Improving Protein Delivery from Microparticles Using Blends of Poly(DL lactide co-glycolide) and Poly(ethylene oxide)-poly(propylene oxide) Copolymers

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Purpose. Microparticles containing ovalbumin as a model for protein drugs were formulated from blends of poly(DL lactide-co-glycolide) and poly(ethylene oxide)-poly(propylene oxide) copolymers (Pluronic). The objectives were to achieve uniform release characteristics and improved protein delivery capacity.

Methods. The water- in oil -in oil emulsion/solvent extraction technique was used for microparticle production.

Results. A protein loading level of over 40% (w/w) was attained in microparticles having a mean diameter of approximately 5 μm. Linear protein release profiles over 25 days *in vitro* were exhibited by certain blend formulations incorporating hydrophilic Pluronic F127. The release profile tended to plateau after 10 days when the more hydrophobic Pluronic L121 copolymer was used to prepare microparticles. A delivery capacity of 3 μg OVA/mg particles/ day was achieved by formulation of microparticles using a 1:2 blend of PLG:Pluronic F127.

Conclusions. The w/o/o formulation approach in combination with PLG:Pluronic blends shows potential for improving the delivery of therapeutic proteins and peptides from microparticulate systems. Novel vaccine formulations are also feasible by incorporation of Pluronic L121 in the microparticles as a co-adjuvant.

KEY WORDS: poly(lactide-co-glycolide); pluronic; controlled-release; microparticles; protein.

INTRODUCTION

The availability of biotechnology-derived, therapeutic proteins and peptides such as analogs of luteinizing hormone releasing hormone (LHRH) and interleukin-2 has increased considerably over the past decade. This in turn has presented major challenges in delivery system design and formulation to extend the often short half-life of protein drugs in the body, to increase bioavailability after parenteral administration or delivery to mucosal tissues and to target protein molecules to specific tissues so as to limit systemic drug levels (1). Microparticulate delivery systems for proteins and peptides have attracted considerable attention because of the wide scope for controlling loading and release characteristics by varying the (coating) polymer and formulation approach. In particular the biodegradable lactide polymers such as poly(DL lactide co-glycolide) [PLG] have been widely investigated since they have regulatory approval for use in humans, and the resorption time can be controlled from several weeks to over a year, which potentially allows fine tuning of protein release.

The water-in oil-in water (w/o/w) double emulsion, solvent evaporation method (2) is regularly applied in investigations of protein delivery systems and vaccine formulation. However, the partition of hydrophilic drug species into the aqueous continuous phase leads to low entrapment levels (generally much less than 10% w/w). Non-uniform, multi-phase release profiles (3, 4) are common, heralded by a rapid 'burst' release of surface protein, and often followed by an extended lag phase. In the absence of adverse structural changes to the protein, the lag phase is expected to persist until breakdown of the microparticle matrix allows release of entrapped protein. Another limitation of the w/o/w method is that protein delivery capacity (defined as the cumulative release: μg protein per mg microparticles per day) is usually low and less than 1 (4, 5). We have recently described a water-in oil-in oil technique (6) which replaces the aqueous phase with an organic continuous phase. This approach resulted in substantial increases in protein loading to over 50% (w/w) in microparticles larger than 10 μm diameter if blends of PLG and PEG were used in the process. Furthermore, a protein release characteristic which was linear with time over 30 days was obtained. Importantly, protein loading levels in excess of 25% were measured in small 2 μm particles which improves prospects for interaction of such microparticles with the gut associated lymphoid tissue (Peyer's Patches) and absorption across the G-I tract. Application of the w/o/o method also enabled the protein delivery capacity to be increased to the level of at least 5 μg/mg/day (6).

Blending of Pluronic poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) copolymers with PLG is a logical progression in the development of the w/o/o microencapsulation technique. Pluronic copolymers are available in a wide range of molecular weights and hydrophilicity and thus provide further opportunities for varying the characteristics of protein release from microparticles. Pluronic copolymers have been used widely in drug delivery research (1) for surface modifying, nanoparticles in order to extend blood circulation times, in gel form (Pluronic F127) for delivery of interleukin-2 and in a blend with poly(L lactic acid) (L.PLA) to produce films for controlled protein delivery.

