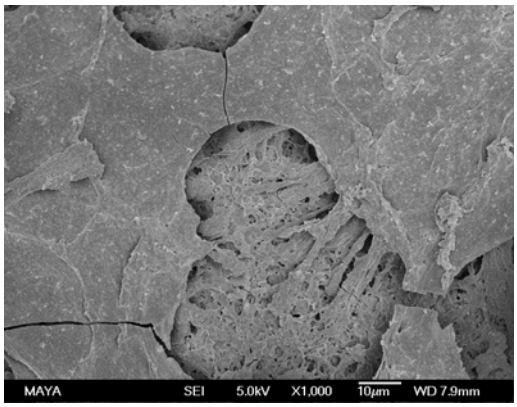
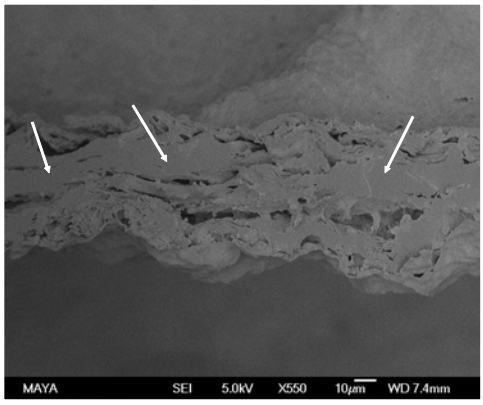


Figure 6. The difference in interior and surface porosity where a piece of the surface has become detached. This PLG film was degraded for 35 days before diffusion measurements, and was not treated with $ZnCl_2$.

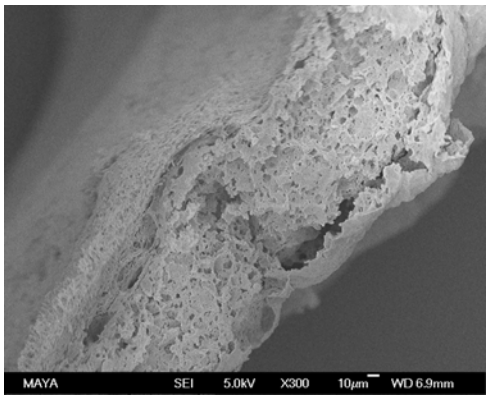
In a previous study, we showed that the mechanism of pore closure at the surface of films of a low-molecular-weight and relatively hydrophilic PLG, degraded at pH 7.4, probably was based on a polymer–water interaction (Fredenberg et al., 2010). The pores seemed to be closed due to the diffusion of mobile polymer chains that healed existing pores, and instead of distinct pores, a swollen and more homogeneous polymer mass was formed. Pore closure at the surface of microparticles of higher-molecular-weight PLGs has also been reported (Wang et al., 2002). Polymer chain mobility seems to be a key issue in the rearrangement of polymer chains leading to pore closure, as pore closure, which did not occur immediately, did occur after a period of degradation, which means shorter and more mobile polymer chains (Huang et al., 2007; Okada, H., 1997). Other indications of the importance of mobility are the fact that increased temperature or the presence of plasticizing agents has been shown to facilitate pore closure (Bouissou et al., 2006; Kang and Schwendeman, 2007). Plasticizing substances are sometimes used in PLG-based formulations and may be present *in vivo* (Reslow et al., 2002; Tracy et al., 1999). Formation of a less permeable skin at the surface of microspheres as a result of heterogeneous degradation, and thus slow pore formation at the surface, has been reported (Lu et al., 1999; Park, 1995). The results in the present experiments show that the transport resistance may be greatest at the surface, which could explain the slow or undetectable diffusion discussed in Section 3.1 and 3.2.

3.3.2. Non-porous areas within the PLG film

SEM analysis of the cross-section of PLG films sometimes showed non-porous areas (Figure 4a), at least when using this low-molecular-weight and relatively hydrophilic PLG. Such PLGs are often used in controlled release formulations. Furthermore, the properties of initially higher-molecular-weight and more hydrophobic PLGs become similar to these PLGs after a period of degradation. It is our experience, having analyzed the cross-section of a vast number of PLG films using SEM, that these non-porous areas are likely to be seen after a relatively short period of degradation, up to approximately 21 days. Longer degradation periods usually result in porous appearances (Figure 7).



a)



b)

Figure 7. Cross sections of two PLG films. a) Non-porous areas are visible after 21 days of degradation (arrows). b) The cross section was porous after 35 days of degradation.

Non-porous areas will lead to high transport resistance. In order to investigate this, confocal microscopy was used to visualize TMR-dextran in thick PLG films degraded for 21 days. It was found that the TMR-dextran did not spread homogeneously inside the PLG films, and that there were areas approximately 20 μm in width, into which the probes seemed unable to diffuse (see Figure 8). The existence of these dark areas indicates that there may be large areas through which there is very little or no diffusion. Upon inspecting the image very closely, very small channels containing fluorescence could be seen in some of these dark areas. Initial experiments and instrument settings confirmed that the fluorescence was not from any other source than the probes. As TMR-dextran is a non-ionic hydrophilic substance, it is expected to spread throughout the whole water-filled space in the polymer, unless the pores are too small. Interactions between PLG and TMR-dextran are considered unlikely. Initial experiments confirmed that TMR-dextran remained stable during the experimental period. The dark areas can thus not be explained by anything other than TMR-dextran not being able to diffuse into these areas. The fluorescent areas in the region scanned, showing the presence of TMR-dextran, varied through the different layers of the PLG film. The hypothesis that the transport resistance is significant inside the film was supported by the results of similar diffusion experiments to those presented in Section 3.3.1., but carried out after 21 days of degradation (non-porous areas inside the film, according to Figure 7a) instead of 35 days (porous interior, according to Figure 7b). It should be noted that only one measurement of diffusion through a $ZnCl_2$-treated PLG film was carried out. The increase in surface porosity after 21 days of degradation did not have any effect on the diffusion resistance. Thus, the transport resistance seemed to be located inside the film. This investigation shows that the interior of the polymer matrix contains areas through which solutes are not able to diffuse into, which may explain the slow diffusion described in Section 3.1 and 3.2.

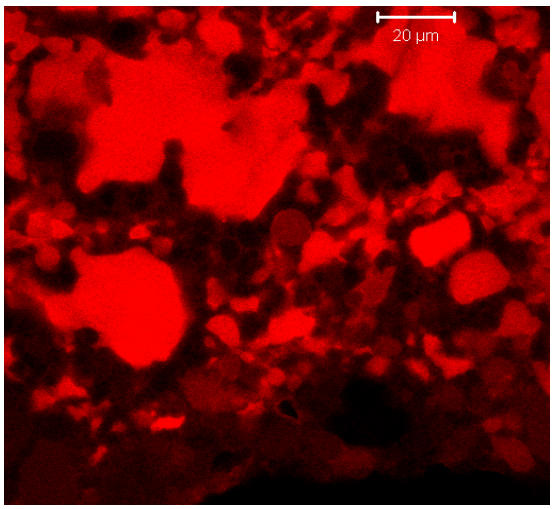


Figure 8. The location of fluorescent probes attached to 10 kDa dextran within a PLG film. The dark areas show regions into which the probes could not diffuse. In contrast to the cross section views in Figure 7, this image was obtained along the plane of the PLG film.

The cause of these non-porous areas may be either that pores have not yet been formed, as pore formation takes place from the surfaces into the film (Fredenberg et al., 2007), or that the pores have closed. Pore closure has been shown to be rapid and extensive at low pH (Fredenberg et al., 2011). Pore formation and pore closure are two simultaneously occurring processes, and the rates of both increase at low pH, at least for this low-molecular-weight PLG with highly mobile polymer chains. The acid-catalyzed hydrolysis that leads to erosion and pore formation is well known, and the hydrophobic nature of the polymer, due to non-dissociated carboxyl acids at low pH, may cause the contraction of the polymer, and thus pore closure. The process that dominates is probably governed by several factors, such as the mobility of the polymer, the mechanical strength of the polymer structure, the molecular weight and the rate of degradation. These conditions may vary in both space and time, and are probably the reason for local, non-porous areas that seem to disappear after a certain period of degradation. Heterogeneous degradation is a result of auto-catalyzed degradation and has been reported in microspheres and films with dimensions of 10 µm (Lu et al., 1999; Park, 1995), although the overall pH gradient should be small in such systems. Thus, pore closure probably also occurs, and the heterogeneous environment of porous and non-porous areas probably

also exists, inside the "thin films" and the coatings on particles, although this was not investigated in this study.

3.3.3. Insufficient pore-size

Another explanation of the slow or undetectable diffusion may be that the pores are not sufficiently large for solutes to diffuse through them. In fact, the effect of size exclusion on diffusion though PLG films was observed in one of our previous studies (Fredenberg et al., 2004), in which the diffusion of hGH was retarded more than that of glucose. As mentioned in Section 3.1, very small area of faint fluorescence could be seen in some of the dark areas. These pores were obviously sufficiently large for diffusion as they contained fluorescent probes, but showed that the pores may differ considerably in size. Pore formation and pore closure occur simultaneously, and after a few weeks of degradation and water absorption by this low-molecular-weight and relatively hydrophilic PLG, some regions should contain pores of sufficient size for diffusion. However, evidence was found in this study that there may also be regions of low porosity where the pores are perhaps too small to allow diffusion. Higher-molecular-weigh and more hydrophobic PLGs may require longer degradation times before the pores become sufficiently large. A continuous path extending from the drug molecule to the surface of particles or films, is of course, also essential for drug release.

4. Conclusions

No diffusion of hGH or glucose was detectable through thin films of PLG degraded for three weeks, despite the fact that these films contained approximately ten times more water than polymer mass, due to water absorption, and had lost approximately 27% of their polymer mass due to degradation. One of our previous studies showed a similar lack of diffusion of lysozyme through even more highly degraded PLG films. The fact that hGH has the opposite charge to lysozyme and that glucose is a small non-charged molecule that does not interact with PLG, demonstrate that our previous findings concerning lysozyme was not an artefact. *In vitro* studies of release of hGH from particles coated with the same PLG also showed a surprisingly slow release. This slow diffusion may be due to: (i) pore closure at the surface, (ii) non-porous areas inside the polymer mass and/or (iii) insufficient pore size. The rate of diffusion through PLG films increased when the porosity at surfaces was

increased. The location of fluorescent probes, attached to dextran, inside PLG films showed the existence of non-porous areas after three weeks of degradation. This was confirmed by SEM analysis. Pores may not have been formed in these areas, or existing pores may have been closed. These results indicate that a significant part of the total transport resistance was located at the surfaces after five weeks of degradation, and inside the films after three weeks of degradation. It should be possible to apply these findings to microparticles and other kinds of DDS of different sizes. Knowledge on the drug release mechanism is important for the development of pharmaceuticals, and identification of the region of highest transport resistance is useful when deciding how to modify the release rate.

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Paper VI

The Mechanisms of Drug Release in Poly(Lactide-co-Glycolide)-based Drug Delivery Systems – a Review

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Abstract

Poly(D,L-lactide-co-glycolide) (PLG) is the most frequently used biodegradable polymer in the controlled release of encapsulated drugs. Understanding the release mechanisms, as well as which factors that affect drug release, is vital in order to be able to modify drug release. Drug release from PLG-based drug delivery systems is however complex. This review focuses on release mechanisms, and provides a survey and analysis of the processes determining the release rate, which may be helpful in elucidating this complex picture. The term release mechanism, the various techniques that have been used to study release mechanisms, and the release mechanisms reported are presented and discussed. The various mechanisms of drug release and the physico-chemical processes that influence the rate of drug release are analyzed, and practical examples are given and discussed. The complexity of drug release from PLG-based drug delivery systems can make the generalization of results and predictions of drug release difficult. However, this complexity also provides many possible ways of solving

problems and modifying drug release. Basic, generally applicable and mechanistic research provides pieces of the puzzle, which is useful in the development of controlled-release pharmaceuticals.

