International Journal of Pharmaceutics 409 (2011) 194–202

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Development of mass transport resistance in poly(lactide-co-glycolide) films and particles – A mechanistic study

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ARTICLE INFO

Article history:
Received 13 January 2011
Received in revised form 26 February 2011
Accepted 28 February 2011

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

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 mass 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 3 days of the degradation period. The results indicated high mass 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 of the reason for transport resistance is important in the development of pharmaceuticals and when modifying the rate of drug release.

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 et al., 2000; Ravivarapu et al., 2000; Zolnik and Burgess, 2008). Other applications of PLG-based formulations are single-shot vaccines (Feng et al., 2006; Jiang et al., 2005; Shi et al., 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

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doi:10.1016/j.ijpharm.2011.02.066

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, and
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., in press). Pore closure is further discussed in Section 3. 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 particle 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 (14,100 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 50 mM 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.

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

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

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 h, 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 3 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):

$$K = \frac{1}{S((1/V_A) + (1/V_B))} \times \frac{\ln((C_{A1} - C_{B1})/(C_{A2} - C_{B2}))}{t_2 - t_1} \quad (3)$$

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:

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{(\varepsilon/\tau)D_{water}} \quad (4)$$

where l is the thickness of the film or filter. The right-hand term in Eq. (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 Eq. (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, 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:

$$\frac{1}{K} = \frac{l_{film}}{D_{e\,film}} + \frac{l_{filter}}{(\varepsilon/\tau) \times D_{e\,film}} \quad (5)$$

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 Eq. (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 2 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.

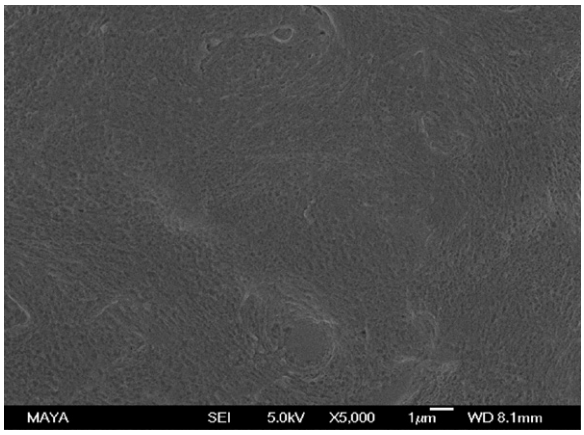

Fig. 1. SEM image of the surface of a thin film after measurements of the diffusion of hGH and glucose. It can be seen that the surface was not porous.

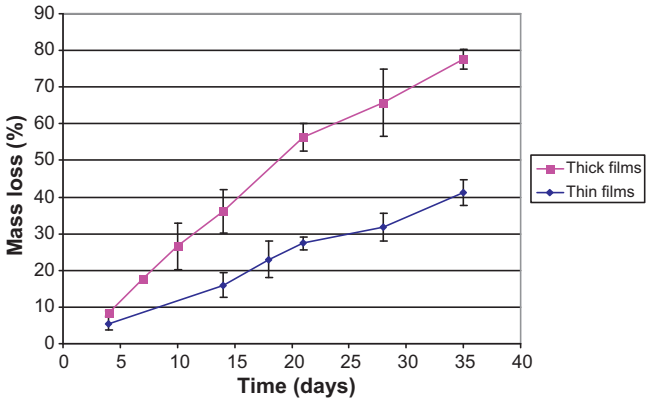

Fig. 2. Mass loss from thick and thin PLG films. The error bars show the standard deviation ($n = 3$).

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 (Fig. 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 (Fig. 2). The rate of diffusion of glucose and

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)	[a]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

[a] He and Niemeyer (2003) and Landolt and Börnstein (1969).

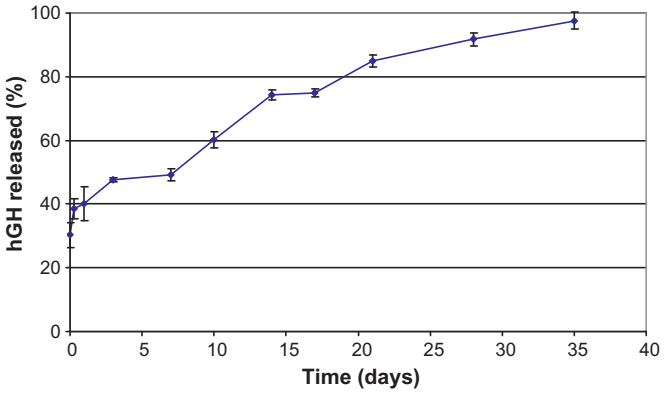

Fig. 3. *In vitro* release of hGH encapsulated in PLG-coated particles. The error bars show the standard deviation ($n = 3$). The manufacturing method used by StratoSphere Pharma AB was modified in order to be able to compare this result to other measurements (see the discussion above).

