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Melted glyceryl palmitostearate (GPS) pellets for protein delivery

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Abstract

Lysozyme was incorporated into glyceryl palmitostearate (GPS) pellets by compression and melting at loadings of 2, 5 and 10% (w/w). Released lysozyme from both compressed and melted pellets showed good retention of enzymatic activity (>80% active). The percentage lysozyme recovered during in vitro release experiments, over 120 h, was significantly lower from the melted pellets (<15%) compared with compressed pellets (71–85%). Scanning electron microscopy suggested this difference in release was due to differences in porosity of the compressed and melted pellets. Inclusion of hydrophilic components, PEG 4000 and Gelucire 50/13, in the melted matrices increased the percentage of lysozyme released in vitro. Lysozyme released from GPS/PEG 4000 matrices showed good retention of enzymatic activity (>88% active) while that from GPS/Gelucire 50/13 showed reduced activity (68 and 51% active). PEG 4000 was not completely miscible with GPS at the concentrations studied and heterogenous systems resulted. At a loading of 20–35% (w/w) PEG 4000 in GPS greater than 80% of the incorporated lysozyme was released, indicating the likely achievement of interconnecting hydrophilic channels throughout the GPS matrix. In conclusion, melted GPS demonstrated potential as a matrix for the controlled release of proteins and release rates could be modified by inclusion of hydrophilic components.

Keywords: Glyceryl palmitostearate; Melted pellets; Lysozyme; Polyethylene glycol; Percolation threshold

1. Introduction

Controlled release implantable systems for the delivery of protein and peptide drugs offer opportunities for localizing bioactive agents to target sites, reducing the frequency of administration and potentially enhancing in vivo stability of the bioactive agents (Cleland and Langer, 1994). Systems can be broadly categorized as non-degradable or biodegradable depending on whether they remain at the site of administration or are absorbed following implantation. Non-degradable materials, such as poly(dimethyl-siloxane) and poly(ethylene-co-vinyl acetate), have been reported to provide release of a drug for long periods (Rhine et al., 1980; Siegel and Langer, 1984) with their major limitation being the need for surgical removal of the drug-depleted matrix. To overcome this, biodegradable materials have received much attention as the basis of implantable controlled release systems. These have included synthetic polymers such as polyesters, polyanhydrides, polyorthoesters (Cleland and Langer, 1994; Göpferich, 1997; Medlicott and Tucker, 1999) and lipid materials such as lecithin, cholesterol and glycosylated fatty acids (Khan et al., 1991; Yamagata et al., 2000). Pro-

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tein drugs are incorporated into these materials using techniques that seek to preserve protein tertiary structure. Typically, formulation processes offer less than optimal conditions for maintaining protein structure, as proteins may undergo denaturation on exposure hydrophobic polymeric surfaces, organic solvents and high shear stresses during emulsion based microencapsulation or excessive heat for prolonged periods in melt encapsulation techniques.

Recently, glycerides have shown potential for use as subcutaneous implants. Glyceryl monostearate implants containing cefazolin were non irritant in rats, indicating biocompatibility (Allababidi and Shah, 1998). Furthermore, lipid microparticles produced from glyceryl tripalmitate showed good biocompatibility when compared with polylactic-co-glycolic acid (Reithmeier et al., 2001). Glyceryl palmitostearate (GPS) is a mixture of mono-, di-, and triglycerides of palmitic and stearic acid and has a melting point of 53–57 °C. It has been used traditionally as a lubricant and an excipient in sustained release preparations in oral dosage forms (Bodmeier et al., 1990; Nokhodchi et al., 1997). However, recently good biocompatibility has been reported on subcutaneous injection of a 10% (w/v) GPS gel (Gao et al., 1995).

In the present study, we report the incorporation of a model protein, lysozyme, into GPS pellets using compression and melting methods. The effect of the preparation method on the in vitro release, content and enzyme activity of lysozyme was compared. Additionally, the characteristics of melted pellets were modified by adding hydrophilic substances, polyethylene glycol (PEG 4000) and Gelucire 50/13.

2. Materials and methods

2.1. Materials

Glyceryl palmitostearate (Precirol ATO5) and Gelucire 50/13 (20% glycerides and 80% PEG esters) were a gift from Gattefossé, (France). Freeze-dried lysozyme (chicken egg white) and lyophilized cells of Micrococcus lysodeikticus, ATCC No. 4698, were purchased from Sigma Chemical CO.(MO, USA). Polyethylene glycol 4000 (PEG 4000) was obtained from BDH Chemical Ltd. (Poole, UK). All other reagents used in this study were of analytical grade.

2.2. Preparation of GPS pellets

GPS and freeze-dried lysozyme were ground gently in a mortar and pestle and sieved through a 355 μm sieve. Mixes consisting of 2, 5, and 10% (w/w) lysozyme in GPS were prepared by geometrically mixing in a glass bottle.

