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Characterization of V3 BRU peptide-loaded small PLGA microspheres prepared by a $(w_1/o)w_2$ emulsion solvent evaporation method

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Abstract

This paper describes the conditions of preparation of poly(lactide-coglycolide) microspheres with a mean diameter lower than 10 μm obtained by a $(w_1/o)w_2$ emulsion solvent evaporation method. Different parameters influencing respectively the size of the inner emulsion and the diameter of the microspheres were determined. V3 BRU, which is a specific immunogenic fraction from GP120 of HIV, was encapsulated in those microspheres. The entrapment efficiency was shown to be superior to that of microspheres prepared according to the single emulsion solvent evaporation method. Electron microscopy observations demonstrated the presence within the microspheres of globules corresponding to the w/o initial inner emulsion in which the peptide was dissolved in the aqueous phase. Analysis of the release kinetics was carried out in phosphate buffer (PBS), in artificial gastric and intestinal medium. V3 BRU release in PBS was slow, reaching a plateau at 24 h corresponding to 25% of drug release. In addition, V3 BRU was not released in gastric medium within 4 h whereas under the same time conditions, 60% of the drug was released in the presence of intestinal medium. These results open up interesting prospects for the use of these microspheres as an oral adjuvant for HIV vaccination.

Keywords: Poly(lactide-co-glycolide); Biodegradable microsphere; Multiple emulsion; V3 BRU peptide; Oral immunization; Release kinetics

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1. Introduction

In the last 5 years, poly(lactide-coglycolide) (PLGA) microspheres have aroused considerable interest in their use as carriers for the oral delivery of peptidic antigens (Eldridge et al., 1991; O'Hagan et al., 1991). Mucosal immunity is stimulated after antigen uptake by Peyer's patches (PPs). This results in the production of secretory IgA in all mucosal sites (gut lamina propia, respiratory tract, mammary glands, salivary glands, lachrymal glands and genito-urinary tract) (Mestecky and McGhee, 1987). Many studies have made evident the uptake of polymeric particulate carriers by the gut associated lymphatic tissue (GALT) (Le Fevre et al., 1980; Eldridge et al., 1990). In addition, after being given via the oral route, microspheres can also provide protection of the entrapped antigen against enzymatic hydrolysis.

Recent studies have shown that, in order to be taken up by Peyer's patches, particles must have a diameter less than 10 μm (Eldridge et al., 1990). The current process for preparing PLGA microspheres is the solvent evaporation method. This procedure involves a o/w emulsion and therefore leads to satisfactory encapsulation rates only for hydrophobic compounds. However, many peptides with immunogenic properties are very soluble in water, and thus their entrapment in microspheres prepared via a single emulsion process remains problematic. In addition, most of the studies (O'Hagan et al., 1989, 1991) using microspheres for the oral delivery of antigens were carried out with model antigens (e.g., ovalbumin). This is of little interest, since specific immunization cannot be achieved by this means. Obviously, the recently described method using $(w_1/o)w_2$ emulsion solvent evaporation was found to be more efficient for entrapping water soluble peptides (Cohen et al., 1991).

In this report, we describe the parameters which may influence the size of PLGA microspheres prepared via a $(w_1/o)w_2$ emulsion solvent evaporation method. The entrapment efficiency of V3 BRU peptide (a specific immunogenic fraction from GP120 of HIV) in microspheres of size below 10 μm and prepared via either a single emulsion solvent evaporation method or the above-mentioned $(w_1/o)w_2$ procedure was determined. These microspheres were additionally characterized with respect to their release kinetics in artificial gastrointestinal medium. Such microspheres could offer interesting prospects in inducing mucosal immunity against HIV after oral immunization.

