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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).

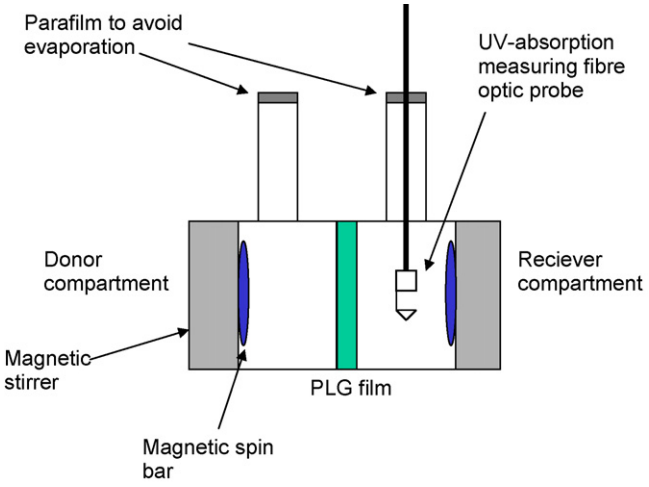

Fig. 1. The diffusion cell and the UV-absorption measuring fibre optic probe.

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).

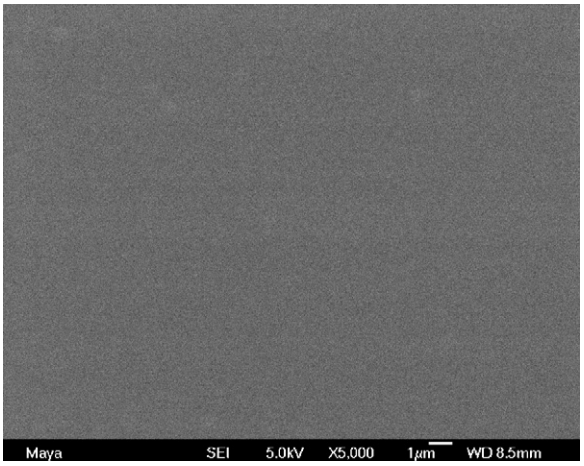

Fig. 2. The surfaces of the sprayed PLG films were smooth and non-porous before incubation in buffer.

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.

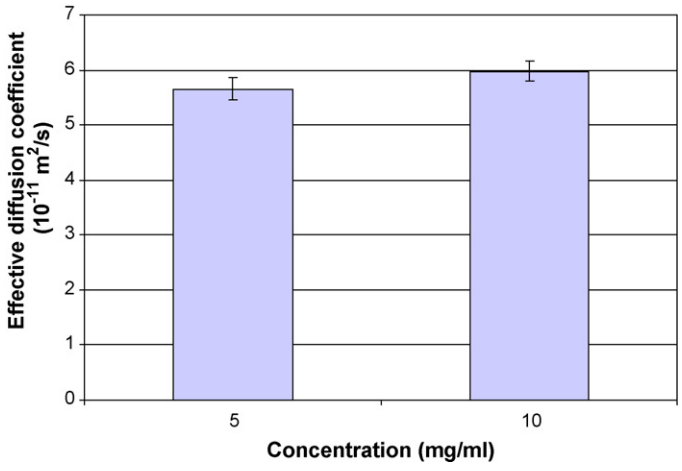

Fig. 3. The effective diffusion coefficient of lysozyme did not depend on the concentration.

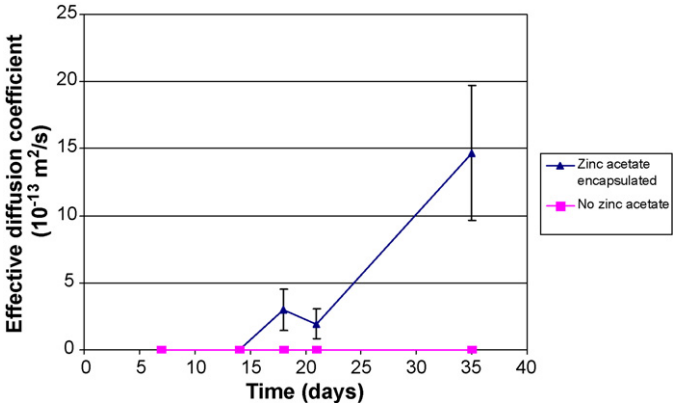

Fig. 4. Encapsulated zinc acetate increased the effective diffusion coefficient of lysozyme after 14 days of degradation. There was no measurable diffusion at 7 and 14 days of degradation. The error-bars show the standard deviation.