2.2.1. Preparation of lysozyme containing GPS pellets by compression or melting

Compressed pellets were prepared by placing 20 mg of each mix into 2.5 mm diameter flat-faced punches and die and pressing to a defined length (4 mm) using an hydraulic press. The mean height of pellets prepared by compression was in the range 4.0–4.1 mm with the relative standard deviation less than 2%.

Melted pellets were prepared by placing 40 mg of each mix into polyethylene tubes (2.5 mm internal diameter) closed at one end by a 2.7 mm diameter glass bead. Tubes were held vertically at 65 °C for 4 min, and then cooled to 45 °C at a rate of 14 °C/min. The solidified pellets were pushed out of the polyethylene tubes and cut to the same length as the compressed pellets (4 mm). The mean weight of pellets prepared by melting was in the range 19.8–20.2 mg with the relative standard deviation less than 2%.

2.2.2. Preparation of PEG 4000 and Gelucire 50/13 containing pellets

PEG 4000 was mixed with GPS at concentrations of 5, 10, 20, 25, 30 and 35% (w/w) and Gelucire 50/13 was mixed with GPS at concentrations of 10 and 20% (w/w). Lysozyme was incorporated into each mix at a concentration of 10% (w/w) by geometric dilution. Pellets were prepared as described for melted pellets above (Section 2.2.1.).

2.3. Determination of lysozyme content in pellets

Pellets were crushed in 5 ml polypropylene tubes. Phosphate buffer saline (5 ml) at pH 7.4 with 0.1% (w/v) sodium azide (PBS) was added to each tube. Tubes were vortexed, incubated in 37 °C for 3 days, then centrifuged at 7840 × g for 10 min, and assayed for lysozyme content using a BCA Protein Assay (Pierce, IL). The assay was linear over the lysozyme concentration range 5–200 μg/ml ($R^2 > 0.99$)

2.4. In vitro release

PBS (pH 7.4 with 0.1% (w/v) sodium azide, 37 °C) was used as the medium. Pellets were weighed (M_o), then wrapped in 0.5 mm diameter stainless steel wire helices, placed into plastic tubes with PBS (5 ml) and shaken at 80 oscillations/min in a shaking water bath (Grant Instruments). Samples (0.5 ml) were collected at various times and replaced with preheated fresh medium (37 °C). The samples were centrifuged at 7840 × g for 10 min and the total protein concentration analyzed using the BCA Protein Assay. At the end of the release study, pellets were blotted dry and weighed (M_r), then, dried in a hot air oven at 37 °C to constant weight (M_d). The remaining lysozyme content in the pellets was determined as previously described (Section 2.3.). The matrix erosion and water uptake were calculated using Eqs. (1) and (2) which were modified from those reported by Sutananta et al. (1995) to allow for protein released during the study.

$$\text{Matrix erosion} = \left(\frac{M_o - M_d - M_c}{M_o}\right) \times 100 \quad (1)$$

$$\text{Water uptake} = \left(\frac{M_r - M_d}{M_d}\right) \times 100 \quad (2)$$

where M_o is the initial pellet mass, M_r and M_d are the wet and dry pellet mass after the release study, and M_c is the total mass of lysozyme released during the study.

2.5. Determination of activity of lysozyme

The activity of released lysozyme was evaluated using *Micrococcus lysodeikticus* as a substrate (Bezemer et al., 2000). This assay was conducted using 96 well microplates. Samples (20 μl) were added to 150 μl of 0.15% (w/v) *Micrococcus lysodeikticus* suspension in PBS and the decrease in turbidity at 520 nm was determined at 30 s intervals for 4 min at 25 °C using SOFTmax PRO microplate analysis software version P1.2.0. The lysozyme activity assay was linear over the range 5–20 μg/ml ($R^2 > 0.99$). Lysozyme activity results are expressed as a percentage of the total protein concentration determined using the BCA Protein Assay.

2.6. Scanning electron microscopy

The surface morphology and internal structure of the pellets were studied using scanning electron microscopy (SEM). Samples were mounted on aluminium stubs and sputter coated with a gold/palladium mixture (BioRad coating system) and viewed using a Cambridge S360 Stereoscan scanning electron microscope.

2.7. Miscibility of PEG 4000 in GPS

Mixtures of PEG 4000 in GPS (10, 20, 30, and 35%, w/w) were prepared as for pellets. Sixty milligrams of each mixture was filled into a 2.5 mm diameter polyethylene tube closed at one end with a 2.7 mm diameter glass bead. The tubes were heated to 65 °C for 8 min during which time two phases separated. Following cooling the solidified material was removed from the tubes and two layers were separated by cutting. Each layer was weighed, crushed and washed several times with 1 ml aliquots of deionized water to extract the water soluble PEG 4000. The washed material was collected and dried to a constant weight. The amount of PEG 4000 in each layer was calculated as the difference between the initial mass and the dry mass after extraction.