Keywords: Release mechanism, Poly(D,L-lactide-co-glycolide), Pore formation, Pore closure, Diffusion, Degradation

Contents

1. Introduction

Poly(D,L-lactide-co-glycolide) (PLG) has been used in various areas, such as the controlled release of encapsulated drugs, tissue engineering (Oh and Lee, 2007; Wang et al., 2010), healing of bone defects (Bertoldi et al., 2008), and in vaccines (Feng et al., 2006; Jiang et al., 2005). Several PLG-based products for the controlled release of encapsulated proteins or peptides are on the market. The use of biopharmaceuticals, such as proteins and peptides, and of hydrophobic drugs with low oral bioavailability, is growing (Närhi and Nordström, 2005; Pisal et al., 2010; Wiscke and Schwendeman, 2008). As the oral bioavailability of both these groups of pharmaceuticals is low, patient compliance is also low due to the necessity of administration by injection. The frequency of injections can be decreased by the use of controlled-release encapsulated drugs, which is very beneficial for patients who require daily and/or long-term treatment.

The reasons for the widespread use of PLG are its biodegradability, its biocompatibility, and the fact that it has been approved for parenteral use by regulatory authorities around the world. The disadvantage associated with PLG is the production of acids upon degradation, as is the case of many other biodegradable polymers. Several techniques for the stabilization of acid-sensitive drugs have been investigated, and this continues to be an area of intense research (Bilati et al., 2005; Houchin and Topp, 2008; Zhu and Schwendeman, 2000). Further advantages of PLGs are that they are commercially available with very different physico-chemical properties, and that the drug release profile can be tailored by selecting PLGs with the appropriate properties, for example, molecular weight (M_w) and the lactide:glycolide ratio (L:G) (Tracy et al., 1999; Ravivarapu et al., 2000; Zolnik and Burgess, 2008). The duration of drug release can be varied from hours (Ratajczak-Emselme et al., 2009) to several months (D'Souza, 2004; Lagarce et al., 2005). Furthermore, pulsed drug release is also possible (Dorta et al., 2002). Blending or co-polymerizing PLG with other materials, or encapsulating PLG microparticles in gels, further extends the possibility of controlling drug release (Cho et al., 2001; Galeska et al., 2005; Mundargi et al., 2008; Vila et al., 2004).

Numerous active pharmaceutical ingredients have been encapsulated in PLG-based drug delivery systems (DDSs) with proven therapeutic effect *in vivo*, or have been released in concentrations considered sufficient for therapeutic effect, for example, siRNA (Murata et al., 2008), proteins (Gu et al., 2007), peptides (D'Souza et al., 2004), anti-cancer drugs (Mo and Lim, 2005), analgesics (Yen et al., 2001), antibiotics (Patel et al., 2008), and vaccines (Cui et al., 2007). Among the different forms of PLG-based DDSs, microspheres or microparticles are the most common. Other types include nanoparticles (Sharma et al., 2007), films (Klose et al., 2008), cylinders (Desai et al., 2010), *in situ* forming implants or microparticles (Dong et al., 2006), scaffolds (Xiong et al., 2009), and foams (Ong et al., 2009). PLG implants may be surgically inserted at the desired location, giving the advantage of local drug delivery of, for example, antibiotics or anti-cancer drugs (Weinberg et al., 2008; Xu and Czernuszka, 2008). Nanoparticles of PLG can also be injected intravenously, and target delivery can be obtained by conjugating an antibody or another molecule with an affinity for a specific target onto the surfaces (Chittasupho et al., 2009), for example, tumor targeting (Patil et al., 2009). Active cellular uptake of nanoparticles is possible, enabling intracellular drug delivery (Cartiera et al., 2009; Hirota et al., 2007), which is an advantage in gene delivery (Cun et al., 2009).

Knowledge of the release mechanisms and the physico-chemical processes that influence the release rate is vital in order to develop controlled-release DDSs. The two main release mechanisms associated with drug release from PLG-based DDSs are diffusion and degradation/erosion. The release rate is often said to be diffusion-controlled initially and degradation/erosion-controlled during the final stage of the release period (D'Souza et al., 2005; Mollo and Corrigan, 2003). However, many processes or events influence the rate of drug diffusion and the degradation kinetics, for example, polymer–drug interactions (Blanco and Alonso, 1997), drug–drug interactions (Kang et al., 2008), water absorption (Desai et al., 2010), and pore closure (Kang and Schwendeman, 2007). Knowledge regarding these more detailed processes is necessary if we are to understand drug release in detail and be able to control the release rate. Drug release is often preceded by a chain of processes (e.g. water absorption, hydrolysis, and erosion). These processes are influenced by many different factors. This increases the complexity of drug release, as discussed in Section 3. The term "release mechanism" is used in different ways in the literature, which further

complicates the picture. Various techniques have been used to study release mechanisms, and the results regarding release mechanisms differ, which is not surprising considering the complexity of drug release from PLG-based DDSs. Although PLG has received much attention as a drug carrier over the past 20 years, new insights into processes that govern drug release and new ways of modifying drug release are still being presented.

This review focuses on the mechanisms of drug release from PLG-based DDSs, and is complementary to previous reviews that have emphasized which factors that effect drug release from mainly poly(lactic acid) (PLA)-based DDSs (Alexis, 2005), the encapsulation and release of hydrophobic drugs (Wiscke and Schwendeman, 2008), and the encapsulation and release of macromolecular drugs in PLG and its derivates (Mundargi et al., 2008). It is also complementary to previous reviews covering other polymers in addition to PLG, and focusing on mathematical modeling of drug release (Siepmann and Göpferich, 2001; Siepmann and Siepmann, 2008). Understanding the release mechanisms is key to developing formulations, and we believe that a deep review focusing solely on release mechanisms will make an important contribution, and help clarify the complex picture of drug release from PLG-based DDSs. This review covers the definition of the term "release mechanism", the release mechanisms that have been reported, different techniques used for the study of release mechanisms, and the physico-chemical processes influencing drug release.

2. Definition of the term "release mechanism"

The term "release mechanism" has been defined in slightly different ways. It has been used as a description of the *way* in which drug molecules are transported or released (Kranz et al., 2000; Sansdrap and Moës, 1997), and as a description of the process or event that determines the release *rate*. Table 1 lists different release mechanisms or processes that have been reported to be the rate-controlling process in drug release. These will be further discussed in Section 5.

Table 1. Processes that have been reported as release mechanisms or rate-controlling processes in drug release.

Mechanism or process	**Reference**
Dissolution of the drug (in combination with diffusion)	Wong et al., 2001
Diffusion through water-filled pores	Kim et al., 2006
Diffusion through the polymer matrix	Sun et al., 2008
Hydrolysis	Bishara and Domb, 2005
Erosion	Shah et al., 1992
Osmotic pumping	Jonnalagadda and Robinson, 2000
Water absorption/Swelling	Mochizuki et al., 2008
Polymer–drug interactions	Gaspar et al., 1998
Drug–drug interactions	Zhu and Schwendeman, 2000
Polymer relaxation	Gagliardi et al., 2010
Pore closure	Kang and Schwendeman, 2007
Heterogeneous degradation	Park, 1995
Formation of cracks or deformation	Matsumoto et al., 2006
Collapse of the polymer structure	Friess and Schlapp, 2002

There are only three possible ways for drug molecules to be released from a DDS: (i) transport through water-filled pores, (ii) transport through the polymer, and (iii) due to dissolution of the encapsulating polymer (which does not require drug transport). Transport through water-filled pores are the most common way of release, as the encapsulated drug is usually a biopharmaceutical, such as a protein or a peptide, which are too large and too hydrophilic to be transported through the polymer phase. The most common way of transport through water-filled pores is diffusion, i.e. random movements of the molecules driven by the chemical potential gradient, which can often be approximated by the concentration gradient. The other way of transport through water-filled pores is convection, which is driven by a force such as osmotic pressure (Cussler, 1997). Osmotic pressure may be created by the influx of water into a non-swelling system. Drug transport driven by this force is called osmotic pumping (Hjärtstam, 1998), and is more common in drug delivery systems utilizing other polymers such as ethyl cellulose (Marucci, 2009). PLGs that absorb a large amount of water also have mobile polymer chains, and are prone to swell. As the volume of water inside increases, any significant increase in pressure will probably be compensated for by swelling and rearrangement of the polymer

chains. Transport through the polymer phase may occur when the drug is small and hydrophobic (Raman et al., 2005). However, the drug must enter the water phase, either at the surface or in the pores inside the DDS, before being released. The encapsulated drug may also be released without any transport due to dissolution of the polymer, i.e. erosion. Erosion also creates pores, thus increasing the rate of diffusion. However, there is a difference between erosion leading to drug release without drug transport, and erosion that increases the rate of drug transport. The latter has been reported as a release mechanism countless times, at least after a lag period, which is often described as diffusion-controlled release (Alexis et al., 2004; Cohen et al., 1991; Goraltchouk et al., 2006; Johnson et al., 1997; Lam et al., 2000; Wang L et al., 2004; Westedt et al., 2006).

The three basic ways of drug release mentioned above, with two types of transport included in the transport through water-filled pores, result in four possible release mechanisms, if the term "release mechanism" is defined as the *way* in which the drug is released:

- diffusion through water-filled pores,
- diffusion through the polymer,
- osmotic pumping, and
- erosion (i.e. no drug transport).

These release mechanisms will be further discussed in Section 5.

However, the most common use of the term release mechanism is in referring to the process that determines the *rate* of release, for example swelling, drug dissolution or polymer–drug interactions. As mentioned above, erosion can be included in both definitions, but with different meanings. Describing the process controlling the release rate is more informative than describing the way of drug release, when it comes to how drug release can be modified. Describing these processes is thus important. However, using these processes as release mechanisms leads to problems: (i) due to the complexity of the system it is not always clear which of the processes is dominating, and (ii) in a chain of processes that leads to drug release it is not obvious which one is the rate-determining process. For example, the drug may be released by diffusion through water-filled pores, and the rate of pore formation may be the rate-controlling process. Polymer erosion, which is determined by the rate of hydrolysis, *probably* determines the rate of pore formation, although the absorption of water also results in pores. Should the release mechanism

be described as "pore formation", "erosion" or "hydrolysis"? And should water absorption be mentioned? How far along the chain of processes should one search for the process mainly responsible for drug release? This is probably one reason why so many different processes have been reported as the release mechanism (Table 1), which does not help clarifying the complex picture of drug release (see Section 3).

In this review, the processes defining the *way* in which the drug is released will be called the *true release mechanisms*, and the processes that control the release *rate* will be called *rate-controlling release mechanisms*. The true release mechanisms are illustrated in Figure 1. In discussions regarding release mechanisms, it is thus recommended to first establish the true release mechanism(s), and then to discuss the rate-controlling release mechanisms in more detail. For example, bovine serum albumin (BSA) was released by diffusion through water-filled pores. The rate of diffusion depended on the degree of polymer erosion, and was slowed down by the adsorption of BSA to the polymer. In this example, the true release mechanism is diffusion through water-filled pores, and polymer erosion and polymer–drug interactions are the rate-controlling release mechanisms. BSA is released by diffusion through water-filled pores during the whole release period no matter if the degradation kinetics, the initial porosity or any other factor determines the release rate, which is the reason why the true and rate-controlling release mechanisms should be discussed separately. Knowing the true release mechanism is useful when trying to identify the rate-controlling release mechanism.

The true and rate-controlling release mechanisms could be compared to the established terms regarding mechanisms of diffusion, namely intrinsic and apparent diffusion (Macarini et al., 2010). Intrinsic is the true mechanism for diffusion, or pure diffusion. The apparent diffusion is the diffusion that can be measured and may depend on other phenomena, such as interaction between the diffusing solute and other materials. There are differences between these couples of terms, through. While diffusion through a porous network, causing an effective diffusion, would fall under the term apparent diffusion, diffusion through a porous network is the *way* in which an encapsulated drug is released and is thus a true release mechanism.