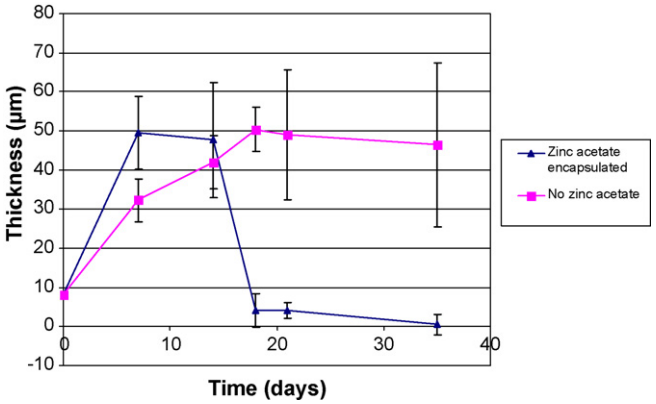

Fig. 5. The thickness of the PLG films with and without zinc acetate.

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).

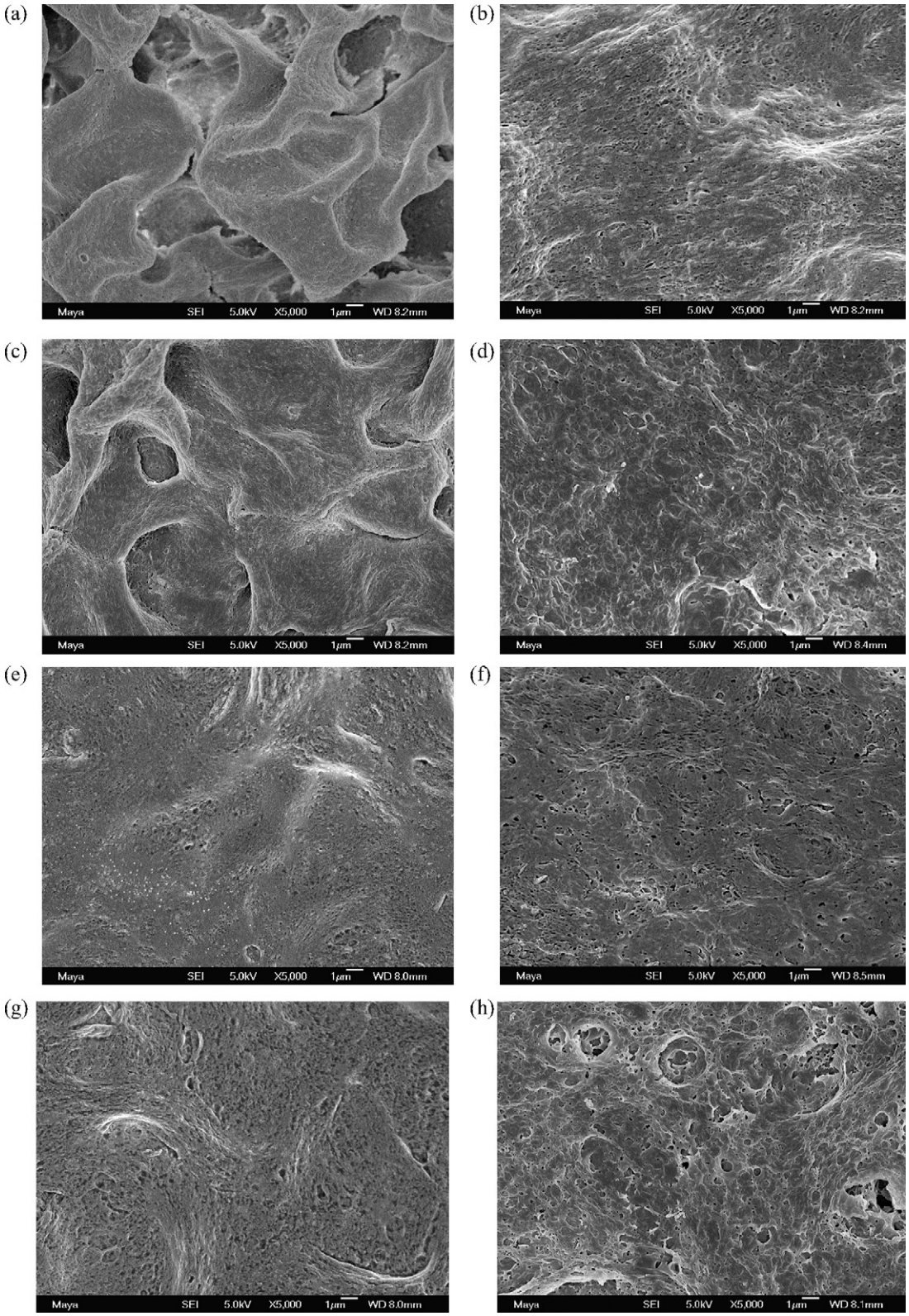

Fig. 6. The porosity of the surface of PLG films after diffusion measurements. The left column: no zinc acetate. The right column: zinc acetate encapsulated. (a and b) 7 days, (c and d) 14 days, (e and f) 18 days, (g and h) 21 days and (i and j) 35 days. PLG films without zinc acetate contained cavities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