2.8. Lysozyme adsorption to GPS

GPS (10 and 20 mg) was accurately weighed into 5 ml plastic tubes and 5 ml of 100 μg/ml lysozyme solution in PBS was added. The tubes were vortexed and incubated at 37 °C in a shaking water-bath (oscillated at 80 times/min) for 9 days. At each sampling time (0, 2, 3, 5, 7 and 9 days) tubes were centrifuged at 2465 × g for 20 min and 0.15 ml of the supernatant collected and centrifuged again at 7840 × g for 10 min. The total lysozyme concentration was analyzed using the BCA Protein Assay.

2.9. Data analysis

Cumulative lysozyme released was plotted against the square root of time. Linear regression was used to determine the constant k in:

$$Q = kt^{0.5} \quad (3)$$

where Q is the amount of lysozyme released and t is time.

Analysis of variance (ANOVA) and Fisher's pairwise comparisons were used to compare content and release data of different pellet formulations. All statistical tests were run on Minitab® Statistical Software Release 12.1.

3. Results

Cumulative release of lysozyme from compressed and melted pellets without additives is shown in Fig. 1 and with hydrophilic additives in Fig. 2. Release characteristics are summarised in Table 1. It can be seen from results (Table 1) that the lysozyme content of melted pellets was apparently lower than those of compressed pellets when compared at the same loading of lysozyme and probably reflects incomplete recovery of the incorporated protein from the melted pellets. At the 10% (w/w) loading, the lysozyme content of melted pellets appeared to increase with increasing incorporation of either hydrophilic components (PEG 4000 or Gelucire 50/13). The activity of lysozyme released during content studies of implants without additives and implants with PEG 4000 was high (>80%) indicating retention of biological activity in the released fraction. In contrast, the activity of lysozyme recovered from implants with 10 and 20% (w/w) Gelucire 50/13 was reduced (68 and 51% active, respectively) (Table 1).

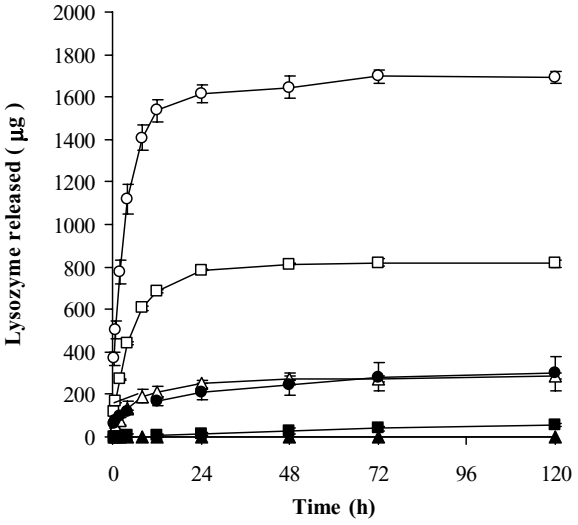

Fig. 1. Effect of preparation methods of GPS pellets and lysozyme contents on release of lysozyme. Compression method: (△) 2; (□) 5; (○) 10% (w/w) lysozyme; melting method: (▲) 2; (■) 5; (●) 10% (w/w) lysozyme. Means are plotted ±S.D., $n = 3$.