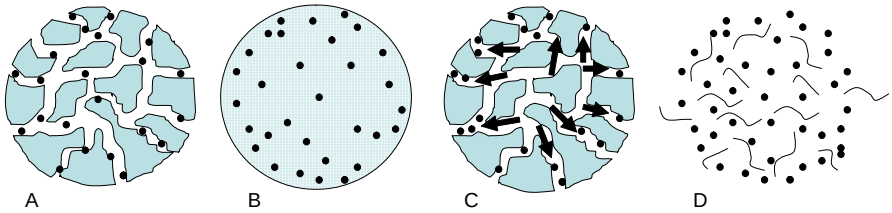


Figure 1. True release mechanisms: (A) diffusion through water-filled pores, (B) diffusion through the polymer, (C) osmotic pumping and (D) erosion.

3. Factors that influence drug release from PLG-based DDSs

3.1. Physico-chemical processes occurring in PLG-based DDSs

Water is absorbed by the polymer immediately upon immersion in water or administration *in vivo* (Figure 2). The rate of water absorption, or hydration, of the DDS is rapid compared to drug release (Batycky et al., 1997; Blasi et al., 2005). Any volume occupied by water inside the polymer matrix can be regarded as a pore, and water absorption is therefore a pore-forming process. These pores are too small for drug transport during the early stage of this process, but as the number and size of water-filled pores in the polymer increase, a porous connected network allowing drug release is formed (Mochizuki et al., 2008; Webber et al., 1998).

Hydrolysis, i.e. the scission of ester bonds and subsequent decrease in M_w, starts immediately upon contact with water. Hydrolysis creates acids, which catalyzes hydrolysis (Shenderova et al., 1999). This auto-catalytic phenomenon is known to cause heterogeneous degradation inside PLG matrices (Li and McCarthy, 1999), i.e. faster degradation at the center of the PLG matrix than at the surface. This effect becomes more pronounced with increasing dimensions of a DDS (Dunne et al., 2000) as the acid gradient increases, but heterogeneous degradation has also been reported in particles and films with dimensions as small as 10 μm (Lu et al., 1999; Park, 1995). The polymer becomes less hydrophobic with decreasing M_w, and at 1100 Da the oligomers become water soluble (Park, 1994).

Erosion, i.e. mass loss of the polymer, starts when the dissolved polymer degradation products are able to diffuse into the release medium. PLG normally undergoes bulk erosion, in contrast to surface erosion, as PLG is relatively rapidly hydrated (Chen and Ooi, 2006). Dissolution of polymer degradation products and erosion create pores. Small pores, formed by water absorption or polymer erosion, grow as contact with water leads to hydrolysis, and the locally produced acids catalyze degradation and causes polymer dissolution inside the pores, leading to subsequent erosion. Small pores consequently grow, and eventually coalesce with neighboring pores to form fewer, larger pores (Batycky et al., 1997). Pores may also be closed (Fredenberg et al., in press; Kang and Schwendeman, 2007). This phenomenon is related to the mobility of the polymer chains, and their ability to rearrange (Yamaguchi et al., 2002), which is further discussed in Section 5. The mobility of polymer chains depends on the glass transition temperature (T_g). The transport resistance is higher for PLGs in the vitreous state, and water absorption and hydrolysis proceed more slowly. The glass transition temperature decreases with decreasing M_w (Zolnik et al., 2006).

The dissolved polymer degradation products affect the system in several ways.

(i) They are acids and thus catalyze hydrolysis.
(ii) They plasticize the polymer, which increases the rate of water absorption and decreases the transport resistance of the polymer (Mauduit et al., 1993).
(iii) They increase the osmolality inside the polymer matrix, and thus the force for water absorption.
(iv) They are known to be able to crystallize, especially if there are many repeating units of the same monomer in a row, i.e. glycolic, L-lactic or D-Lactic monomers (Schliecker et al., 2003; Vert et al., 1991). This crystallization inhibits water absorption, further degradation and transport (Li, 1999).

These dissolved degradation products are released at polymer erosion, which means that their effect on the system ceases upon erosion. The onset of rapid erosion often coincides with a cessation of the decrease in the average M_w and T_g, as the low-M_w fraction of polymer chains is released, and the effects of dissolved degradation products are lost (Yoshioka et al., 2008). The transport resistance is thus important, not only for the release of the encapsulated drug, but also for the polymer

degradation kinetics. Two important processes that influence the transport resistance are pore formation and pore closure. Other processes that influence the rate of drug transport are drug dissolution, polymer–drug interactions and drug–drug interactions.

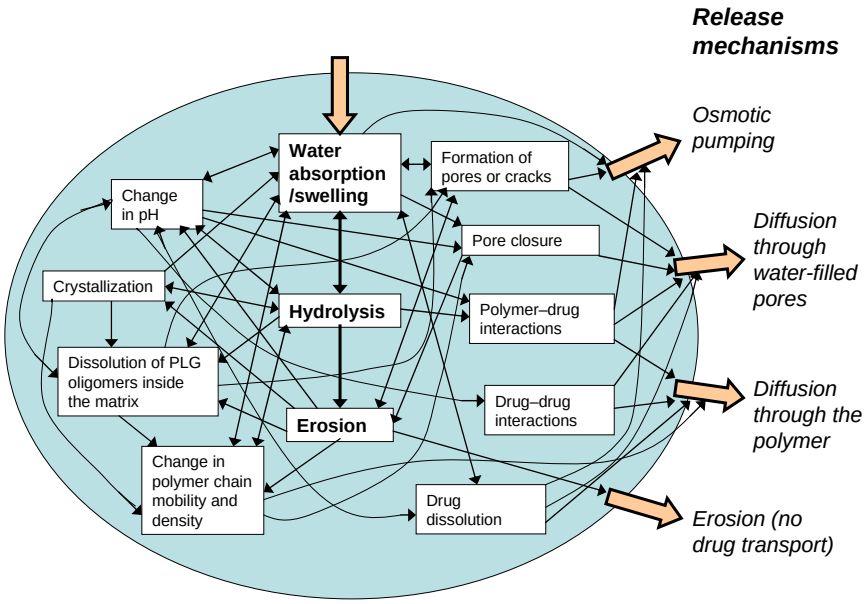


Figure 2. The complex picture of physico-chemical processes taking place within PLG matrices, leading to drug release. The influence of processes on drug release and on other processes is illustrated by arrows. Note that some arrows point in both directions.

3.2. Factors influencing the physico-chemical behavior of PLG

The processes described in Section 3.1 are affected by the properties of the DDS and the surrounding environment, which are listed in Table 2. How these affect the processes are illustrated in Figure 3.

Table 2. Properties of the DDS and the surrounding environment that influence drug release

The polymer Molecular weight L:G ratio End-group capping Semi-crystallinity	***In vitro* conditions** Temperature Stirring Composition of the release medium pH Osmolality
Encapsulated substances The characteristics of the drug Drug load and location The characteristics of additives, such as salts, surfactants and plasticizing agents	***In vivo* conditions** Sink conditions Enzymes Lipids Immune responses
The DDS Size Porosity Density Shape	

One method of controlling drug release is to select PLGs with the appropriate properties. The molecular weights of PLGs used for controlled release are usually relatively low, often less than 50 kDa and very seldom above 150 kDa. PLGs with molecular weights less than 10 kDa are sometimes used. The L:G ratio ranges from 50:50 to 100:0. PLA can be regarded as a 100:0 PLG and will be included in the discussions regarding PLG in this review. The polymer end groups may or may not be capped with a hydrophobic ester group, for example, a stearyl group (Johansen et al., 2000). Low M_w, low L:G ratio and un-capped polymer end groups result in a less hydrophobic polymer with increased rates of water absorption, hydrolysis and erosion (Husmann et al., 2002; Lu et al., 1999; Tracy et al., 1999; Zilberman and Grinberg, 2008). The amount of water absorbed and the duration of drug release is highly dependent on these properties (Alexis et al., 2006; Kim et al., 2005), and the choice of PLGs is perhaps the most important tool in drug release modification. The initial T_g is also dependent on these properties. Polymers with only the L-lactic acid may be semi-crystalline (Alexis et al., 2006). When discussing drug release from PLG-based DDSs it is important to

remember that PLGs with different molecular weights, L:G ratios and end-group capping behave very differently. It is also important to bear in mind the dynamic nature of PLG, as its properties and behavior change with degradation. Hydrophobic, high-M_w and slow-degrading PLGs will eventually become more hydrophilic, low-M_w and fast-degrading PLGs.

The encapsulated drug and additives may affect many of the processes listed above. Salts consisting of a divalent cation and a basic anion are common protein stabilizers (Takada et al., 2003; Zhong et al., 2007). Basic anions neutralize acids (Li and Schwendeman, 2005), and divalent cations can be used to stabilize proteins by complex binding, or by inhibiting acylation (Johnson et al., 1996; Sophocleous et al., 2009). Divalent cations may also be pore forming, as they probably catalyze hydrolysis (Fredenberg et al., 2007; Fredenberg et al., 2009). Other common additives are plasticizing or surface active substances. The encapsulated drug or the co-encapsulated additives may affect drug release in several ways:

(i) enhanced or inhibited water absorption and hydrolysis due to increased hydrophilicity/hydrophobicity, osmolality, or due to surface active substances (Chung et al., 2006; Kang and Schwendeman, 2002),
(ii) increased or decreased rate of hydrolysis due to acid or base catalysis, or acid neutralization (Wang L et al., 2004; Zhang et al., 1997),
(iii) plasticization of the polymer (Blasi et al., 2007; Kranz et al., 2000), or
(iv) constitution of crystalline parts of the DDS.

The amount of drug encapsulated, i.e. the load, may be important as the space left vacant after drug release will probably constitute pores, facilitating further drug release (Perugini et al., 2001). The release profile may also be affected by the location of the drug inside the DDS (Berkland et al., 2003). The location of the drug may be affected by the physico-chemical properties of the drug (Sandor et al., 2001).

The characteristics of the DDS, such as the porosity and the polymer chain density, are important (Duvvuri et al., 2006; Kim and Park, 2004; Ricci et al., 2005). Large DDSs result in an increased pH gradient, and the auto-catalytic effect on degradation is enhanced (Fu et al., 2000). The shape of the DDS, in particular the ratio of surface area to volume,

affects the release of the drug and the PLG degradation products. The size of particles may effect the drug distribution within the particles (Berkland et al., 2003). Most of the properties characterizing the DDS are influenced by the manufacturing method (Yushu and Venkatraman, 2006).

The local environmental conditions also affect the processes and drug release. Increased temperature increases all chemical reactions, but also increases the mobility of the polymer and, thus, possibly the rate of pore closure. An unstirred surface layer surrounding the DDS inhibits drug release. Salts, plasticizing agents and surfactants in the release medium may affect the processes in the same way as if they were encapsulated, but with the exception that high osmolality in the release medium would decrease the rate of water absorption by the DDS (Faisant et al. 2006; Li, 1999; Okada, 1997). The pH or buffering capacity is important for the rate of degradation (Park et al., 1995), but also for the rate of pore formation and pore closure, as will be discussed in Section 5.2. The conditions must therefore be considered when designing an *in vitro* release method. Faster polymer degradation and drug release, and a shorter drug release lag-phase, have been reported *in vivo* (Spenlehauer et al., 1989; Zolnik and Burgess, 2008), and have been attributed to the effects of enzymes, lipids, non-sink conditions, possible merging of microparticles and immune responses (Grayson et al., 2004; Pratt et al., 1993; Zeng et al., 2005). The collection of macrophages around the DDS is an immune response, and the phagocytosis of small microparticles, and the release of acidic products by these cells may increase the rate of degradation (Anderson and Shive, 1997). The formation of a fibrous capsule around injected particles, which may decrease the pH due to acidic degradation products, has also been reported (Sastre et al., 2007).