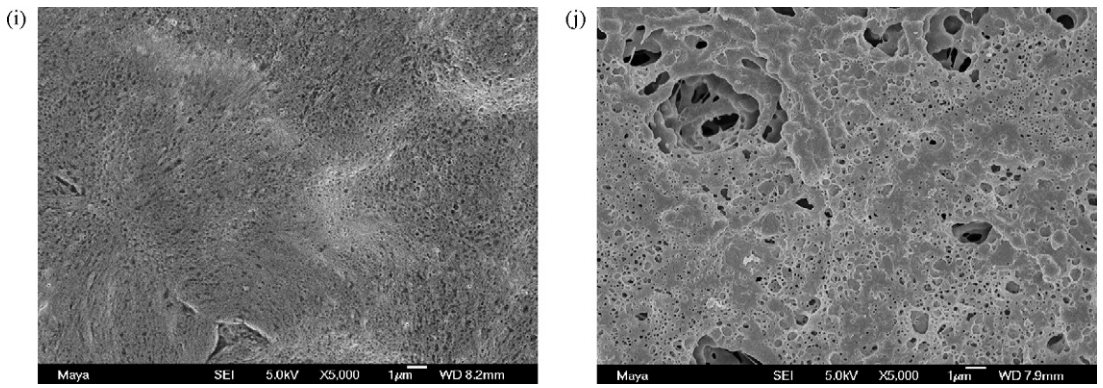

Fig. 6. (*Continued*).

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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References

Batycky, R.P., Hanes, J., Langer, R., Edwards, D.A., 1997. A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. J. Pharm. Sci. 86, 1464–1477.

Berkland, C., Pollauf, E., Raman, C., Silverman, R., Kim, K., Pack, D.W., 2007. Macromolecule release from monodisperse PLG microspheres: control of release rates and investigation of release mechanism. J. Pharm. Sci. 96, 1176–1191.

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

Borgquist, P., Nevsten, P., Nilsson, B., Wallenberg, R., Axelsson, A., 2004. Simulation of the release from multiparticulate system—single pellet and dose release. J. Controlled Release 97, 453–465.

Carino, G.P., Jacob, J.S., Mathiowitz, E., 2000. Nanosphere based oral insulin delivery. J. Controlled Release 65, 261–269.

Cleland, J.L., Johnson, O.L., Putney, S., Jones, A.J.S., 1997. Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development. Adv. Drug Deliv. Rev. 28, 71–84.

Duvvuri, S., Janoria, K.G., Mitra, A.K., 2005. Development of a novel formulation containing poly(D,L-lactide-co-glycolide) microspheres dispersed in PLGA–PEG–PLGA gel for sustained delivery of ganciclovir. J. Controlled Release 108, 282–293.

Fredenberg, S., 2004. PLG films in controlled release pharmaceuticals—diffusion and degradation. Licentiate Thesis. Lund University, Sweden.

Fredenberg, S., Reslow, M., Axelsson, A., 2004. Measurement of protein diffusion through poly(D,L-lactide-co-glycolide). Pharm. Dev. Technol. 10, 299–307.

Fredenberg, S., Reslow, M., Axelsson, A., 2007. Effect of divalent cations on pore formation and degradation of poly(D,L-lactide-co-glycolide). Pharm. Dev. Technol. 12, 563–572.

Houchin, M.L., Neuenswander, S.A., Topp, E.M., 2007. Effect of excipients on PLGA film degradation and the stability of an incorporated peptide. J. Controlled Release 117, 413–420.

Ishihara, T., Izumo, N., Higaki, M., Shimada, E., Hagi, T., Mine, L., Ogawa, Y., Mizushima, Y., 2005. Role of zinc in formulation of PLGA/PLA nanoparticles encapsulating betamethasone phosphate and its release profile. J. Controlled Release 105, 68–76.