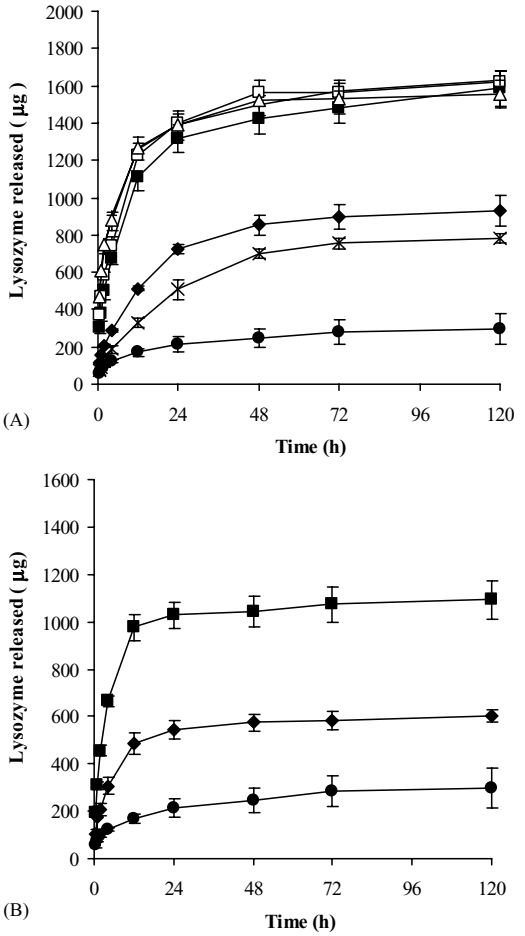

Fig. 2. Effect of PEG 4000 (A) and Gelucire 50/13 (B) on the release of lysozyme from melted pellets containing 10% (w/w) lysozyme: (●) 0; (×) 5; (◆) 10; (■) 20; (□) 25; (+) 30; (△) 35% (w/w) PEG 4000 and (●) 0; (◆) 10; (■) 20% (w/w) Gelucire 50/13. Means are plotted ±S.D., $n = 3$.

In vitro release studies showed virtually complete release from compressed pellets (71–85%) within two days while less than 15% was released from melted pellets (Fig. 1). Adsorption studies (Fig. 3) showed loss of lysozyme from solution on incubation with GPS, suggesting the balance of lysozyme in the release studies may be adsorbed onto the lipid surface.

Table 1
Characteristics of compressed and melted glyceryl palmitostearate (GPS) pellets

Formulation	Lysozyme content[a] (μg per pellet)	Lysozyme activity[a] (%)	Lysozyme release			
			Release rate[a] ($\mu g\,h^{-0.5}$)	R^{2b}	Initial burst release at 0.5 h[a,c] (%)	Total release[a,c] (%)
Compression						
2% (w/w) LSZ	238.0 ± 7.3	92.2 ± 2.1	78.6 ± 18.3	0.988	8.0 ± 2.1	71.3 ± 3.0
5% (w/w) LSZ	752.3 ± 25.9	84.1 ± 5.4	255.0 ± 5.6	0.989	11.9 ± 0.3	81.6 ± 2.0
10% (w/w) LSZ	1733.1 ± 74.9	94.1 ± 6.4	591.0 ± 32.5	0.998	18.4 ± 1.6	84.6 ± 1.4
Melting						
2% (w/w) LSZ	33.9 ± 11.2	124.6 ± 10.9	–	–	–	–
5% (w/w) LSZ	181.5 ± 9.4	87.8 ± 12.5	–	–	–	5.5 ± 1.7
10% (w/w) LSZ	440.4 ± 26.7	89.9 ± 11.0	35.8 ± 9.2	0.991	3.0 ± 0.7	14.9 ± 4.1
10% (w/w) LSZ						
+5% (w/w) PEG 4000	888.2 ± 41.1	88.6 ± 3.4	103.9 ± 10.7	0.993	3.5 ± 0.4	39.0 ± 1.3
+10% (w/w) PEG 4000	936.8 ± 54.5	88.5 ± 5.1	144.3 ± 4.7	0.998	5.8 ± 0.1	46.5 ± 4.0
+20% (w/w) PEG 4000	1631.5 ± 41.1*	89.6 ± 1.6	288.3 ± 17.0*	0.999	15.4 ± 1.6	79.4 ± 5.1
+25% (w/w) PEG 4000	1778.2 ± 56.3*,**	96.8 ± 2.9	324.0 ± 26.3*,**	0.992	18.4 ± 1.5	81.3 ± 2.9
+30% (w/w) PEG 4000	1715.3 ± 93.3*,**	91.7 ± 0.7	308.7 ± 19.4*,**	0.994	23.3 ± 0.1	81.4 ± 2.6
+35% (w/w) PEG 4000	1669.5 ± 45.9*,**	94.7 ± 3.7	309.0 ± 10.5*,**	0.989	23.4 ± 2.6	77.9 ± 3.8
+10% (w/w) Gelucire 50/13	580.0 ± 21.2	68.8 ± 9.4	145.7 ± 12.9	0.991	5.1 ± 1.1	30.2 ± 1.3
+20% (w/w) Gelucire 50/13	983.9 ± 94.7	51.1 ± 17.8	361.0 ± 13.0	0.999	9.8 ± 0.6	54.7 ± 4.1

(–) No determination.
[a] Data are means ± S.D. of three determinations.
[b] Coefficient of correlation for the relationship between the amount of lysozyme released and square root of time.
[c] (Amount of lysozyme released/theoretical lysozyme loading) × 100.
* $P < 0.05$ compared with 5 and 10% (w/w) PEG 4000.
** $P > 0.05$ compared with 20% (w/w) PEG 4000.

SEM showed the melted pellets had a smoother surface (Fig. 4A) than the compressed pellets (Fig. 4B) prior to release. Following in vitro release the surface of melted pellets remained relatively non-porous (Fig. 4C) whereas the surface of compressed pellets became highly porous (Fig. 4D) probably due to incomplete welding of the GPS particles during compression. Water uptake and matrix erosion measurements also showed significant differences ($P < 0.05$) for pellets produced by compression or melting (Fig. 5) with melted pellets taking up only small amount of water and undergoing minimal erosion during the study period of 120 h.