PLG microparticles have already demonstrated considerable promise for formulating single dose vaccines for subcutaneous and intramuscular administration (7), and have also been investigated for nasal (8) and oral delivery (9) of antigens to exploit particulate uptake by lymphoid tissue as a means of inducing both mucosal and systemic immune responses. Certain (hydrophobic) Pluronics such as Pluronic L121 have been included in oil-in water emulsions to modulate the presentation of protein antigens and thereby improve the immune response (10). The incorporation of Pluronic L121 in microparticle-based vaccines could therefore produce a co-adjuvant effect.

The present paper describes the production and characterisation of protein-loaded microparticles using the water-in oil-in oil (w/o/o) technique and blends of PLG with Pluronic, triblock PEO-PPO-PEO copolymers. The hydrophilic Pluronic F127 and the more hydrophobic Pluronic L121 copolymer were

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selected to investigate the capacity for varying the protein release amount and release characteristics.

MATERIALS AND METHODS

Materials

50:50 poly(DL-lactide-co-glycolide):(50:50 PLG), (Weight average molecular weight (Mw) 34,000, Number average molecular weight (Mn) 12,200 RG503), 75:25 PLG, (Mw 63,000, Mn 20,400 RG755), 85:15 PLG, (Mw 232,000, Mn 131,300 RG858), poly(D.L-lactide):(D.L.PLA), (Mw 348,000, Mn 170,000 R208), were obtained from Boehringer Ingelheim, Ingelheim, Germany. Mw and Mn data were provided by the manufacturer. Poly(vinyl pyrrolidone) (PVP) MW 40,000 was obtained from Aldrich Chemical Co., Gillingham. U.K. Dichloromethane (DCM) and methanol (HPLC grade) were supplied by Fisons, Loughborough, U.K. Ovalbumin (OVA) (grade V), Span 60 and BCA protein assay reagents were obtained from Sigma Chemical Co., Poole. U.K. Pluronic PEO-PPO-PEO copolymers (Pluronic L104, L121, L123 and F127) were obtained from BASF Co., Parsippany. N.J. U.S.A. All materials were used as supplied. The composition of the Pluronics used is presented in Table I.

PREPARATION OF OVA-LOADED MICROPARTICLES

The Effect of PLG:Pluronic F127 Solution Composition

An OVA aqueous solution (1 ml, 30 mg/ml) was emulsified with a solution of Span 60 (2 ml, 0.5% w/v) in DCM for 30 seconds using a Silverson homogeniser (Silverson Machines, Chesham, Bucks, U.K) to provide the primary emulsion. The resulting emulsion was then mixed at high speed for 2 minutes with 5 ml of a 6% (w/v) polymer solution produced by co-dissolving PLG and Pluronic F127 in DCM in various ratios: 3:1, 1:1, 1:2 and 1:3. The resulting w/o emulsion was mixed for 4 minutes with 20 ml of a continuous phase solution, methanol, containing 15% w/v PVP as an emulsion stabilizer and the resulting w/o/o emulsion was stirred with a magnetic stirrer for 3–4 hours under ambient conditions to extract DCM. The microparticles were cleaned by centrifuging (10,000 g, 5 minutes) and resuspension in distilled water a total of three times and then freeze dried. The final product was stored in a desiccator below 4°C.

Table I. Composition of the Pluronic PEO-PPO-PEO Block Copolymers

Pluronic	Mn	Number of PO units/chain	Number of EO units/chain	%w/w PEO	HLB Value
L 104	2200	21	11	40	1–7
L 121	4400	69	5	10	1–7
L 123	5750	69	20	30	7–12
F 127	12600	69	98	70	18–23

Note: Mn: number-average molecular weight; PO: propylene oxide; EO: ethylene oxide.

Samples are designated in the text in terms of the ratio of PLG to Pluronic in the starting polymer solution.

The Effect of Lactide/Glycolide Ratio

An OVA aqueous solution was emulsified with a solution of Span 60 in DCM. The resulting emulsion was then mixed at high speed with different types of PLG co-polymer in solution with Pluronic F127 in DCM (6% w/v, 1:1 PLG : Pluronic F127). Microparticle formulation then proceeded as described above.