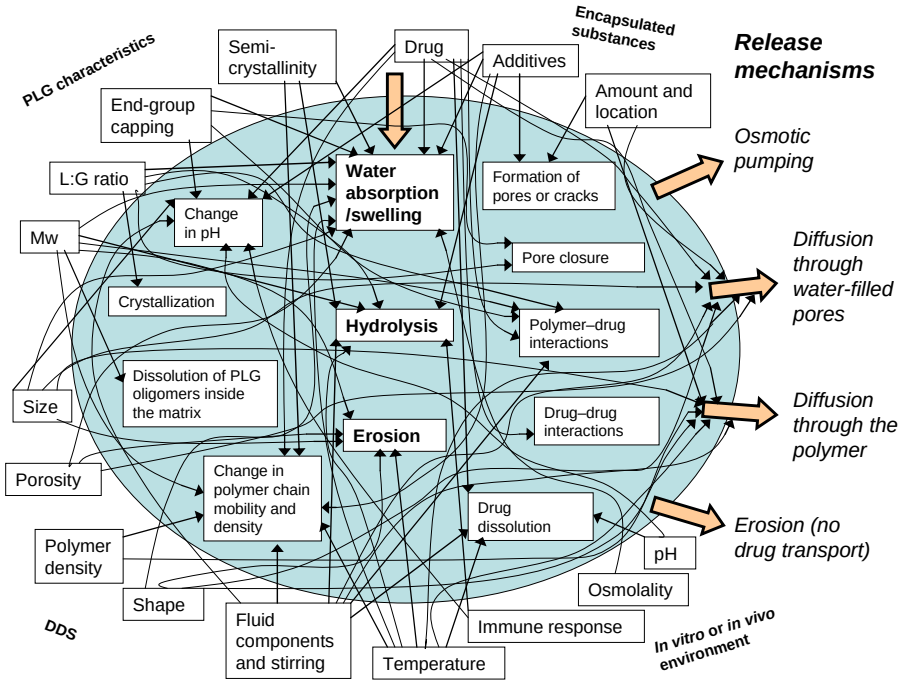


Figure 3. The complex picture of the different factors that influence drug release from PLG matrices. The effects of the properties of the DDS and the surrounding environment on the processes that, in turn, influence drug release are illustrated by arrows.

4. Studies of release mechanisms

4.1. The shape of the release profile

The release profile is sometimes used as the basis for mechanistic evaluation. Although zero-order release is the most commonly preferred profile, mono-phasic release from PLG-based DDSs is rare. Drug release is sometimes bi-phasic, but a tri-phasic profile is probably most common. Large particles or DDSs often exhibit this tri-phasic release profile due to heterogeneous degradation (Berchane et al., 2007; Berkland et al., 2003). Small particles and particles coated with a thin PLG film often exhibit a bi-phasic release profile with a relatively rapid

second phase (Fredenberg, 2011; Sansdrap and Moës, 1997). Combining particles of different sizes has been shown to offer a means of altering the drug release profile, from a Fickian diffusion profile and a sigmoidal profile to a zero-order profile (Berkland et al., 2002).

Phase I in the classic tri-phasic release profile is usually described as a burst release, and has been attributed to non-encapsulated drug particles on the surface or drug molecules close to the surface easy accessible by hydration (Wang et al., 2002). Other reasons for burst release may be the formation of cracks and the disintegration of particles (Huang and Brazel, 2001). Phase II is often a slow release phase, during which the drug diffuses slowly, either through the relatively dense polymer or through the few existing pores, while polymer degradation and hydration proceed. Phase III is usually a period of faster release, often attributed to the onset of erosion. This phase is sometimes called the *second burst*. However, all release profiles do not follow the traditional tri-phasic release profile. If the second phase is rapid, there may be a slower phase at the end of the release period (Bae et al., 2009; Han et al., 2010). The release profile may not exhibit any burst release (Pan et al., 2006). Some examples of different release profiles are given in Figure 4, while Table 3 summarizes some of the explanations of the different release profiles. The term *degradation* is used with slightly different meanings in different studies: i.e. both hydrolysis and erosion of the polymer, or the combination of the two processes. In this paper, except in Table 3, *degradation* refers to the M_w-decreasing process of hydrolysis, which is the most common use of the term.

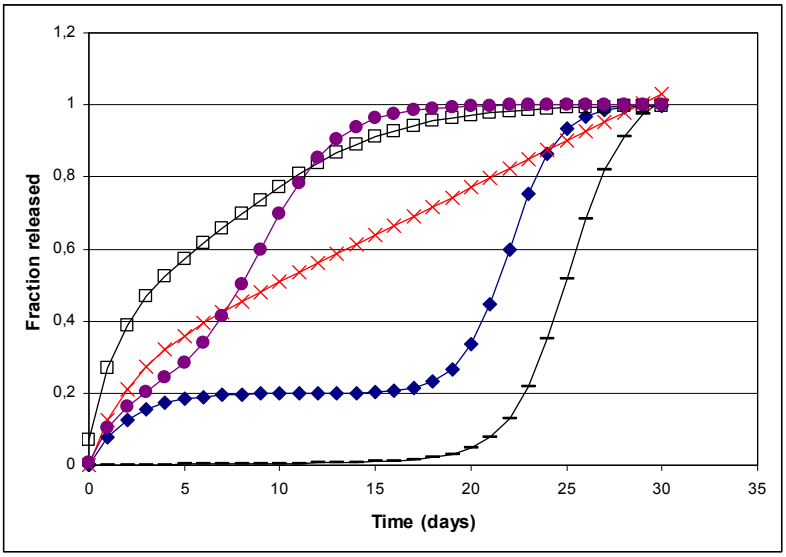


Figure 4. Release profiles consisting of different phases. Open squares: burst and a rapid phase II. Filled circles: tri-phasic release with a short phase II. Crosses: burst and zero-order release. Filled diamonds: tri-phasic release. Dashes: bi-phasic release, similar to tri-phasic but without the burst release.

Table 3. Explanations of the origins of the phases observed during drug release.

Phase I	**Phase II**	**Phase III**	**Reference**
Burst	Slow diffusion-controlled release	Rapid erosion-controlled release	Loo et al., 2010
No burst	Slow diffusion-controlled release	On-set of degradation. Erosion-controlled release	Alexis et al., 2004
Diffusion-controlled release of drug molecules at the surface or in pores initially connected to the surface	Dependent on diffusion and erosion	Dependent on diffusion and erosion	Zolnik et al., 2006
Similar to the row above	Lag-phase, as the first and second phase did not overlap	Second phase, erosion-controlled	Johnson et al., 1997
Similar to the row above	Slow and minimal release	Rapid release. Rapid water absorption associated with sudden mass loss	Duvvuri et al., 2006
Similar to the row above	Degradation and erosion.		Capan et al., 2003
Burst. Drug molecules on or with access to the surface	Slow diffusion-controlled release	Onset of bulk degradation	Chen and Ooi, 2006
Burst	Diffusion governed by water absorption and swelling	Erosion phase at which degradation occurs	Xu and Czernuszka, 2008
Burst	Diffusion due to hydration	Faster diffusion due to erosion. The onset of this phase depends on the rate of hydration	D'Souza et al., 2005
Burst. Surface-bound and poorly encapsulated drugs may diffuse through pores and cracks	Slow diffusion, which may be attributed to binding of the drug to the polymer	Faster diffusion through the eroding matrix. Decrease in polymer M_w increases the gaps in the matrix	Janoria and Mitra, 2007
Burst. Solvent penetration and glass transition	Limited drug dissolution. Polymer degradation and relaxation	Diffusion through water-filled pores	Lao et al., 2008

There are many possible explanations of the different phases, as can be seen in Table 3. The complexity of the processes or events that enhance or inhibit drug release (illustrated in Figure 2) makes it difficult to draw any conclusions merely from the release profile. A slow second phase, or lag-phase, may not necessarily be caused by a dense polymer with low porosity, which is the common explanation. It may also be caused by pore closure, polymer–drug interactions or drug–drug interactions that inhibit the release of the drug (Blanco and Alonso, 1997; Kang et al., 2008; Kang and Schwendeman). In a study on the release of leuprolide acetate from PLG microparticles, the interior became porous while the surface remained non-porous at an early stage of the slow second phase of a tri-phasic release pattern. It is logical to assume that diffusion inside the particle was rapid and that the low porosity at the surface was the reason for the slow release. Adding medium chain triglycerides to the microparticles made the surface porous, in addition to increasing the porosity inside, and the slow second phase disappeared (Luan and Bodmeier, 2006). The second burst, or rapid phase III, is commonly attributed to the onset of polymer erosion. However, it may also be caused by cracks or the disintegration of particles (Matsumoto et al., 2006). As the pH and other microenvironmental characteristics change with time, the conditions causing the slow release may have been altered, for example, such that the process of pore formation dominates over pore closure. Friess and Schlapp (2002) found that the rapid release phase could be phase II or III depending on the type of PLG. The onset of rapid drug release was found to be correlated with massive swelling, erosion and deformation of the microparticles, and the increase in release rate was ascribed to the accessibility of new surfaces. One problem with visual analysis of the release profile is that the start and end-point of each phase is not always obvious. Phases may also have their origin in superimposing processes or events that counteract each other. Attributing a second burst release to pore formation caused by degradation/erosion is probably often accurate, however, caution should be exercised when drawing conclusions merely from the release profile.

4.2. Mathematical modeling

A variety of mathematical models have been used to describe drug release from PLG-based DDSs. Mathematical models can be divided into two categories: empirical/semi-empirical models and mechanistic

mathematical models (Siepmann and Siepmann, 2008). Empirical/semi-empirical models are purely mathematical descriptions, and are not based on any real chemical, physical of biological phenomenon. These do not provide any insight into which factors that control drug release, and their predictive power is low. However, they may still be useful, for example, in describing different phases of the drug release, which can be helpful in product development (Duvvurvi et al., 2006). Mechanistic mathematical models, on the other hand are based on real phenomena, such as diffusion, degradation and erosion, and are useful tools in the mechanistic understanding of the release process. The values of some parameters may be determined in complementary experiments, or fitted using experimental data. Several parameters may be fitted simultaneously. The validity of a model increases if its predictions are in good agreement with independent experimental data. Predictability has been demonstrated for some models (Faisant et al., 2003; Guse et al., 2006a; Raman et al., 2005; Wang et al., 2007), however, tests of predictability have not been performed in many studies. Several techniques can be used for mathematical modeling. Some examples are: exponential models (Mollo and Corrigan, 2004), models based on percolation theory (Batycky et al., 1997; Ehtezazi and Washington, 2000), compartment models (Murty et al., 2004), Monte Carlo simulations (Barat et al., 2008), models based on convolution (Guse et al., 2006a), and Fourier analysis (Raiche and Puleo, 2006). Some examples of models used to describe drug release from PLG-based DDSs are mentioned in this section, but as these techniques are not the subject of this review, the reader is referred to other review articles on this topic (Arifin et al., 2006; Siepmann and Göpferich, 2001; Siepmann and Siepmann, 2008).

The most famous of the empirical/semi-empirical mathematical models is the Peppas equation (Peppas, 1985):

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{Eq. 1}$$

where M_t is the amount of drug released at time t, M_∞ is the total amount of drug encapsulated, k is a constant incorporating characteristics of the system and n is the release exponent. The value of n may be indicative of the release mechanism. For a purely diffusion-controlled, non-swelling, and non-degrading system, and a constant diffusion coefficient, n=0.5 for a thin film, 0.45 for a cylinder and 0.43 for a sphere. Other values of

n are indicative of purely swelling-controlled systems. Exponents between the values consistent with purely one-factor-controlled systems describe a form of transport referred to as anomalous transport, which may include other types of phenomena than swelling and diffusion. The Higuchi equation and the Hopfenberg model are other examples of empirical models (Siepmann and Göpferich, 2001; Siepmann and Siepmann, 2008). The Weibull equation is another example, and is suitable for sigmoidal drug release profiles (D'Souza et al., 2005). Duvvuri et al. (2006) used three different empirical equations to describe sigmoidal or tri-phasic release from microspheres with different PLG blends. They obtained good fit between experimental data and the model, and the results indicated that some PLG blends had a higher density. These and similar empirical/semi-empirical equations have been used in discussions on the release mechanism (Gagliardi et al., 2010; Liu et al., 2003; Yen et al., 2001; Zidan et al., 2006) and to calculate the diffusion coefficient (Alexis et al., 2004) However, conclusions can only be drawn if the assumptions associated with the equations are fulfilled, for example, constant diffusion coefficient and no erosion. This may be the case when using a hydrophobic and high-M_w PLG that swells and degrades at a negligible rate compared to the rate of diffusion through an initially continuous porous network. However, more dynamic PLGs are commonly used, and after a period of degradation, slowly swelling and degrading high-M_w PLGs become rapidly swelling and degrading low-M_w PLGs. In such cases, the diffusion coefficient can not be assumed to be constant.