Johnson, O.L., Cleland, J.L., Jung Lee, H., Charnis, M., Duenas, E., Jaworowicz, W., Shepard, D., Shahzamani, A., Jones, A.J.S., Putney, S.D., 1996. A month-long effect from a single injection of microencapsulated human growth hormone. Nat. Med. 2, 795–799.

Johnson, O.L., Jaworowicz, W., Cleland, J.L., Bailey, L., Charnis, M., Duenas, E., Wu, C., Shepard, D., Magil, S., Last, T., Jones, A.J.S., Putney, S.D., 1997. The stabilization and encapsulation of human growth hormone into biodegradable microspheres. Pharm. Res. 14, 730–735.

Kang, J., Schwendeman, S.P., 2007. Pore closing and opening in biodegradable polymers and their effect on the controlled release of proteins. Mol. Pharm. 4, 104–118.

Kim, H.K., Park, T.G., 2004. Comparative study on sustained release of human growth hormone from semi-crystalline poly(L-lactic acid) and amorphous poly(D,L-lactic-co-glycolic acid) microspheres: morphological effect on protein release. J. Controlled Release 98, 115–125.

Lagarce, F., Renaud, P., Faisant, N., Nicolas, G., Cailleux, A., Richard, J., Menei, P., Benoit, J.-P., 2005. Baclofen-loaded microspheres: preparation and efficacy testing in a new rabbit model. Eur. J. Pharm. Biopharm. 59, 449–459.

Lam, X.M., Duenas, E.T., Daugherty, A.L., Levin, N., Cleland, J.L., 2000. Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes. J. Controlled Release 67, 281–292.

Li, L., Schwendeman, S.P., 2005. Mapping neutral microclimate pH in PLGA microspheres. J. Controlled Release 101, 163–173.

Liu, F.-I., Kuo, J.H., Sung, K.C., Hu, O.Y.P., 2003. Biodegradable polymeric microspheres for nalbuphine prodrug controlled delivery: in vitro characterization and in vivo pharmacokinetic studies. Int. J. Pharm. 257, 23–31.

Liu, W.H., Song, J.L., Liu, K., Chu, D.F., Li, Y.X., 2005. Preparation and in vitro and in vivo release studies of Huperzine A loaded microspheres for the treatment of Alzheimer's disease. J. Controlled Release 107, 417–427.

Mattisson, C., 1999. Diffusion studies in gels using holographic interferometry. Ph.D. Thesis. Lund University, Sweden.

Park, T.G., 1995. Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition. Biomaterials 16, 1123–1130.

Pratt, L., Chu, C., Auer, J., Kim, J., Zollweg, J.A., Chu, C.C., 1993. The effect of ionic electrolytes on hydrolytic degradation of biodegradable polymer: mechanical and thermodynamic properties and molecular modeling. J. Polym. Sci., Part A: Polym. Chem. 31, 1759–1769.

Reslow, M., Jönsson, M., Laakso, T., 2002. Sustained-release of human growth hormone from PLG-coated starch microspheres. Drug Deliv. Syst. Sci. 2, 103–109.

Ross Colvin, J., 1952. The size and shape of lysozyme. Can. J. Chem. 30, 831–834.

Shenderova, A., Burke, T.G., Schwendeman, S.P., 1999. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. Pharm. Res. 16, 241–248.

Takada, S., Yamagata, Y., Misaki, M., Taira, K., Kurokawa, T., 2003. Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique. J. Controlled Release 88, 229–242.

Takenaga, M., Yamaguchi, Y., Kitagawa, A., Ogawa, Y., Kawai, S., Mizushima, Y., Igarashi, R., 2004. Optimum formulation for sustained-release insulin. Int. J. Pharm. 271, 85–94.

Tracy, M.A., Ward, K.L., Firouzabadian, L., Wang, Y., Dong, N., Qian, R., Zhang, Y., 1999. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in vitro. Biomaterials 20, 1057–1062.

Walsh, G., 2003. Pharmaceutical biotechnology products approved within the European Union. Eur. J. Pharm. Biopharm. 55, 3–10.

Webber, W.L., Lago, F., Thanos, C., Mathowitz, E., 1998. Characterization of soluble, salt-loaded, degradable PLGA films and their release of tetracycline. J. Biomed. Mater. Res. 41, 18–29.
Westrin, B.A., 1991. Diffusion measurement in gels: a methodological study. Ph.D. Thesis. Lund University, Sweden.
Zhang, Y., Zale, S., Sawer, L., Bernstein, H., 1997. Effects of metal salts on poly(D,L-lactide-co-glycolide) polymer hydrolysis. J. Biomed. Mater. Res. 34, 531–538.
Zhu, G., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives. Pharm. Res. 17, 351–357.