The Effect of Pluronic Type

An OVA aqueous solution (2 ml, 30 mg/ml) was emulsified with a solution of Span 60 (4 ml, 0.5% w/v) in DCM. The resulting emulsion was then mixed at high speed with different types of Pluronic copolymer co-dissolved with 50:50 PLG in 10 ml of DCM (3% w/v, 1:2 PLG : Pluronic). The mixture was emulsified with 40 ml of a continuous phase solution, methanol, containing 10% w/v PVP as an emulsion stabilizer. Microparticle formulation then proceeded as described above.

MICROPARTICLE CHARACTERIZATION

The OVA loading of microparticles was determined using a BCA protein assay (Sigma Chemical Co.) after particle disruption in 0.1M NaOH containing 5% w/v SDS as previously described (6). Each sample was assayed in triplicate. The entrapment efficiency was subsequently calculated by comparing the total weight of protein in the microparticle batch with the starting weight of protein.

Particle Size

Microparticles were sized by laser diffractometry using a Malvern 2600D laser sizer. Average particle size was expressed as volume mean diameter (vmd) in μm. Particle diameter d(90) (90% below this size) and particle diameter d(10) (10% below this size) were also recorded.

Morphology of Microparticles

Aqueous suspensions of microparticles were dropped onto metal stubs and allowed to dry in air under ambient conditions. Samples were coated with gold prior to examination by scanning electron microscopy (SEM) (JEOL 6400, Tokyo, Japan).

Analysis of Microparticle Composition by Infrared Spectroscopy

The composition of microparticles produced from various blended polymer solutions was analyzed by infrared spectroscopy. A Bruker IFS 88 fourier transform infrared (FTIR) spectrometer was used to obtain the spectra (50 scans per sample, over the 600–4000 cm^{-1} range) for the various PLG:Pluronic microparticles. A series of physical mixtures of PLG and Pluronic were used to construct a calibration curve. Microparticle samples were dissolved in chloroform and injected through a 0.25 μm polypropylene filter (to remove protein) into a FTIR liquid cell with a sodium chloride window. The composition of the microparticles was estimated by comparing peak height ratios corresponding to the carbonyl (C=O) band of PLG at 1759 cm^{-1} (11) and the CH_2 band at 2870 cm^{-1} (12) due to

the PEO component of the Pluronic copolymer, and assuming a negligible content of PVP surfactant in the microparticles.

In-vitro *Release of Protein from Microparticles*

Approximately 20 mg of freeze-dried microparticles (accurately weighed) were dispersed in 2.0 ml phosphate buffered saline (PBS pH 7.4), containing 0.02% sodium azide as a bacteriostatic agent. Samples were retained in a water-bath at 37°C and shaken intermittently at 3, 6, 9 and 24 hours. After 1 day and then at three day intervals, the microparticle samples were centrifuged (2000 g, 5 minutes), the supernatant was removed and the protein content of three samples was analyzed using a BCA protein assay. Fresh PBS was added to the microparticles and incubation was continued. Release profiles were calculated both in term of cumulative release (% w/w) with incubation time and cumulative release (μg OVA/mg microparticles) with incubation time.

Analysis of Protein Integrity

Samples of ovalbumin released from PLG:Pluronic microparticles by incubation in phosphate buffered saline at 37°C overnight, and native ovalbumin respectively were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (6).

RESULTS AND DISCUSSION

The Effect of PLG:Pluronic Solution Ratio on Microparticle Characteristics

Blending of Pluronic F127 in solution with PLG to produce microparticles resulted in an improvement of protein loading relative to PLG microparticles. The maximum loading level achieved of approximately 30% w/w (1:2 PLG:Pluronic F127) was more than twice that obtained with PLG alone (13% w/w). The protein entrapment efficiency was characteristically high for the w/o/o method used in excess of 90%. The mean microparticle size was found to decrease with decreasing PLG:Pluronic F127 ratio from 17.7 μm (3:1) to 6.4 μm (1:3) in line with the lower concentration of PLG in the polymer solution and indicating the dominant role of the PLG copolymer in development of the microparticle matrix. Microparticle formation was not achieved when the proportion of PLG in the blend solution was less than 25%. Similar trends in microparticle size and protein loading were recorded when PLG:PEG solutions were used to prepare protein loaded microparticles by the w/o/o method (6).