2. Materials and methods

2.1. Materials

Poly(dl-lactide-co-glycolide) (copolymer composition, 75:25; Mol. Wt 128000) was obtained from Birmingham Polymers, U.S.A. Poly(vinyl alcohol) (Mowiol®) was supplied by Aldrich Chemical, France and methylene choride (HPLC grade) by Prolabo, France. V3 BRU peptide (TRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAH) (33 amino acids and molecular weight of 3846.44) was synthesized using an automated peptide synthesizer (Applied Biosystems, CA, U.S.A.) according to the Boc-benzyl technique. Side chain protections were as follows: serine (benzyl); threonine (benzyl); histidine (N^{im}dinitrophenyl); arginine (tosyl); lysine (N^{ϵ}-2-chloro-benzyloxycarbonyl) and methionine (sulfoxide). Amino acids were double coupled using dicyclohexylcarbodiimide and hydroxybenzotriazole followed by capping with acetic anhydride. After treatment with hydrogen fluoride, the crude peptide was purified on a C_{18} reversed-phase column eluted with an acetronitrile-water-0.05% TFA gradient. The amino acid composition of the purified peptide, determined by using an automated Beckman 6300 amino acid analyzer (Beckman, CA, U.S.A.) after total acid hydrolysis at 110°C for 24 h in 6 M HCl, was in good agreement with the expected values.

2.2. Micropshere preparation

Microspheres prepared by the o/w solvent evaporation method were obtained by first dissolving 1.0 g of PLGA in 10 ml of methylene

chloride (MC). The resulting solution was dispersed in 100 ml of an aqueous phase containing 1% of poly(vinyl alcohol) (PVA) and 0.025% of methylcellulose. The emulsion was homogenized for 5 min using an Ultraturax® at a speed of 8000 rpm. Evaporation was carried out by mechanical stirring (700 rpm) for 3 h at room temperature. In encapsulation studies, V3 BRU peptide (1.0 mg) was dispersed in methylene chloride before emulsification in the aqueous phase.

The $(w_1/o)w_2$ solvent evaporation technique was performed by first dissolving 0.8 g of the polymer in 8 ml of methylene chloride. Then, 0.5 ml of distilled water was dispersed in the organic phase with an Ultraturax® at a speed of 24000 rpm for 2 min at room temperature to form the inner emulsion (w_1/o) (the inner emulsion refers to the first emulsion). This emulsion was poured, under mechanical stirring (700 rpm), into 80 ml of the external aqueous phase containing 2% of PVA. Stirring was maintained for 3 h in order to achieve complete solvent evaporation. For encapsulation studies, V3 BRU peptide (1–10 mg) was dissolved in the inner water phase.

The influence of the composition of the inner aqueous, organic and outer aqueous phases on the size and the aspect of the microspheres was studied. For each experiment, the viscosity of the medium was determined using a Control Stress Rheometer Carri-Med CSL 100 (RHEO, S.A. France).

After preparation (single solvent evaporation process or $(w_1/o)w_2$ method), the microspheres were collected by centrifugation (3000 rpm for 5 min), then washed three times with distilled water and freeze-dried for 24 h in the presence of 0.1% of PVA.

2.3. Microsphere characterization

The diameter of microspheres was measured by light microscopy (Olympus BH-2, France) and using a Coulter counter (Coultronics, France) both during preparation and after its completion.

Drug content in microspheres was determined as follow: 100 mg of V3 BRU loaded microspheres were dissolved in 1 ml of methylene chloride and V3 BRU peptide was extracted with 1 ml of an aqueous solution of acetic acid 5%. Peptide was assayed in the aqueous phase by HPLC (column C_{18} Novapack 3.5 × 150 mm, Waters, France). The elution phase consisted in a gradient of A (trifluoroacetic acid/distilled water, 0.05:99.95) and B (acetonitrile/distilled water/trifluoroacetic acid, 74.95:25:0.05). Detection was achieved using a UV spectrometer at 215 nm. HPLC assay allowed us to measure V3 BRU encapsulated into microspheres as well as to verify its integrity.

The partition coefficient was determined in a methylene chloride/water system and expressed as log K_p where K_p equals C_{MC}/C_W (C_{MC}, concentration of peptide in methylene chloride; C_W, concentration of peptide in water).