Mechanistic models describing drug release are often based on diffusivity as described by Fick's law. Some models utilize a constant effective diffusion coefficient, while in others, the effective diffusion coefficient is a function of another parameter. Wang et al. (2007) used a constant diffusion coefficient, but included the processes dissolution, drug crystallization and drug-excipient complex binding. Lemaire et al. (2003) used two different diffusion coefficients: one for diffusion from micropores to initially existing larger pores and one for diffusion in these larger pores. The latter coefficient was much higher than the first. According to this theory drug release was determined by the rate of transport to the larger pores, which was governed by either diffusion or erosion. Hsu et al. (1996) used the Roseman-Higuchi model for a cylindrical system to calculate the constant diffusion coefficient, as they argued that polymer erosion had little influence on drug release during

the release period. They encapsulated isoniazid in two different DDSs: dry-mixed matrices, in which the drug particles were connected like drug-filled channels, and in PLG foams, in which the drug particles were separated by polymer regions. The release was studied *in vitro* at different temperatures. When diffusion occurs through a solid phase, the diffusivity can be related to the Arrhenius expression, and the natural logarithm of the diffusion coefficient is proportional to $1/T$ (where T is the temperature in Kelvin). Diffusion in a liquid can instead be described by the Stokes-Einstein equation, combined with the Carrancio equation, which describes the relation between viscosity and temperature. The natural logarithm of the diffusion coefficient is then instead proportional to D/T. Plotting ln (D) against D/T and $1/T$ led to the conclusion that isoniazid diffused through water-filled pores in the dry-mixed matrices and through the polymer phase in the foams.

The mechanistic models describing erodible systems often utilize a chemical reaction to describe the effect of polymer degradation and/or erosion. Many of these models include a non-constant diffusivity or permeability parameter, which is an advantage. Siepmann et al. (2005) simulated the effective diffusion coefficient as a function of particle size to illustrate the effect of auto-catalysis on diffusion. The classical Higuchi model was modified by Heller and Baker who introduced a permeability parameter that increased with time as more pores were created (Arifin et al., 2006). The same time-dependent effective diffusion coefficient and the constant k characterizing the polymer degradation rate (Equation 2), was used to describe the drug release from PLG films and microspheres in two separate studies (Berkland et al., 2004; Charlier et al., 2000). In a study of 5-fluorouracil release from microparticles of PLG, a relationship was found between the diffusion coefficient and the polymer M_w (Equation 3) (Faisant et al., 2002). Another such mathematical relationship has been found in a study on a small hydrophobic drug (Equation 4) (Raman et al., 2005).

$$D_{eff}(t) = D_0 \times e^{kt} \qquad (D_0 \text{ is the initial diffusion coefficient}) \qquad \text{Eq. 2}$$

$$D(Mw) = D_0 + \frac{k}{Mw} \qquad (k \text{ is a constant}) \qquad \text{Eq. 3}$$

$$\ln(D) = -0.347x^3 + 10.394x^2 - 104.950x + 316.950 \quad (x = \ln(M_w)) \qquad \text{Eq. 4}$$

The Monte Carlo technique has been used to simulate polymer erosion and, combined with diffusive mass transfer, this technique could describe the release of 5-fluouracil from PLG-based microparticles, and showed good agreement with experimental data (Figure 5) (Siepmann et al., 2002). Limited drug solubility in the system was also taken into account. This model allowed the simulation of a time- and position-dependent diffusion coefficient, which is a great advantage. The model did not include a description of swelling or processes such as pore closure. However, these processes may be insignificant, considering that the high-M_w PLG (104 kDa) used in the study exhibits a low degree of water absorption and polymer chain mobility during the first three weeks of degradation (Fredenberg, 2004), which was the duration of drug release.

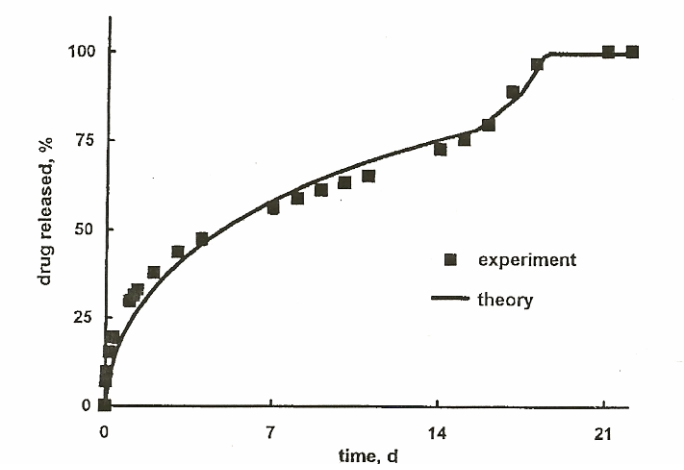


Figure 5. Comparison of experimental data and a simulation of drug release based on the Monte Carlo technique of polymer erosion in combination with diffusive mass transfer. Originally published by Siepmann et al., A new mathematical model quantifying drug release from bioerodible microparticles using Monte Carlo Simulations, Pharm. Res. 2002, vol. 19, pp. 1885-1893.

The use of mathematical models in the evaluation of the physico-chemical processes governing drug release makes it possible to explain and predict the release process. A prerequisite is that the model is properly validated experimentally. If the model fits the experimental data, and particularly if its predictive power can be demonstrated, it is very probable that the conclusions drawn from the simulations are

accurate. Another advantage is that it is possible to perform quantitative predictions of drug release. The disadvantage is that other possible explanations can not be completely excluded. A model based on many parameters, which is often necessary for an accurate description, can be made to fit many different release profiles. There may be more than one set of parameters or equations that fit the experimental data. For example, a zero-order release pattern may depend on, and be modeled by, the rate of diffusion and the rate of polymer degradation/erosion. The decreasing concentration gradient and increasing diffusion distance, leading to a decrease in the rate of transport, may be counteracted by the increase in porosity resulting from erosion, leading to an increased rate of transport. However, the zero-order release may also be due to pore closure at a rate that counteracts the effect of pore formation. Another possible explanation may be that the transport resistance increases in one part of the system and decreases in another part (Fredenberg, 2011; Park, 1995). A study of leuprorelin release from one-month depot microspheres provides a good example of apparently zero-order release, attributed to superimposed first-order phases of diffusion and erosion is (Okada, 1997). In addition to diffusion and erosion, drug release was affected by the interaction between the cationic leuprorelin and the anionic PLG, and the magnitude of this effect depended on the degree of degradation. Scanning electron microscopy (SEM) images showed increasing pore closure in the microspheres with time, which could affect the rate of drug release, although this was not mentioned. Furthermore, as the osmolality of the release medium was decreased, the rate of drug release increased, due to faster water absorption and possibly the formation of cracks due to osmotic pressure. This is an example of drug release being affected by many processes simultaneously, which would be difficult to simulate, although zero-order release was easily described mathematically, showing a good fit to the experimental data. Tests of predictability using independent experimental data are therefore an advantage.

Unfortunately, swelling is often ignored in mathematical modeling. DDSs have been classified as diffusion-controlled systems, swelling-controlled systems or erosion-controlled systems (Arifin et al., 2006). PLG-based systems are mostly considered to be erosion-controlled systems, sometimes diffusion-controlled, while possible swelling often is ignored. The amount of water absorbed is highly dependent on the properties of the PLG, and swelling is sometimes insignificant. However,

a large amount of water may be absorbed (Fredenberg et al., 2009; Kim et al., 2005), leading to the formation of pores, apart from erosion (Mochizuki et la., 2008; Webber et al., 1998).

A model is a simplification of the real system, and its applicability and suitability are restricted (Siepmann and Siepmann, 2008). As chemical reactions, mass transfer and other kinds of processes influencing drug release depends strongly on the characteristics of the DDS, it is crucial to choose an appropriate model for each DDS (Arifin et al., 2006; Siepmann and Göpferich, 2001). A suitable model with proven predictive power is an important tool in pharmaceutical development. Mathematical models can be very useful for the mechanistic understanding of drug release, but the assumptions made in modeling are very important, and the general application of mathematical models should be undertaken with care and preferably be substantiated by predictability tests.

4.3. Studying processes that enhance or hinder drug release

A third way of elucidating true and rate-controlling release mechanisms is to study processes that influence drug release. The drug release profile can be compared to the results of studying processes such as erosion, swelling, pore closure, pore formation, drug–drug interaction, and changes in the T_g. Another example of the way in which insight into drug transport can be gained is to study the heterogeneity/homogeneity of the polymer mass and the location of high transport resistance (Fredenberg et al., 2011). Examples of studies and the conclusions drawn are presented below.

Park (1995) found that a surface layer with low porosity controlled the rate of mass transfer until it cracked. The PLG microspheres had two glass transition temperatures: one decreased with time while the other remained constant, until it disappeared. This suggests that there were two regions degrading at different rates, and it is likely that the rapidly degrading area was in the interior due to the pH gradient. The fact that the microspheres retained their shape and integrity until they disintegrated completely indicates that the slow-degrading region was at the surface, which suddenly broke. This coincided with the disappearance of crystallized degradation products, indicating that the

surface acted as a semi-permeable diffusion barrier, allowing small molecules such as water to enter, but not the crystallized degradation products to be released, until the build-up of osmotic pressure was so high that the surface broke.

Pore closure, ending burst release and probably affecting subsequent drug release, has been demonstrated in a study of porous microspheres (Wang et al., 2002). The permeability was studied using fluorescent probes and confocal microscopy. The encapsulated drug exhibited a burst release on the first day. SEM analysis clearly showed the closure of pores at the surface. In addition, fluorescent probes in the release medium were initially able to diffuse into the microsphere, but not after 24 hours. In another, but methodologically similar, study this pore-closing phenomenon was found to be affected by the incubation temperature, as temperature influences the mobility of the polymer chains and their ability to rearrange (Kang and Schwendeman, 2007).

The ability of the additives to act as porogens and affect the porous structure appeared to determine the release rate in a study on the effect of different additives on drug release using SEM analysis and erosion measurements (Song et al., 1997). Without additives, the drug release profile showed a slow release period followed by a faster release period, which coincided with an increasing rate of erosion. Thus, the release mechanism seemed to be diffusion through water-filled pores, with increased rate of diffusion at a later stage due to pore formation caused by erosion. Water-soluble additives increased the porosity and changed the release profile to one approaching zero-order, while a water-insoluble additive decreased the rate of drug release, making it more similar to the erosion profile. The additives affected the porous structure in addition to polymer erosion, and the rate of drug diffusion was determined by the porous structure.

Polymer–drug interactions were found to affect drug release in a study on the release of amoxicillin from cylinders of PLG or PLA. Polymers with different molecular weights and L:G ratios were studied together with measurements of erosion, decrease in M_w, and nuclear magnetic resonance (NMR) (Mollo and Corrigan, 2003). The analyses showed that the presence of the drug decreased the rate of hydrolysis, and the effect was greater for the high-M_w and lactide-rich polymers. The fraction of intact drug released increased with polymer M_w and with the drug load.

These findings indicate that the drug, or its degradation products, may bind or cross-link to the polymers. This phenomenon was less pronounced with higher-M_w PLGs, as there are fewer polymer chain end groups, and at higher drug load, as there were probably more drug molecules than polymer end groups. Interactions between the drug and PLG were supported by NMR.

Drug release seemed to be predominantly governed by connected channels formed by the presence of the drug, in a study of the release of ovalbumin (OVA) from PLG microparticles (Zhao and Rodgers, 2006). A rapid release phase was followed by a period of zero-order release, and there was no lag phase, which is common for 75:25 PLG with an M_w of 68 kDa, as it swells and degrades slowly (Fredenberg, 2004). Initial high porosity would cause rapid release. However, the surface of the microparticles seemed to be non-porous when analyzed with SEM. OVA was stained and its location within the microparticles was monitored during drug release using transmission electron microscopy (TEM). It was found that connected pores filled with OVA existed initially (Figure 6). These pores may initially have been too small for detection using SEM analysis, and there may have been few connections with the surface. This investigation showed that swelling and degradation/erosion did not play an important role in drug release. It was also found that the protein distribution was not completely homogeneous initially, and release from some parts of the particle was faster than from others.