Incorporation of the hydrophilic additives, PEG 4000 and Gelucire 50/13, into melted pellets allowed increased release of lysozyme (Table 1 and Fig. 2). Greater than 80% of the incorporated lysozyme was released at PEG 4000 loadings of 25% (w/w) and above. Release rates of lysozyme increased with increasing PEG 4000 loadings from 5 to 20% (w/w). Then, above 20% (w/w) loadings of PEG 4000 there

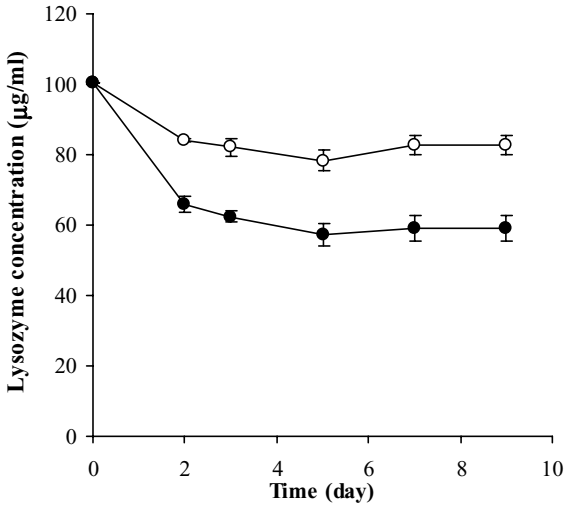

Fig. 3. Adsorption profiles of lysozyme on (○) 10 mg and (●) 20 mg of GPS particles. Means are plotted ±S.D., $n = 3$.