The higher protein loading obtained by using PLG:Pluronic F127 blend solutions rather than PLG alone to produce microparticles may be due to the combined use of a non-aqueous continuous phase, and interaction between OVA and Pluronic F127 (13) which results in efficient protein containment within the microparticle core. An increase in viscosity of the PLG:Pluronic disperse phase in the present w/o/o system could also account for the high protein loading levels achieved. A sharp increase in the viscosity of blended solutions of poly(DL lactide) and Pluronic F68 in DCM relative to solutions of the individual polymers has been measured by Nihant *et al.* (13), which was considered to contribute to the primary emulsion stability in w/o/w systems and consequently to favour a high loading of hydrophilic drugs. Evidence for chain entanglement and/or complex formation between L.PLA and PEO-PPO copolymers in solution in chloroform (based on GPC derived increases in molecular weight) has also been reported by Park *et al.* (12).

Table II. The Effect of PLG:Pluronic F127 Solution Composition on Microparticle Characteristics (6% w/v polymer solution concentration)

PLG: Pluronic Ratio	Protein Loading (% w/w)	Microparticle Size (μm)		
		vmd	d(90)	d(10)
1:0	13.3 ± 1.2	8.1	13.5	1
3:1	22.4 ± 0.9	17.7	42.7	1.9
1:1	25.1 ± 1.3	11.9	26.8	2.0
1:1[a]	16.4 ± 0.7	3.1	4.4	1.5
1:2	29.7 ± 2.1	9.5	25.8	1.9
1:3	27.8 ± 1.9	6.4	14.8	1.5

[a] Volume of each reagent increased by a factor of 4.

The effect on microparticle characteristics of increasing the volume of reagents, for the same volume ratio of OVA/Span/polymer solution/continuous phase, (1/2/5/20) is also shown in Table II. The mean particle size decreased significantly with increasing volume of reagents from approximately 12 μm to 3 μm, which can probably be attributed to a more uniform mixing condition as indicated by the corresponding narrower particle size distribution.

Analysis of the PLG content of 3:1, 1:1, 1:2 and 1:3 PLG:Pluronic F127 microparticles using infrared spectroscopy yielded estimates of the PLG content of the matrix of 92.4, 77.5, 72.5 and 69.0% w/w respectively. Thus the substitution of PLG in the microparticle matrix by hydrophilic Pluronic F127 appears to be limited to approximately 30% w/w.

The Effect of PLG and Pluronic Type on Microparticle Characteristics

Blending of Pluronic F127 in solution with different types of lactide polymers resulted in significant differences in protein loading and microparticle size. A maximum OVA loading of approximately 40% w/w was achieved using a 1:1 solution blend of 75:25 PLG and Pluronic F127 (Table III). This was

Table III. The Effect of Lactide Polymer Type and Pluronic on the Characteristics of PLG:Pluronic Microparticles

Lactide/ Glycolide Ratio	Pluronic Type	Protein Loading (% w/w)	Microparticle Size (μm)		
			vmd	d(90)	d(10)
50:50[a]	F127	25.1	11.9	26.8	2.0
75:25[a]	F127	40.5	10.1	22.4	2.3
85:15[a]	F127	16.3	19.6	38.5	3.0
100:0[a,c]	F127	—	—	—	—
50:50[b]	L104	44.2	6.2	11.9	1.2
50:50[b]	L121	40.3	4.5	8.6	1.3
50:50[b]	L123	41.2	3.9	7.3	1.2
50:50[b]	F127	45.9	4.8	9.5	1.4

[a] 1:1 PLG:Pluronic F127 solution ratio; 6% w/v concentration.
[b] 1:2 PLG:Pluronic solution ratio; 3% w/v concentration.
[c] Microparticles not formed.

more than 1.5 times that obtained using 50:50 PLG (25.1% w/w) in similar sized microparticles. The microparticle size was also found to change with different lactide polymers. The smallest particles (10.1 μm) were obtained using a blend of 75:25 PLG with Pluronic F127. Microparticle formation was not obtained using the high molecular weight D.L.PLA investigated, which suggests that the viscosity of the primary emulsion is important for successful particle production in the w/o/o method. In comparison Prieto *et al.* (14) showed recently that peptide loaded PLG microparticles were not formed, using the w/o/w technique, if the viscosity of the PLG solution used in the primary emulsion was excessive. No firm relationship can be established, however, between lactide:glycolide ratio and microparticle characteristics based on the results presented in Table 3 due to molecular weight differences among the polymers investigated.