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 and Westrin, 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 ($70/27 \times 3.5$). 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).

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 Fig. 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).

As can be seen in Fig. 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 autocatalytic hydrolysis in large PLG matrices, which explains the faster mass loss from the thicker films, shown in Fig. 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 Fig. 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 Eq. (6) (Cussler, 1997).

$$M = \frac{D_e}{l} c_{sat} A t \tag{6}$$

M denotes the mass released, D_e is the effective diffusion coefficient and l is 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, the *in vitro* release data from days 1 to 35, 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. Fig. 4 shows that pores were formed close to the surface

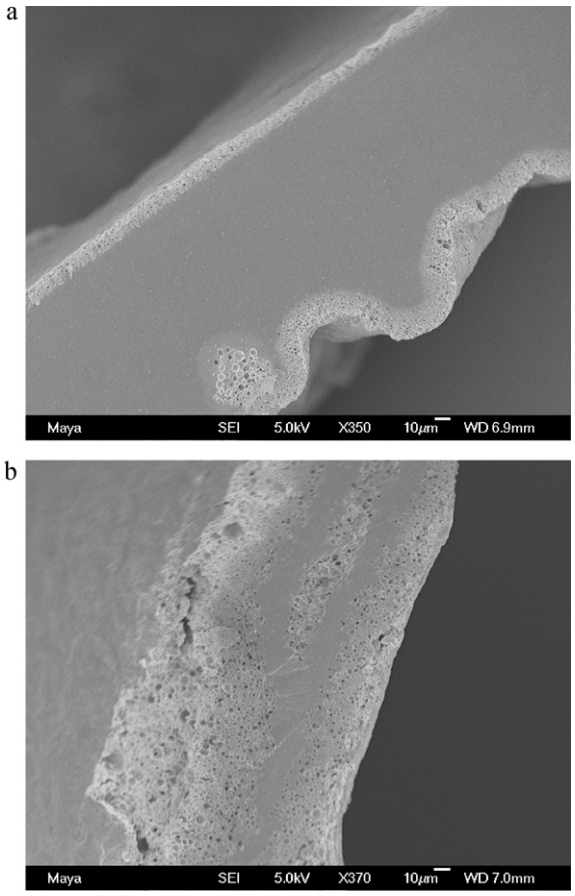

Fig. 4. Pore formation after 4 days of degradation. (a) $ZnCl_2$ dissolved in the buffer resulted in pore formation close to the surfaces. (b) Zinc acetate encapsulated inside the PLG film resulted in pore formation inside the film.

after 4 days in the presence of $ZnCl_2$ in the buffer, while pores were formed inside the film after 4 days when zinc acetate was encapsulated 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 (Fig. 5). These films had lost almost 80% of their polymer mass, according to the mass loss profile in Fig. 2, and contained about six 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

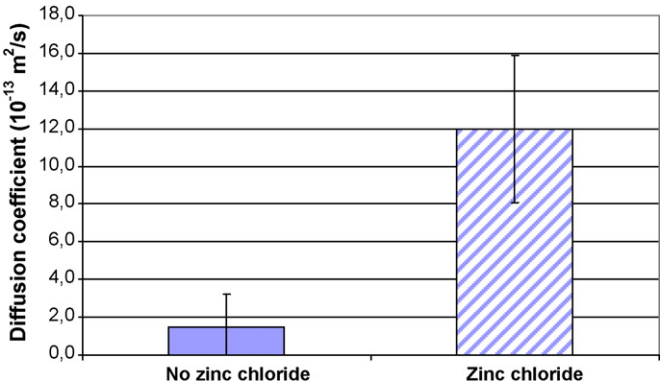

Fig. 5. A significant increase in diffusion coefficient was observed when the surfaces of the PLG films were made more porous by the presence of $ZnCl_2$ in the buffer during the last 3 days of the 35-day degradation period. The error bars show the standard deviation ($n = 3$).

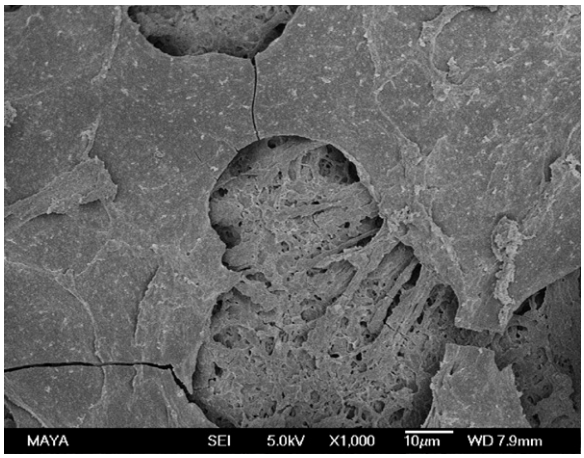

Fig. 6. The difference in interior and surface porosity where a piece of the surface has become detached. This PLG film was degraded for 35 days before diffusion measurements, and was not treated with $ZnCl_2$.

the polymer. The increased porosity at the surfaces increased the diffusion coefficient eight times (Fig. 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 Fig. 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.