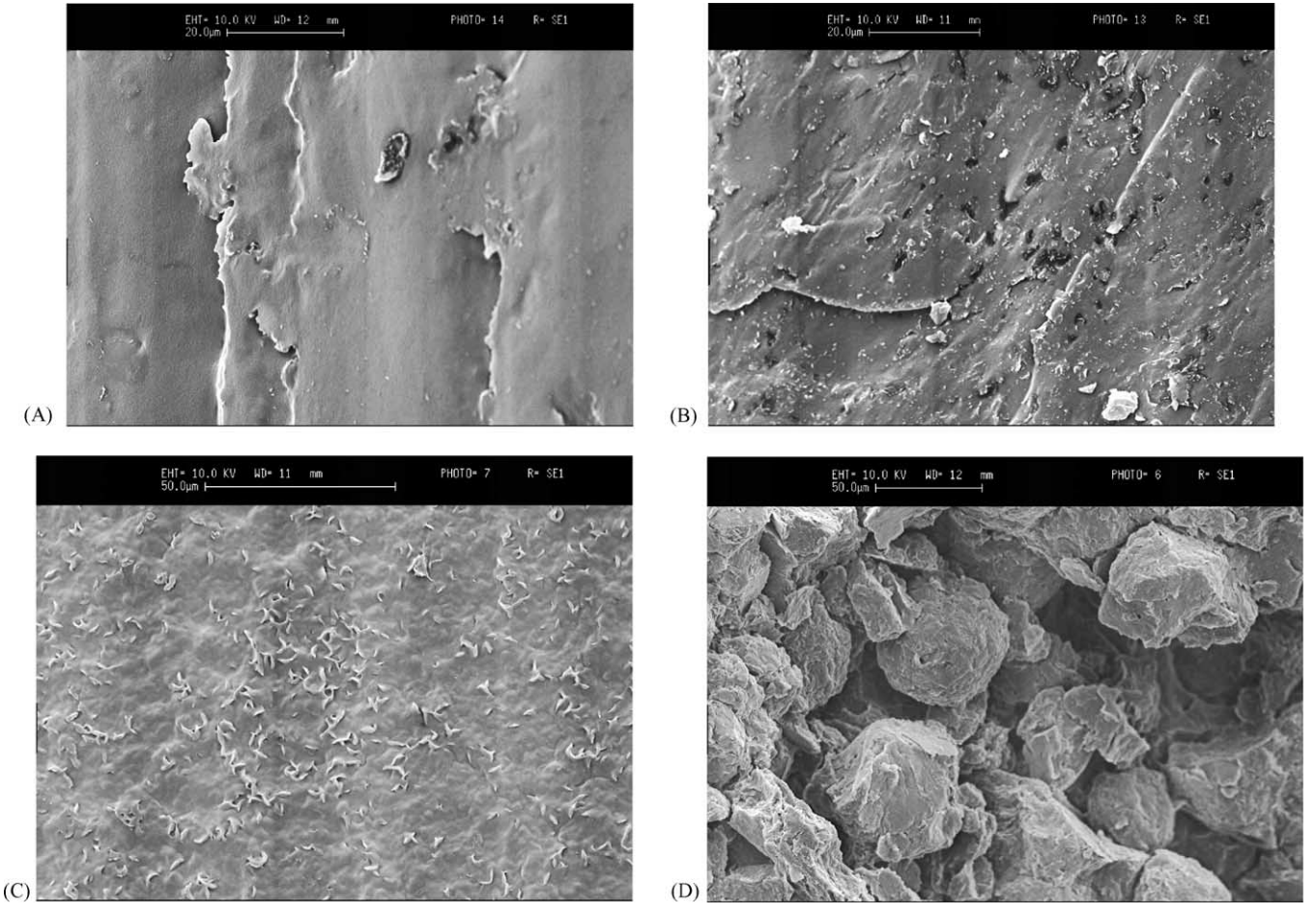

Fig. 4. Scanning electron micrographs of surface morphology of GPS pellet containing 10% (w/w) of lysozyme: melted pellets before (A) and after (C) release, and compressed pellets before (B) and after (D) release study.

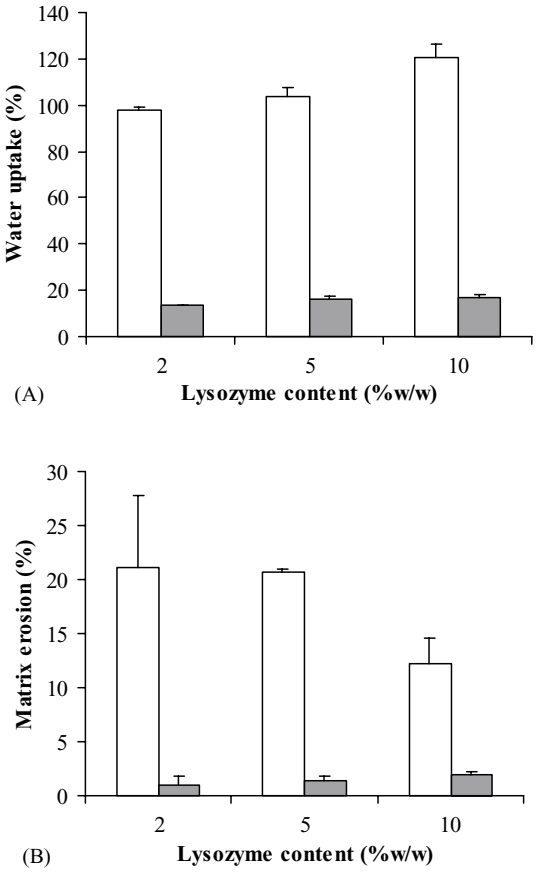

Fig. 5. Water uptake (A) and matrix erosion (B) of GPS pellets prepared by compression (open bars) and melting (close bars) methods. Means are plotted ±S.D., $n = 3$.

were no further significant increases in the release rate ($P > 0.05$). Examination of the internal structure of GPS/PEG 4000 pellets by SEM showed the appearance of a two-phase system (Fig. 6A). Following in vitro release the areas suspected to be PEG 4000-rich appeared as pores in the GPS matrix, indicating these area represent a water soluble components (Fig. 6B). The porous surface morphology of melted pellets containing PEG 4000 following the release studies also confirmed improved water access into melted pellets by incorporation of PEG 4000 (Fig. 6C). Miscibility experiments (Table 2) showed partial miscibility of PEG 4000 in GPS, with PEG 4000 concentrations of 83.3 ± 2.7% (w/w) in the PEG 4000-rich layer and 11.7 ± 1.6% (w/w) in the GPS-rich layer.

Table 2
Miscibility of PEG 4000 in glyceryl palmitostearate (GPS)

PEG 4000 content (%, w/w)	Miscible PEG 4000 (%, w/w), $n = 3$	
	PEG 4000-rich layer (lower layer)	GPS-rich layer (upper layer)
10	81.8 ± 1.8	9.5 ± 0.2
20	82.3 ± 3.6	11.8 ± 0.3
30	83.3 ± 2.9	12.7 ± 1.2
35	85.8 ± 0.8	10.7 ± 0.5
Overall mean[a]	83.3 ± 2.7	11.7 ± 1.6

[a] $n = 12$.