Blending of 50:50 PLG in solution with different types of Pluronics (1:2 blend ratio) resulted in a high and fairly constant protein loading level of approximately 40% (Table III). The mean microparticle size was also found to lie in a fairly narrow range between 4 and 6 μm. Pluronic L121 is of interest because of its potential to function as a co-adjuvant in microparticles. The Pluronic L121 content of PLG:Pluronic microparticles, analysed by FTIR, was estimated at 42% w/w.

Protein Release Characteristics

Protein release studies using PLG:Pluronic F127 microparticles revealed a burst phase of approximately 8% (which is generally considered to result from loss of surface protein) followed by a short lag phase which lasted for 5 days. A steady rate of OVA release then occurred up to 30 days (Figure 1). The cumulative release of OVA, in terms of % w/w, provides an indication of delivery efficiency and can be seen to be limited at 30 days to approximately 40% of the OVA load.

The changes in release pattern and amount released (or delivery capacity) obtained by blending PLG with Pluronic F127 to produce the carrier matrix are more evident in Figure 1 which shows the cumulative release profiles in terms of μg OVA/mg of microparticles. Blending resulted in at least a three fold increase in protein release relative to PLG in similar sized microparticles. This could be advantageous, for example, for increasing the local concentration of antigen after vaccination. A cumulative release amount in excess of 100 μg OVA/mg microparticles may be achieved after one month in PBS at 37°C by increasing the Pluronic content of the starting solution.

A considerable 'burst effect' of loosely bound surface protein in the first 24 hours of release testing (amounting in some cases to 60% of the initial protein load) is generally associated with protein-loaded microparticles prepared by the o/w and w/o/w techniques (3, 4) and is often accentuated for small microparticles less than 10 μm due to a large surface protein component (15). A lag phase is then often observed when little or no protein is released. A final phase of protein release is considered to occur when bio-erosion or resorption of the polymer matrix and consequent pore formation (resulting from the removal of water soluble, degraded chain fragments), permits protein release from the microparticle core (3, 4).

The release characteristics of the PLG:Pluronic F127 microparticles described here are considered to arise from a combination of rapid dissolution of the Pluronic component of the microparticle matrix and extraction of the protein component which basically eliminates the lag phase of protein release. The resulting, extensive pore structure of the microparticles is clearly shown in Figure 2. The surface of PLG:Pluronic F127 microparticles at 0 and 2 weeks incubation is characteristically rough in contrast to PLG:PEG microparticles (6). This feature may be caused by embedded or adherent protein and Pluronic F127 which resists the washing and incubation process.

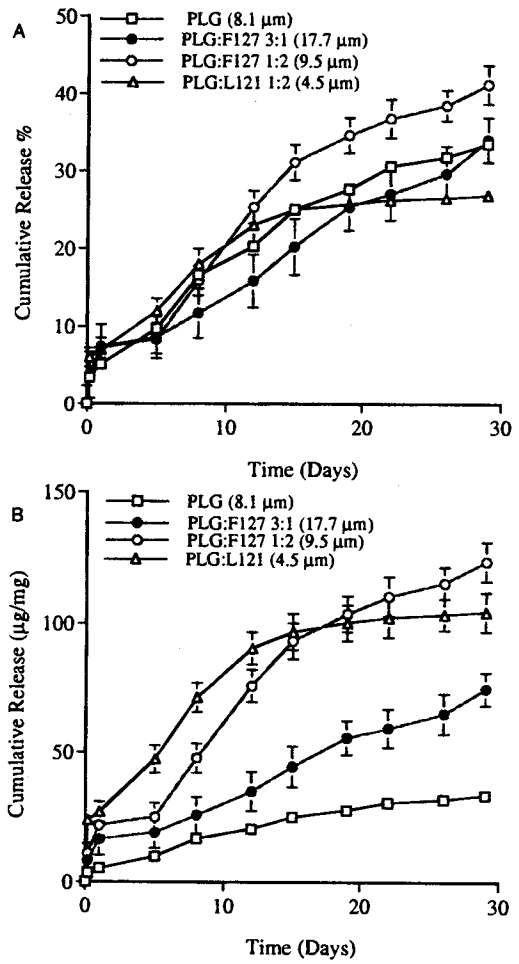

Fig. 1. Cumulative release of OVA from PLG:Pluronic microparticles. (A) % OVA release. (B) μg OVA/mg microparticles.