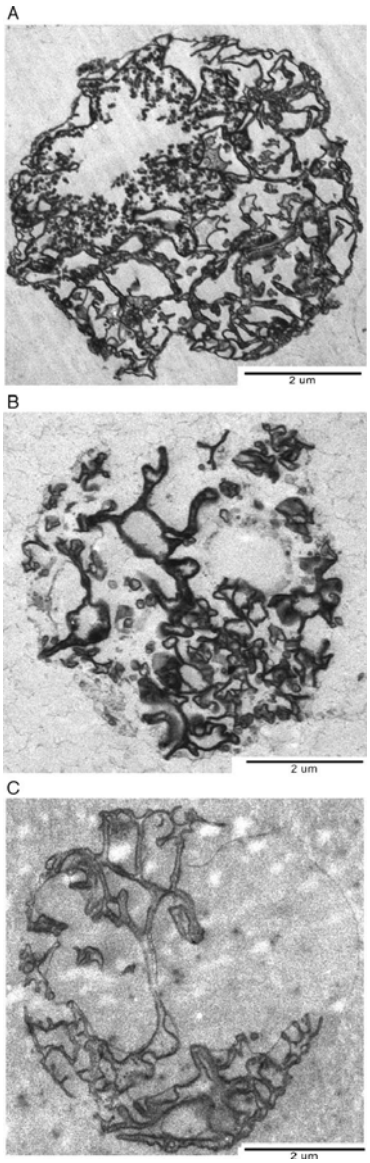


Figure 6. TEM images of OVA-loaded microparticles during degradation. The samples were stained with osmium and then post-stained with a mixture of uranyl acetate and lead citrate before TEM analysis. The 80 nm slice was cut from approximately half the diameter of the particle. Protein distribution is represented by dark areas. (A) 20 days; (B) 40 days; (C) 60 days. Originally published by Zhao and Rodgers, Using TEM to couple transient protein distribution and release for PLGA microparticles for potential use as vaccine delivery vehicles, J. Control. Release, 2006, vol. 113, pp. 15-22.

The transport resistance and rupture of the shell structure seemed to govern the release of cisplatin from microparticles consisting of a core of PLG and cisplatin, and an outer shell of PLA (Matsumoto et al., 2006). Different release profiles were found depending on the M_w of the PLA shell. An almost-zero-order drug release, which was identical to the profile of the erosion of the PLG core, was found for 10 kDa PLA. This result alone indicates erosion-controlled drug release. However, when the shell consisted of a mixture of 10 and 110 kDa PLA (5:1), the drug release surprisingly increased between days 4 and 7. This mixture absorbed less water, as measured in the study, and is known to degrade slower. However, microscopic studies revealed rupture of the higher-M_w shell, probably due to high water absorption and swelling of the PLG core. The ruptures were observed at the same time as the drug release increased. These findings suggest that the shell constituted a significant part of the total transport resistance. The transport resistance in the PLA shell, in contrast to the degradation kinetics of the PLG core, may have determined the release of PLG core degradation products, which would result in the similar profiles for drug release and PLG erosion. The molecular weight of cisplatin (300.1 Da) is in the same range as water-soluble PLG degradation products (up to 1100 Da), which means that the rate of diffusion should be similar, based on the M_w. In a similar study, PLA shells of a different M_w that did not rupture were used (Matsumoto et al., 2005). From studies of drug release, polymer erosion and SEM analysis, drug release was concluded to consist of four steps: (i) a burst of drug molecules at the surface, (ii) drug release through pores in the PLA shell, (iii) erosion of the PLG core and (iv) diffusion through more effective pores in the PLA shell.

Studying processes that enhance or inhibit drug release could, of course, also be combined with mathematical modeling in order to elucidate the true and rate-controlling release mechanisms. Faisant et al. (2002) used different analyses, such as differential scanning calorimetry, size exclusion chromatography and SEM, to identify the processes taking place before developing a mathematical model that allowed the quantitative description of drug release.

The third technique that can be used to obtain a mechanistic understanding discussed in this section, i.e. studying specific processes that may influence drug release, has the advantage that it provides detail knowledge on drug release. The disadvantage of this technique is that

pre-knowledge regarding the system may be required in order to decide what to study. As with mathematical modeling, identifying some processes that explain drug release does not exclude the possibility of other processes affecting the system. However, as in the case with mathematical modeling, when there is agreement between different results, for example, a simultaneous increase in the rate of drug release and the appearance of cracks, it is very unlikely that another unobserved process governs drug release. When there is no detailed knowledge of the system, it is a good idea to perform a general analysis of the DDS and follow, for example, drug release, degree of native drug release, water absorption, polymer M_w, erosion, the porosity, and the size and shape of the DDS. Most of these analyses are inexpensive and easy to perform. The knowledge gained is important as it contributes small pieces to the complex puzzle of drug release and is helpful in pharmaceutical development.

5. True and rate-controlling release mechanisms

As mentioned in Section 2, many physico-chemical processes have been reported as the dominating release mechanism or rate-determining process. One reason for this is the different use of the term "release mechanism" by researchers. Another reason is the complexity of drug release from PLG-based DDSs, as discussed in Section 3 and illustrated in Figure 2 and 3. The true and rate-controlling release mechanisms are discussed in this section, and examples of studies that support or disprove them are given.

5.1. True release mechanisms

As mentioned in Section 2, there are four true release mechanisms:

- diffusion through water-filled pores,
- diffusion through the polymer,
- osmotic pumping, and
- erosion (no drug transport).

Diffusion through water-filled pores has been mentioned as the release mechanism countless times (Gao et al., 2007; Yushu and Venkatraman,

2006; Zidan et al., 2006). In many studies, this release mechanism has only been used to describe the first stage of the release period, before the onset of polymer erosion (Alexis et al., 2004; Johnson et al., 1997; Lam et al., 2000). However, diffusion describes the *way* in which the drug is released, while in these cases erosion is a process that influences the *rate* of diffusion. There are also examples of complete drug release before any significant polymer erosion (Liu et al., 2005; Patel et al., 2008; Sansdrap and Moës, 1997). The burst release phase is sometimes said to be diffusion dependent. In a study on the release of a highly water-soluble drug from microspheres, the drug release was reported to be proportional to the square root of time, during the burst phase, which is indicative of diffusive transport (Lee et al., 2002).

Diffusion through water-filled pores is very dependent on the porous structure of the polymer, and is therefore dependent on the processes that promote pore formation and pore closure. The effective diffusion coefficient is dependent on the diffusion coefficient in the fluid in the pores, the porosity and the tortuosity (Cussler, 1997). Pores must also be continuous from the drug molecule to the surface of the DDS and sufficiently large for the solute to pass through. Dead-end pores, too small pores and the degree of connection between pores influence the porosity and the tortuosity. Constant diffusion coefficients for drugs encapsulated in PLG-based DDSs are more likely to be found in cases of small and initially porous particles consisting of high-M_w, hydrophobic and slowly swelling and degrading PLGs, with low polymer chain mobility. Pore-forming processes, i.e. erosion and swelling, will have greater effects on low-M_w and less hydrophobic PLGs, and on large or non-porous particles. For example, in a study of the release of human growth hormone (hGH) encapsulated in porous microspheres of slow-degrading semi-crystalline PLA, it was found that hGH was completely released before any significant erosion had taken place. However, the release of hGH encapsulated in non-porous PLG particles was slower and more dependent on pore-forming processes (Kim and park, 2004). Mathematical modeling has been used to confirm purely diffusion-controlled drug release from PLG-based DDSs. In a study on the release of pyranine encapsulated in a core of tri-glycerides, and coated with PLG, the release was found to be purely diffusion-controlled, as the release profile could be described by an analytical solution of Fick's second law for cylinders, after a lag phase of 20 days (Guse et al., 2006b). The lag phase could be due to the time before polymer erosion or water

absorption had formed continuous pores of sufficient size. However, as the release could be described using a constant diffusion coefficient, further enlargement or formation of more pores did not seem to be dominating processes. Perhaps the rates of pore formation and pore closure were equal. In another study of 5-fluorouracil release from PLA fibers, diffusive drug release was also concluded by the use of mathematical modeling. Polymer erosion was much slower than drug release in this study (Gao et al., 2007).

There are many different factors that may influence the rate of drug diffusion. However, as long as the drug molecules are released due to transport, diffusion through water-filled pores is the true release mechanism throughout the whole release period, unless diffusion takes place in the polymer or the drug transport is driven by osmotic pressure. As the encapsulated drug is often a large hydrophilic molecule, not able to diffuse through the polymer, and the osmotic pressure is often compensated for by polymer swelling, diffusion through water-filled pores is usually the main true release mechanism.

Diffusion through the polymer is possible for small hydrophobic drugs (Raman et al., 2005; Wiscke and Schwendeman, 2008). For example, the small hydrophobic drug ropivacaine, was completely released from PLG microspheres *in vivo* after 8 hours, which is before the onset of polymer erosion. Some of these molecules could have been detached from the surface, but it is unlikely that none of the drug molecules would have been properly encapsulated using the spray-drying method (Ratajczak-Emselme et al., 2009).

Unlike diffusion through water-filled pores, diffusion through the polymer is not particular dependent on the porous structure. However, the drug must be dissolved in water before being released, and this process could decrease the overall release rate. High porosity increases the surface area for drug dissolution and could thus enhance drug release. Kang and Schwendeman (2003) used confocal microscopy to determine the diffusion coefficient of bodipy, a small hydrophobic molecule, which partitioned strongly to the polymer. The diffusion coefficient did not increase as PLG degraded or when the pore-forming substance $MgCO_3$ was encapsulated together with bovine serum albumin (BSA). However, the diffusion coefficient varied considerably with the temperature. These results clearly indicate that most diffusion took place

in the polymer, although pores were created and diffusion was faster in the pores. The strong partitioning of bodipy to the polymer explained the constant diffusion coefficient, and it was concluded that the porous structure was not important.

The rate of diffusion through a polymer is very dependent on the physical state, and for a small molecule, may increase by several orders of magnitude at the transition from the vitreous to the rubbery state (Karlsson et al., 2001). T_g is above 37°C for the original polymer. However, upon immersion in water at 37°C, the plasticizing effect of water usually transfers the polymer into the rubbery state (Blasi et al., 2005; Ricci et al., 2005). A very high-M_w PLG may remain in the vitreous state for a while before degradation and water absorption affect the polymer. The glass transition temperature of PLG in a DDS may also be lower than that of the original polymer due to degradation during the manufacturing process and the plasticizing effects of additives or residual water (Passerini and Craig, 2001; Spenlehauer et al., 1989). Drug diffusivity through the polymer is often higher in lower-M_w polymer, as the polymer chains are more flexible (Faisant et al., 2002; Ricci et al., 2005; Wiscke and Schwendeman, 2008). As mentioned in Section 4.2, different mathematical relationships have been found between the diffusivity and polymer M_w. It has also been reported that PLG microparticles may undergo structural relaxation after transition to the vitreous state during manufacturing, which means that the polymer chains become closer, and the microparticles become denser (Allison, S.D., 2008). This process, which often takes place during storage, may be a source of batch-to-batch variability. As in the case of diffusion through water-filled pores, the diffusion coefficient will be less variable in high-M_w PLGs, with small particles. Degradation will play a greater role in low-M_w PLGs, and with large particles. For example, the release of estradiol by diffusion through low-M_w PLGs was found to follow zero-order, as a result of increasing diffusion coefficient due to degradation. The use of high-M_w PLGs lead to release profiles which could be described by the Higuchi equation, i.e. a constant diffusion coefficient (Mittal et al., 2007).