4. Discussion

GPS has been used as a tablet lubricant and matrix for controlling drug release in oral drug delivery systems (Malamataris et al., 1991). The potential of this and related glycerides as the basis of an implantable system is beginning to be investigated (Gao et al., 1995; Allababidi and Shah, 1998; Reithmeier et al., 2001). It can be fabricated into the pellets by compression or melting (m.p. = 50–55 °C) methods. Retention of lysozyme activity following formation into pellets suggests both preparation methods are suitable for producing protein-containing matrices. However, only a small fraction of the incorporated lysozyme could be recovered from melted pellets during content assays and release studies (<25%), so the quality of remaining protein is unknown. This is in agreement with poor release of drugs from non-porous matrices when the loading is below the percolation threshold (Bonny and Leuenberger, 1993; Leuenberger et al., 1995). On incorporation of PEG 4000 in the melted pellets, at 25% (w/w) and above, greater than 80% of incorporated lysozyme was recovered with excellent retention of enzymatic activity (92–97% active), indicating that exposure of the lysozyme to 65 °C for the short time required to produce the pellets (4 min) did not adversely affect protein quality. In contrast, lysozyme activity was reduced if pellets were prepared with Gelucire 50/13.

In granular matrices, the release of drug involves the simultaneous penetration of release medium, dissolution of drug particle and leaching out of the drug through channel or pores by diffusion (Martin, 1993). SEM of pellet surfaces indicated that pellets prepared by compression yielded a porous, erodible

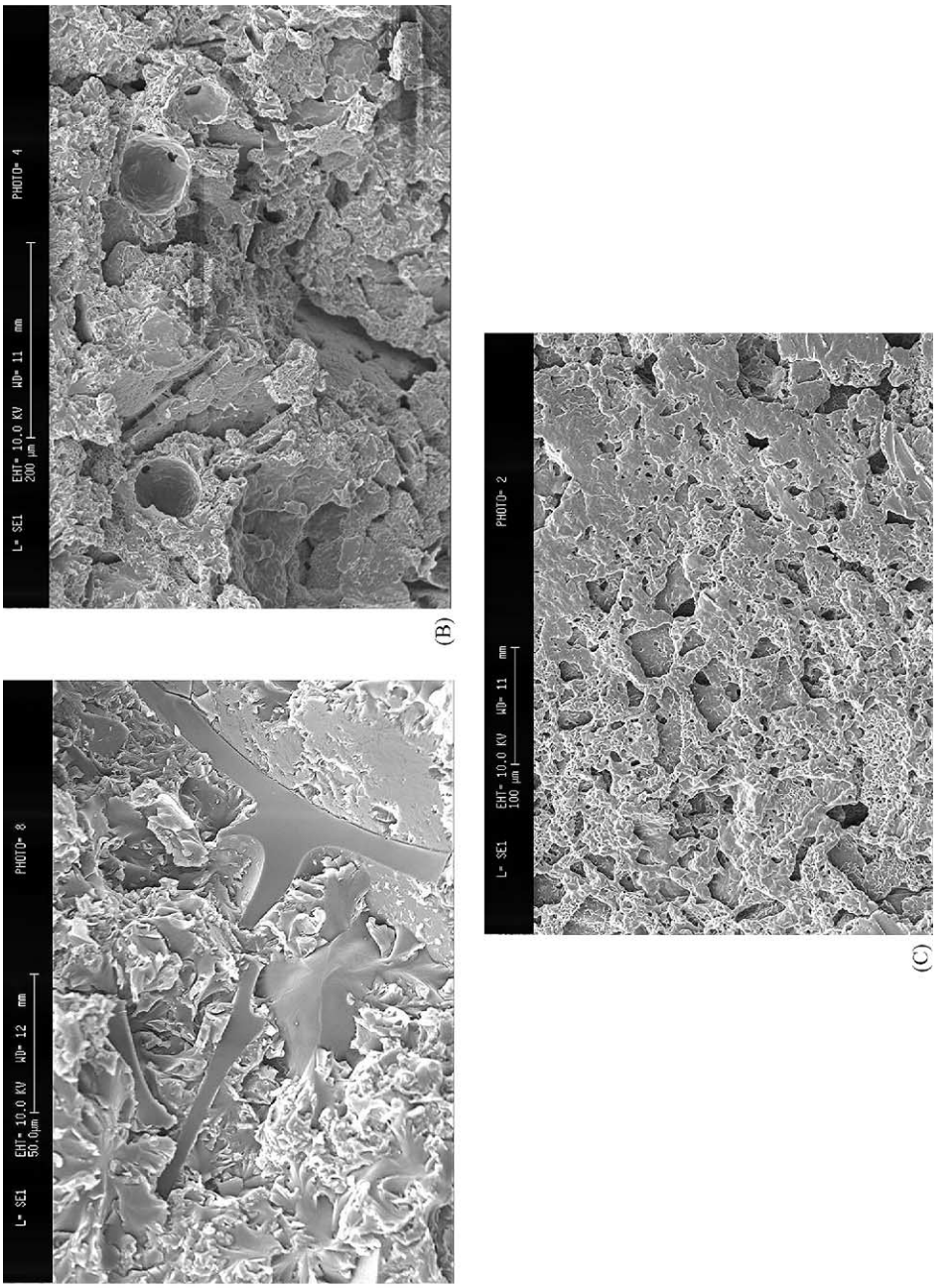

Fig. 6. Scanning electron micrographs of 10% (w/w) lysozyme melted pellet containing 35% (w/w) of PEG 4000: internal structure before (A) and after (B) release and surface morphology after release (C).