As discussed by Park *et al.* (12) in relation to L.PLA:Pluronic film matrices, dissolution and extraction of the Pluronic component is expected to be retarded by physical entanglements and by complex formation (due to hydrogen bonding) between PLG and Pluronic chains. Protein release from the microparticles, may however, be assisted by Pluronic modified internal surfaces (pores and channels) which would modulate protein/polymer interactions (12). The high loading of OVA can be expected to affect the structure (fine or coarse domains) and distribution of the soluble phase comprising the PLG:Pluronic microparticles and in turn exert, an influence on protein release. The development of 80–150 nm pores in the surface of PLG:Pluronic F127 microparticles, for example, (Figure 2) suggests a fairly uniform dispersion but coarse structure of the soluble phase. At present the nature of this phase (Pluronic or protein) is unknown.

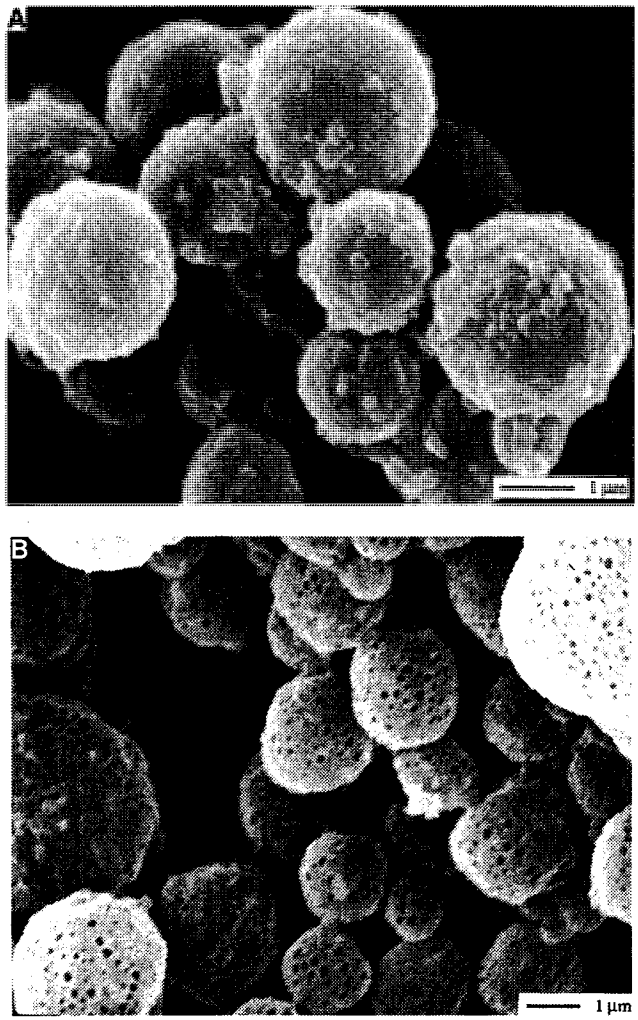

Fig. 2. Scanning electron micrograph of OVA-loaded PLG:Pluronic F127 microparticles produced from a 1:1 PLG:Pluronic F127 blend solution. a) after 2 weeks incubation in PBS at 37°C. b) after 4 weeks incubation in PBS at 37°C.