Osmotic pumping is a phenomenon that occurs when osmotic pressure, caused by water absorption, drives the transport of the drug. The nature of the transport is then convection and not diffusion, as discussed in Section 2. This release mechanism is more common for DDSs using

materials other than PLG. However, there have been some reports of osmotic pumping from PLG-based DDSs. One example is a hollow cylindrical DDS of PLA using polyethylene glycol (PEG) as a porogen to create pores (Jonnalagadda and Robinson, 2000). The cylinder was filled with either 5 fluorouracil (5FU) or fluorescein isothiocyanate (FITC) dextran and then sealed with a viscous PLA solution. Release of 5FU followed an equation describing diffusion-controlled transport. However, the release of FITC dextran was not dependent on the dextran M_w, as is the case for diffusive transport, and a linear relationship was seen between the release rate and the osmotic gradient. From the analyses of this DDS it was concluded that the system functioned mainly as an osmotic pump. Another example is a DDS of PLG (85:15) containing a reservoir space filled with the drug basic fibroblast growth factor (bFGF) and the osmotic agent PEG (Ryu et al., 2007). Narrow channels connected the reservoir and the surface of the DDS. Water was taken up through the channels and an osmotic pressure was built up in the reservoir as PEG was dissolved, and this pumped the drug out through the channels. Osmotic transport was found to depend on the length of the channels, while diffusive transport depended on both the length and the area. Osmotic transport dominated when the channels were longer than 60 μm. In both of these examples, osmotic agents and pores or channels were used for drug transport. A requirement for transport to be driven by osmotic pressure is that the influx and efflux of water are equal, after an initial period of water content adjustment. The polymers used in both examples were very hydrophobic, as the L:G ratios were high (100:0 and 85:15), and the molecular weights were very high (324 kDa and inherent viscosity (IV) = 2.3). The rate of water absorption through such polymers and thus the swelling are minimal, and it is possible to maintain an equal water influx and efflux and osmotic pressure. These polymers degrade very slowly, and degradation and erosion are negligible during the drug release period. However, most PLG-based DDSs consist of lower-M_w PLGs, which swell significantly sooner or later, and any osmotic pressure will then be compensated for by the increase in volume. Osmotic pressure caused by water absorption may result in rupture of the polymer. However, osmotic pumping is not a common release mechanism for PLG-based DDSs.

Erosion, as a true release mechanism, i.e. drug release without drug transport, results in identical profiles of drug release and polymer erosion, assuming that the drug is homogeneously distributed

throughout the DDS. Identical drug release and polymer erosion has been reported although such reports are rare. In a study on the release of testosterone and BSA from PLG films, the drug release profiles were identical to the polymer mass loss profile (Figure 7), at least up to 60% release of BSA, after which the release of BSA leveled off (Shah et al., 1992). In another study, the release profiles of levamisole from PLG discs and polymer erosion were reported to be almost identical (Fitzgerald and Corrigan, 1996). As mentioned in Section 2, degradation/erosion is frequently reported as a *rate-controlling* release mechanism, i.e. the process controlling the rate of diffusion, often during the final period of drug release (Grayson et al., 2004; Wang L et al., 2004; Westedt et al., 2006; Zilberman and Grinberg, 2008). Polymer erosion could cause drug molecules very close to the surface to be released without transport, and the release mechanism would then be erosion. However, as hydration is normally much faster than erosion, it is more probable that the drug will diffuse through pores formed by water absorption. Erosion could be the main release mechanism for low-M_w PLG formulations, in which a significant part of the polymer has a molecular weight just above the limit for water solubility. However, as remnants of the polymer are commonly reported after complete drug release (Cleland et al., 1997; Faisant et al., 2002), erosion is rarely the dominating true release mechanism.

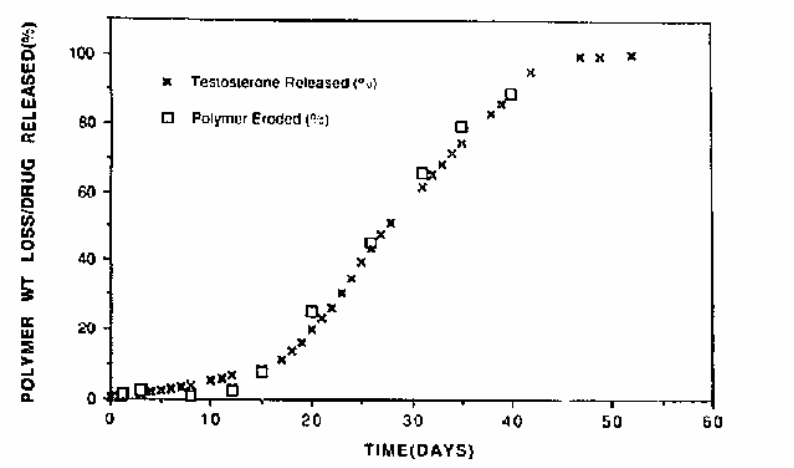


Figure 7. Comparison of the release of testosterone and polymer erosion of PLG films. Originally published by Shah et al., Poly(glycolic acid-co-DL-lactic acid): diffusion or degradation controlled drug delivery? J. Control. Release, 1992, vol. 18, pp. 261-270.

The encapsulated drug may be released by more than one true release mechanism simultaneously, and the dominating mechanism may change with time. Diffusion is not only dependent on the diffusion resistance inside the polymer matrix. Diffusion through an unstirred layer surrounding the DDS may lead to a significant transport resistance. The concentration in the surrounding bulk can also inhibit drug diffusion from the DDS. Sink conditions and sufficient stirring are of special concern for hydrophobic drugs with low water solubility, as the concentration may easily reach saturation (Wiscke and Schwendeman, 2008).

5.2. Rate-controlling release mechanisms or processes that enhance or inhibit drug release

Water absorption or *swelling* occurs immediately upon immersion in water or administration *in vivo*. This has been found to create pores in the polymer matrix (Mochizuki et al., 2008; Webber et al., 1998), increasing the rate of drug diffusion. However, swelling may also cause pore closure in low-M_w and relatively hydrophilic PLGs with high polymer chain mobility, as swelling may enable the rearrangement of the polymer chains and the formation of a homogeneous swollen polymer mass without distinct pores (Fredenberg et al., in press). Water absorption causes hydrolysis, but it also increases the pH inside a DDS and reduces the acid catalytic effect on hydrolysis. Swelling was found to cause burst release in a study in which drug release was monitored using confocal microscopy, pore size was analyzed using SEM and the diffusion of water was measured using NMR (Messaritaki et al., 2005). Desai et al. (2010) found a relationship between drug release and water absorption. In a study on the effect of $Mg(OH)_2$ on the release of BSA from PLG millicylinders, it was found that the salt increased the release rate, due to increased water absorption and porosity (Zhu and Schwendeman, 2000). However, water absorption does not always have a significant effect on drug release. Song et al. (1997) found no direct correlation between the water absorption capacity and drug release.

Dissolution of the drug could determine the rate of release, if it is slower than the rate of transport. Wong et al. (2001) found that a model describing both drug diffusion and dissolution fitted the experimental

data for human immunoglobulin G release from PLG microspheres during the first 50 days better than a model describing diffusion only. Dissolution is, however, rarely reported as the rate-controlling process, probably as encapsulated drugs have, to date, usually been relatively hydrophilic, for example, proteins and peptides. However, the trend towards more pharmaceutical substances of very low solubility will make the dissolution process more important.

Hydrolysis has been found to be important regarding drug diffusion through the polymer (Charlier et al., 2000; Raman et al., 2005). The release rate and the diffusion coefficients have been linked to the M_w in several studies, as described in Section 4.2. In a study on the release of hGH encapsulated in PLG films, which normally takes place in water-filled pores, mathematical relationships were found between the polymer M_w and the release of hGH (Santoveña et al., 2006). Relationships could only be established for a certain period of the drug release, and these periods differed for different formulations. During diffusion through water-filled pores, it is likely that hydrolysis affects another process which, in turn, affects the rate of diffusion, for example, water absorption and erosion. Both these processes are pore forming, and depend on the M_w of the polymer. Diffusion through the polymer depends on polymer chain mobility and density, which are affected by the M_w. Hydrolysis is a process that strongly influences other processes that may enhance or inhibit drug release, as shown in Figure 2, and discussed further below.

Heterogeneous degradation due to the auto-catalytic effect is well known. As mentioned in Section 4.3, Park (1995) reported two glass transition temperatures, one originating from the rapidly degrading interior, and one originating from a slowly degrading region close to the surface. The former decreased with time while the latter remained constant. This surface layer did not become porous as the interior, and acted as a diffusion barrier, until the barrier appeared to have burst due to the build-up of osmotic pressure. The formation of a less porous layer, due to heterogeneous degradation, thus controlled drug release. The microspheres investigated were about 10 μm in diameter, and heterogeneous degradation, with a porous interior and a less porous surface layer, has also been observed in thin films 10 μm thick in another study (Lu et al., 1999). Berkland et al. (2007) found a surprisingly slow release of fluorescein-dextran from non-porous PLG microspheres,

which is attributable to heterogeneous degradation. The interior became hollow, while the surface remained non-porous, or showed low porosity. This morphological development was also observed in another study on the release of a hydrophobic model drug (Mao et al., 2008).

Changes in polymer chain mobility and density affect the rate of diffusion through the polymer, as discussed in Section 5.1. Polymer chain mobility and density is affected by hydrolysis and plasticization of the polymer, and by crystallization of oligomers trapped inside the matrix.

The crystallization of oligomers decreases the rate of transport of the drug, the dissolved degradation products and water. The crystallization of oligomers has been reported to occur (Vert et al., 1991) but, to the best of our knowledge, no study has been performed demonstrating that this process actually determines the rate of drug release.

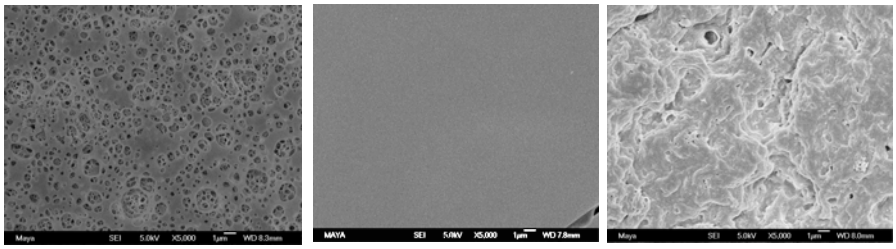
Erosion, or polymer mass loss, has been reported to start at an average M_w of 15 kDa (Husmann et al., 2002). Erosion as a true release mechanism has been discussed in Section 5.1. Erosion as a rate-controlling release mechanism leads to pore formation, which increases the rate of diffusion. Dissolved degradation products trapped inside the DDS can affect the processes influencing drug release in many ways, for example, by catalyzing hydrolysis, by increasing the rate of water absorption due to increased osmolality, and by plasticization of the polymer. As these degradation products are lost during erosion, so are their effects, which means that erosion could theoretically inhibit drug release. However, the dominating effect of erosion is increased drug release and, as mentioned in Section 2, there are numerous reports of erosion governing drug release, especially during the later part of the release period.

Pore formation is a process governed by water absorption and polymer erosion, as mentioned in Section 3, or may be caused by the release of a porogen. The rate of drug release from PLA films has been found to be associated with the presence of open pores at the surface (Mochizuki et al., 2008). In a study on the effect of morphology on drug release, porous, non-porous and porous particles with covered pores at the surface were prepared. Drug release was found to be governed by the initial porous structure during the first period of drug release (Bae et al., 2009). Pore formation is an important process, as the encapsulated drug

is often a large hydrophilic substance, usually released by diffusion through water-filled pores.