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., in press). The PLG samples absorbed a great amount of water and swelled. The pores seemed to be closed due to the diffusion, and thus spreading, 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, 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 Sections 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 (Fig. 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

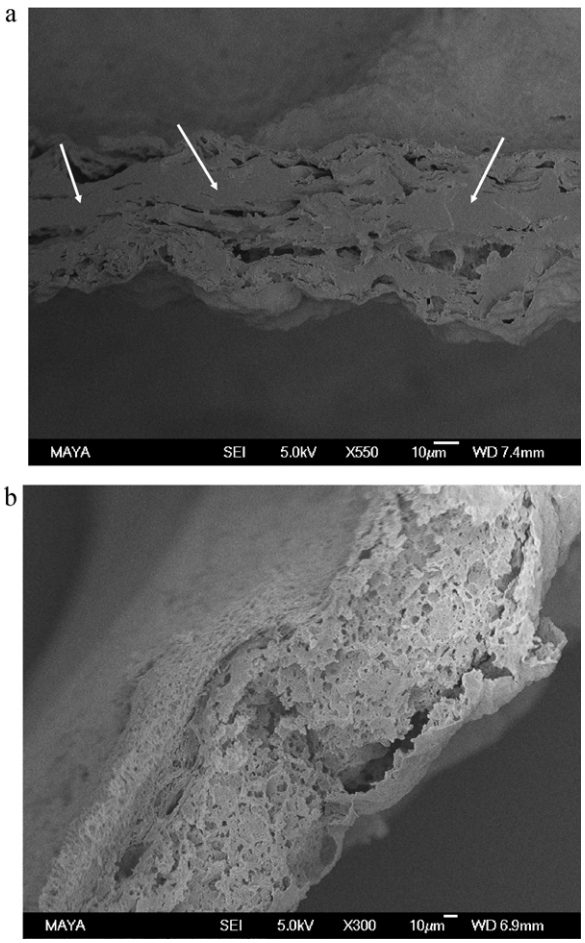

Fig. 7. Cross sections of two PLG films. (a) Non-porous areas are visible after 21 days of degradation (arrows). (b) The cross section was porous after 35 days of degradation.

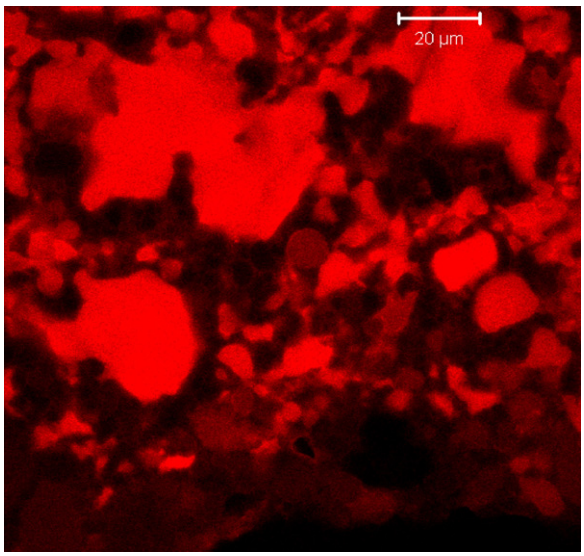

Fig. 8. The location of fluorescent probes attached to 10 kDa dextran within a PLG film. The dark areas show regions into which the probes could not diffuse. In contrast to the cross section views in Fig. 7, this image was obtained along the plane of the PLG film.

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 (Fig. 7).

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 Fig. 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 an anionic 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 Fig. 7a) instead of 35 days (porous interior, according to Fig. 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 Sections 3.1 and 3.2.

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 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. The hydrophobic nature of the polymer at low pH, due to non-dissociated carboxyl acids, may cause the contraction of the polymer. In our previous study (Fredenberg et al., in press), pore closure was found to be rapid and extensive at pH 3.0. Porous PLG samples, which absorbed a large amount of water at pH 7.4, hardly absorbed any water at pH 3.0, and they contracted instead of swelling. It is likely that the polymer chains were rearranged during this contraction and separation from water, and that pores were closured. Which one of the processes pore formation and pore closure 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 10 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 DDSs of different sizes. SEM and diffusion studies based on confocal microscopy could be used to study regions of high mass transport resistance in different forms of DDSs. 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.

Acknowledgements

The authors would like to thank the Swedish Research Council for financial support. We also want to express our gratitude to the Division of Microbiology, Immunology and Glycobiology at Lund University for kindly allowing us to use their confocal microscope. Thanks also to Maria Trulsson for help in the use of the microscope.

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