Porosity development and protein release characteristics are heavily influenced by the nature of the Pluronic copolymer used for microparticle production. In contrast to PLG:F127 microparticles, only isolated pores were visible after 4 weeks incubation of microparticles prepared using the more hydrophobic Pluronic L121 copolymer (Figure 3) (A population of particles less than 0.5 μm in size is also apparent in Figure 3.). Analysis of the Pluronic content of 1:2 PLG:Pluronic F127 microparticles by IR spectroscopy after incubation in PBS solution for 1, 2, 3 and 4 weeks yielded estimates of the Pluronic F127 content of the microparticle matrix of 12.5, 6.0, 4.0 and 2.0% respectively. In contrast, IR analysis of 1:2 PLG:Pluronic L121 microparticles yielded estimates of the Pluronic L121 content of the microparticle matrix of 23.5, 22.2, 22.0 and 20.4% respectively. Thus the more hydrophobic Pluronic L121 tends to be retained in the microparticle matrix. In comparison, Park *et al.* (12) measured a 12.6% weight loss from 80:20 blend films of L.PLA and hydrophilic Pluronic F108 after 1 week in PBS at 37°C and only a 4.5% weight loss from blend films containing the more hydrophobic Pluronic L121 copolymer.

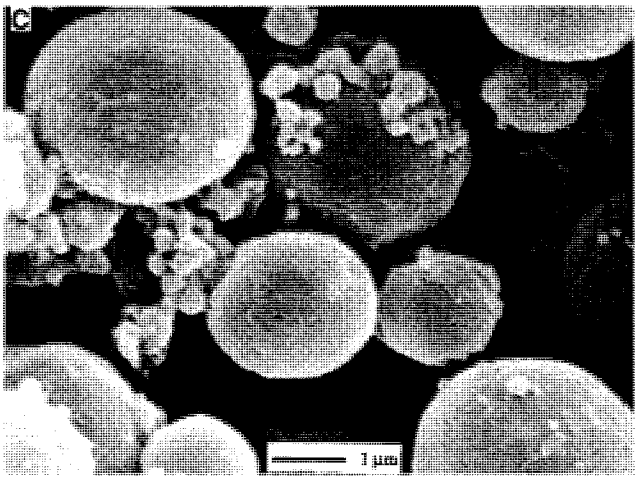

Fig. 3. Scanning electron micrograph of OVA-loaded PLG:Pluronic L121 microparticles produced from a 1:2 PLG:Pluronic blend solution after 4 weeks incubation in PBS at 37°C.

The latter system was also more effective in limiting the initial protein burst and extending the time of protein release from film matrices therein emphasising the major influence of the hydrophilicity of the Pluronic on release characteristics. In the present case the absence of developed porosity in PLG:Pluronic L121 microparticles indicates that protein release is mainly due to loss of surface protein rather than release of encapsulated protein. The tendency of the release profile to plateau for PLG:Pluronic L121 microparticles (Figure 1) supports this proposal.

Protein Integrity

SDS-PAGE analysis showed that the bands relating to ovalbumin released from PLG:Pluronic microparticles, coincided with the bands for native ovalbumin. There were no additional bands to indicate the presence of high molecular weight aggregates or fragments greater or less than 45 kDa, respectively. This would suggest that the structural integrity of ovalbumin was not significantly affected by the entrapment process. Specific testing for retained bioactivity and immunogenicity is required in order to fully gauge the merits of the technique for encapsulation of therapeutic proteins and peptides. Such investigations are in progress and will be reported separately.

SUMMARY AND CONCLUSIONS

OVA-loaded microparticles were prepared from blends of PLG and Pluronic copolymers using the water- in oil -in oil technique. Protein loading levels of over 40% w/w were measured in microparticles having a mean diameter of approximately 5 μm. The high protein loading may be attributed to employment of a methanol as a continuous phase which prevents protein partition and to effective confinement of protein within the microparticles due to the presence of Pluronic in a polymer blend with PLG. The content of 50:50 PLG in the microparticles was reduced to a minimum of approximately 70% w/w of the matrix by blending with Pluronic F127. An *in-vitro* release characteristic of OVA which was almost linear with time over 25 days, following a brief (5 days) lag phase, was exhibited by certain blend formulations

containing the hydrophilic Pluronic F127 copolymer. A delivery capacity of 3 μg OVA/mg particles/day was attained. The w/o/o technique, for preparing PLG:Pluronic microparticles, shows considerable potential for efficiently encapsulating and releasing therapeutic proteins and peptides. In addition the hydrophobic copolymer, Pluronic L121 was incorporated in microparticles with PLG which may provide a co-adjuvant function in vaccine formulations.

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