Pore closure has been observed in several studies, and is likely to affect the release rate. This phenomenon has been demonstrated, by the use of confocal microscopy together with fluorescent probes and SEM analyses, to be the explanation of the cessation of burst release (Wang et al., 2002). Kang et al. (2007) suggested that pores may open and close during the release period, and thus alternately trap and release drug molecules. They argued that the diffusivity in water should lead to rapid drug release, even for large molecules, due to the short diffusion pathways in microspheres, even those with high tortuosity. Disregarding polymer–drug or drug–drug interactions, diffusion through water-filled pores can be inhibited by low porosity, insufficient pore size (Fredenberg et al., 2004) or pore closure. In one of our recent studies, the diffusion of glucose, a small, inert hydrophilic molecule, through a highly swollen PLG film was found to be very slow (Fredenberg et al., 2011). The explanation of this slow diffusion therefore seems to lie in the transport properties of the DDS, and not in the properties of the diffusing molecule. Pore closure is related to polymer chain mobility and rearrangement. Examples of different factors that have been found to induce or affect pore closure, and also polymer chain mobility, are polymer degradation, plasticizing agents and increased temperature (Badri Viswanathan et al., 2001; Berkland et al., 2003; Bouissou et al., 2006; Huang et al., 2007; Kang and Schwendeman, 2007; Okada, H., 1997). The collapse of porous microparticles, and thus pore closure, has been observed when the (constant) incubation temperature had reached the so-called critical softening point, which was 10-20°C higher the decreasing T_g (Friess and Schlapp, 2002). In one of our studies we observed pore closure at the surface of porous PLG films being degraded under different conditions. Pore closure was especially rapid at low pH (3.0) (see Figure 8) (Fredenberg et al., in press). The pH may be important as it may be low inside PLG matrices, and *in vitro* and *in vivo* (Anderson and Shive, 1997; Díez and de Ilarduya, 2006; Ding and Schwendeman, 2008; Sastre et al., 2007). The polymer contracted and separated from water at low pH, and we suggested that pore closure was caused by a hydrophobic effect, due to the higher hydrophobicity of PLGs with a low degree of polymer carboxyl acid dissociation at low pH. The more hydrophobic nature of the polymer was confirmed by measurements of water absorption and wettability (contact angles). This

result is in agreement with findings in a study on burst release from microspheres incubated in a buffer of pH 4. Water absorption was slower, pore closure was more rapid, and the burst release was decreased upon co-encapsulation of a small amount of glucose in porous microspheres (Wang J et al., 2004). According to the authors, polyols are known to increase the surface tension of water which, according to our findings, was part of the mechanism of pore closure at low pH. Our findings could also explain the results of a study on the release of Huperzine A from PLG microspheres. The rate of drug release and the rate of water absorption were slower during incubation in a buffer of pH 4.0 than of 7.4 (Liu et al., 2005). In our study, pore closure also occurred at pH 7.4, although it was slower. At pH 7.4, the polymer was more hydrophilic and swelled considerably. We suggested that pore closure was caused by the diffusion of mobile polymer chains, forming a homogeneous, swollen polymer–water mass, instead of distinct regions of either polymer or pores. It should be noted that the M_w of this PLG was relatively low, and that the rate of pore closure, or lack of detectable closure, was related to the M_w and the degree of hydrophobicity of the polymer (Fredenberg et al., in press). Berkland et al. found a surprisingly slow release of BSA from initially porous microspheres. The microspheres became hollow with time, while the pores at the surface closed. This was probably one reason for the slow drug release, although drug–polymer interactions or drug–drug interactions could not be ruled out (Berkland et al., 2007). Pore closure and pore formation are two simultaneously ongoing processes, and in our study we found that pore closure occurred rapidly at pH 3.0 and pH 7.4, while pore formation dominated at pH 5-6 (Fredenberg et al., in press). The complexity of the processes taking place in PLG matrices result in microenvironmental heterogeneity throughout the matrices. The difference in polymer chain mobility and pH may be the cause of porous and non-porous regions. Another factor that may affect the processes on a submicron level is the curvature at polymer–water interfaces, which are known to affect physico-chemical properties, such as solubility, according to the Ostwald ripening phenomenon (Ratke and Voorhees, 2002)



A B C

Figure 8. Porosity at the surface of PLG films after two days of pore-forming pre-treatment (A), and after five more days of degradation at pH 3.0 (B) or pH 7.4 (C). The left picture constitutes a part of a figure in a paper by us that has been accepted for publication in J. Control. Release (Fredenberg et al., in press, DOI 10.1016/j.jconrel.2010.11.020).

Polymer–drug interactions have been found to influence the release rate (Okada, H., 1997). In two separate studies, the release of L-asparaginase in one and the release of BSA in the other, were found to be slower from nanoparticles of an uncapped polymer than from a capped, but otherwise identical PLG, although capping decreased the rate of degradation (Blanco and Alonso, 1997; Gaspar et al., 1998). This was attributed to the interaction between the drugs and the uncapped terminal carboxyl groups. Ionic interaction between lidocaine and PLG was also proposed as the probable explanation of the slower release of lidocaine than ibuprofen in a study on PLG particles and films (Klose et al., 2008). The adsorption of drug molecules to the polymer is undesirable, as it may lead to incomplete release (Butler et al., 1999; Crotts et al., 1997). A protein may also lose its biological function due to chemical reactions, such as deamidation and acylation in acidic environments (Houchin et al., 2006; Ibrahim, M.A., 2005; Zhang et al., 2009). Ketoprofen was found to plasticize PLG by hydrogen binding (Blasi et al., 2007), which may enhance or inhibit drug release. As the environment inside PLG matrices varies with time and position, so may the degree of polymer–drug interactions. These interactions may be responsible for the release of only a certain proportion of the drug molecules and perhaps only for part of the release period.

Drug–drug interactions, such as the formation of physical or covalent aggregates, have been suggested to be the cause of slower and incomplete drug release (Wong et al., 2001; Zhu and Schwendeman, 2000). Such aggregates are also the result of an acidic environment (Kan

et al., 2008). As in polymer–drug interactions, the influence of drug–drug interactions may vary with time and position according to the microenvironment.

The formation of cracks in the DDS may affect the release rate. Rapid water absorption could result in polymer rupture, which should of course increase the release rate. The above mentioned study regarding heterogeneous degradation, during which a surface diffusion barrier was formed, is another example of the probable formation of cracks. The surface barrier allowed water penetration, and then seemed to disappear (Park, 1995). Another example is the study mentioned in Section 4.3, in which rupture of the PLA shell surrounding the drug–PLG core increased the rate of drug release. Before rupture, drug release followed the course of polymer erosion (Matsumoto et al., 2006).

Collapse of the DDS may enhance drug release, as new surfaces may be created and fragments of the DDS may fall off (Friess and Schlapp, 2002). It may also inhibit drug release, due to a decrease in porosity (Díez and de Ilarduya, 2006). Collapse is often the result of degradation and the decrease in T_g, and is often associated with particle aggregation (Park et al., 1995). Aggregation could lead to slower drug release due to a decrease in surface area, or faster drug release, as the acid gradient and the catalytic effect on degradation would increase. However, it is not obvious that aggregated particles are particularly densely packed. The diffusion pathway of high transport resistance may still be short, and the surface area for drug release inside the agglomeration of aggregated particles may still be sufficient, and therefore only have a minor effect on drug release.

Many of the rate-controlling release mechanisms may affect drug release simultaneously, and the dominant mechanism may alter during the release period. The dominant mechanism may also differ between different microparticles in the same system. Particles of different sizes are prone to different degrees of auto-catalytic degradation. Cracks may be formed on some particles but not on others. As mentioned in Section 4.3, the release rate of OVA from different regions of a microparticle differed, according to TEM (Zhao and Rodgers, 2006). This demonstrates the heterogeneous nature of PLG matrices. When a process is taking place at a particular place in the matrix, the effect will

be local, and as one process may influence others, regions with different characteristics may arise.

Some of these processes affect drug release in more than one way. For example, hydrolysis leads to erosion and pore formation, and thus an increase in drug release. However, hydrolysis also leads to a lower T_g, possible rearrangement of polymer chains, and pore closure, and thus possibly a decrease in drug release. More opposing effects are given in Table 4. The impact of one process on drug release may be altered when other processes or the environment are changed. For example, the solubility of the drug, drug–drug interactions, polymer–drug interactions, hydrolysis, pore formation and pore closure, all depend on the pH, which depends on the rate of hydrolysis, water absorption and transport out of the system. The different factors that influence these processes, sometimes in more than one way, add to the complexity. For example, a soluble basic salt with divalent cations may: (i) decrease the rate of hydrolysis by neutralizing acids, (ii) create pores due to the pore-forming effect of divalent cations, probably caused by the catalysis of hydrolysis, (iii) create pores due to water absorption caused by increased osmolality, and (iv) act as a porogen. The situation becomes even more complicated due to the fact that it may be difficult to predict the actual *in vivo* environment. However, the complexity of the system also means that there are many possible ways to solve a particular problem. Each arrow in Figures 2 and 3, demonstrating the complexity, is also a potential way of modifying drug release, and there are thus many ways of obtaining a suitable DDS.

Table 4. Processes that may increase or decrease the rate of drug release

Process	Possible effect	Effect on the release rate
Hydrolysis	Auto-catalysis Erosion and pore formation Plasticizing effect of oligomers	Increase
	Crystallization of oligomers Polymer chain mobility and pore closure Drug–drug and polymer–drug interactions	Decrease
Erosion	Pore formation	Increase
	Loss of catalytic effect of acidic degradation products	Decrease
Water absorption	Hydrolysis Pore formation	Increase
	Increased pH Polymer chain mobility and pore closure	Decrease
Collapse of the polymer structure	Cracks and new surfaces	Increase
	Decreased porosity	Decrease

6. Conclusions and future outlook

PLG has attracted much interest due to its potential as a drug carrier in the controlled release of encapsulated drugs, and is currently the most frequently used biodegradable polymer for this application. It is important to understand the release mechanisms, and which factors that influence the release rate, in order to be able to modify drug release. Many studies have been carried out on this subject. The term release mechanism has been used with different meanings, and the definition of the term has been discussed in this review. The term can refer to the *way* in which a drug is released or to a process that determines the *rate* of drug release. We suggest that processes describing the way the drug is released should be denoted *true* release mechanisms. Processes influencing drug release are important, but should be discussed in terms

of processes or *rate-controlling* release mechanisms, as they provide important information regarding the rate of drug release. True and rate-controlling release mechanisms have been studied in different ways, which are generally based on the shape of the release profile, mathematical modeling or studies on processes that influence drug release. All of these techniques have their advantages and disadvantages. Mathematical modeling gives a rapid general view and fundamental insight into the dominating release mechanism, or the processes influencing drug release. However, as PLG systems are complex, models require a substantial experimental effort for model validation to make full use of the approach. Studying specific processes that influence drug release, for example, polymer erosion, pore closure or polymer–drug interactions, provides detailed knowledge of the system from which conclusions can be drawn regarding the release mechanisms and the dominating processes influencing the release rate. However, this method may be more time consuming than mathematical modeling, and the complexity should be considered when drawing conclusions.

We have discussed the release mechanisms and processes influencing drug release that have been reported in the literature. Controlled drug release from PLG-based DDSs is complex, and many processes that influence drug release affect each other in many ways. The effects of different factors on drug release may vary in time and position through a polymer matrix. There are four true release mechanisms: (i) diffusion through water-filled pores, (ii) diffusion through the polymer, (iii) osmotic pumping, and (iv) polymer erosion (i.e. no drug transport). Diffusion through water-filled pores is the most common, as the encapsulated drugs used so far have mainly been large, relatively hydrophilic biopharmaceuticals, for example proteins and peptides.

The complexity of drug release from PLG-based DDSs makes it difficult to generalize results obtained with specific DDSs. Although research with specific DDSs is necessary for product development, and insuring that controlled-release products actually reach the market, the findings may not be applicable to other DDSs. Simplified systems have the advantage of including fewer parameters, enabling studies on a specific parameter or process which should be applicable in several situations, although the dominant parameter or process may differ.

PLGs with a wide range of physico-chemical properties are commercially available, and it is possible to tailor the release profile by the choice of PLG. PLGs can also be blended with other materials, and formulations can be mixed, for example, formulations displaying a slow sigmoidal release and a faster Fickian diffusive release. It may be difficult to predict drug release due to the complexity of the system, but there are many possible ways of modifying drug release. General, basic and mechanistic research can provide pieces of the full puzzle improving the possibility of rapidly solving problems during the development of controlled-release pharmaceuticals.

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