matrix, which allowed entry of water and virtually complete release of the incorporated protein. In comparison, pellets produced by melting retained a smooth, slightly porous surface after release, which suggested restricted entry of water caused the low fractional release of lysozyme. Pellets were studied at protein loads of 2–10% (w/w), which is less than generally accepted as the percolating threshold (Bonny and Leuenberger, 1993; Leuenberger et al., 1995; Caraballo et al., 1996), so that although an increasing release rate was observed with increasing drug load, it is unlikely that protein particles were able to produce interconnected pore networks through the matrices. Hence, the mechanism of lysozyme release from compressed pellets is probably controlled by the rate of water uptake into the porous matrix. Whereas, release from the melted pellets represents only lysozyme associated with or in close proximity to the pellet surface as described for fatty acid pellets by Kaewvichit and Tucker (1994).

In order to manipulate the release of protein from melted GPS matrices, hydrophilic components of similar melting points were investigated. The melting points of PEG 4000 and Gelucire 50/13 are reported to be about 55–59 and 46–51 °C, respectively (Lloyd et al., 1997; Craig, 1995). Both substances completely melted at 65 °C during preparation of the melted implants. Sutananta et al. (1995) reported that matrices of Gelucire 50/13 could take up water to 300% (w/w) of the dry base weight within 12 h to form a swollen matrix, hence it was incorporated into GPS melted implants to improve water access into the pellets. Pellets prepared from GPS/Gelucire 50/13 blends were softer than GPS alone and when incubated during the release study were observed to swell and break-up within 24 h. Since the lipid material from these implants dispersed in the release media, it is thought that the loss of lysozyme activity on release from the GPS/Gelucire 50/13 pellets may have resulted by binding of the protein to the dispersed lipid.

The matrices produced by GPS/PEG 4000 blends, in contrast, showed good retention of enzymatic activity of the incorporated lysozyme. However, in these matrices a heterogeneous system resulted because the PEG 4000 was not completely miscible in the GPS. Polyethylene glycols have often been incorporated into controlled release matrix systems to alter porosity and drug release (Kim et al., 2000). In these systems, PEG is expected to leach rapidly from the matrix to create pores and channels through which release medium can enter, thus accounting for increases in the initial burst release and release rate (Cleek et al., 1997; Lin and Yu, 2001). The concentration of hydrophilic components (lysozyme + PEG 4000) in the melted GPS matrix appears critical in determining the release rate and extent of lysozyme release. Bonny and Leuenberger (1993) define the parameter, critical porosity, of inert matrices to be the matrix porosity below which the drug is mainly encapsulated by the inert material. In ethylcellulose and hydrogenated castor oil matrices, the critical porosity was estimated to be 0.30–0.36 (Bonny and Leuenberger, 1993). Above this porosity, a complete pore network is thought to be formed through the matrix and the drug loading at which this critical porosity is reached has been described as the percolation threshold of this system (Bonny and Leuenberger, 1993; El-Arini and Leuenberger, 1995; Caraballo et al., 1999). In the GPS/PEG 4000 matrices described in this paper, partial miscibility of PEG 4000 in GPS may affect the percolation threshold. The miscibility of PEG 4000 in GPS was estimated as 11.7% (w/w) and is expected, at this concentration, to increase the hydrophilic character of the GPS, whereas the immiscible PEG-rich fraction (83.3% PEG 4000, 16.7% GPS) is likely to contribute to the network of hydrophilic material through the GPS matrix. From release experiments, the amount of PEG 4000 required to reach the percolation threshold in melted GPS pellets containing 10% (w/w) lysozyme could be estimated to be around 20% (w/w). However, once the percolation threshold was reached, it appeared further increases in the hydrophilic component (PEG 4000) did not further increase lysozyme release rates.

5. Conclusions

Melted GPS has demonstrated potential as a matrix material for the controlled release of proteins. Exposure to the GPS melting temperature (65 °C), for a short duration (4 min) did not greatly affect the activity of lysozyme, a model protein, suggesting processes with modest heating may be suitable for preparation of protein-containing controlled release systems. Release rates could be manipulated by incorporation of

hydrophilic components, such as PEG 4000. However, the effects of these additives on protein activity must be considered.

Acknowledgements

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