For freeze-fracture electron microscopy, a small drop of a pelleted aqueous suspension of microspheres was deposited on a thin copper planchette and rapidly frozen in liquid propane. Fracturing, etching and replication were conducted with a Balzers BAF 301 freeze-etching unit with platinium-carbon shadowing. The replicas, after digestion of organic material with methylene chloride and washing with alcohol, were observed using a Philips 301 electron microscope.

2.4. Peptide release kinetics

In vitro V3 BRU release kinetics from microspheres prepared by the multiple emulsion method were determined in: (i) phosphate buffer; (ii) artificial gastric medium; and (iii) artificial intestinal medium.

2.4.1. Phosphate buffer

PLGA microspheres (100 mg) were suspended in 4 ml of phosphate-buffered saline (pH 7.4) into a glass tube and incubated at 37°C. The dispersion was magnetically stirred. At different time intervals (0.02, 0.25, 0.50, 1, 2, 4, 6, 8, 24, 48 h), an aliquot of 200 μl was taken from the acceptor medium and replaced by 200 μl of fresh buffer to maintain sink conditions. The samples were centrifuged for 10 min at 4500 rpm and the V3 BRU concentration in the acceptor medium was determined by HPLC.

2.4.2. Artificial gastric medium

Samples of 25 mg of microspheres were incubated, under magnetic stirring, with 1 ml of artificial gastric medium (HCl, pepsin and sodium chloride, pH 1.2) (USP XXII/NF XVIII) at 37°C. At different time intervals (5, 30, 60, 90, 120, 150, 180, 210, 240 min) 50 μl of perchloric acid (2 N) was added to the microspheres in order to stop the enzymatic reaction. The mixture was centrifuged (10 min at 4500 rpm), and the acceptor medium was separated from microspheres containing the unreleased peptide. Due to the possible degradation of the peptide by the enzymes, only the microsphere content was determined as described above. The percentage of peptide remaining in the microspheres at time t was calculated on the basis of the initial amount of V3 BRU encapsulated into the microspheres before incubation.

2.4.3. Artificial intestinal medium

Samples of 25 mg of microspheres were incubated with 1 ml of artificial intestinal medium (pancretin, monobasic potassium phosphate, NaOH 0.2 N, pH 7.5) (USP, XXII/NF XVIII). The release kinetics were determined in the same way as for the artificial gastric medium.

2.5. Peptide degradation studies

Degradation studies were performed by placing 1 mg of V3 BRU in (a) 3 ml of artificial gastric medium and (b) 3 ml of artificial intestinal medium. The tubes were incubated at 37°C. Periodically (5, 15 30, 45, 60, 90, 120, 150, 180, 240 min), aliquots were taken and the enzymatic reaction was stopped by adding perchloric acid (2 N). The aliquots were centrifuged for 10 min at 4500 rpm and the peptide was assayed by HPLC.

3. Results and discussion

3.1. Size measurements

Using a water phase containing PVA (1%) and methylcellulose (0.025%), the single evaporation method led to the formation of microspheres with a diameter below 10 μm as shown by Coulter counter measurements (Fig. 1). To prepare microspheres with a mean diameter of less than 10 μm by the $(w_1/o)w_2$ method, the influence of different parameters on the size and appearance of the inner emulsion was studied. For all these experiments the stirring rate was kept constant. The results are summarized in Table 1. The data show that with an aqueous phase containing no PVA and with an organic phase consisting in 0.8 g of polymer dissolved in 8 ml of methylene chloride, individual globules were obtained with a diameter less than 10 μm. This emulsion was only stable in the presence of PLGA. This is probably because the PLGA polymer plays the role of a surfactant, thus stabilizing the initial emulsion. Increasing PVA concentration in the inner phase up to 2% resulted in increased viscosity of the inner water phase (7.64 mPa s) so that it was not possible to prepare an emulsion under these conditions. Similarly, increasing amounts of PLGA up to 1.6 g in the organic phase led to the same results. Using PVA at a concentration of 2 or 5% in the outer water phase resulted in microspheres with a mean diameter lower than 10 μm (Table 2). However, at a concentration of 5%, the elimination of PVA became much more difficult during the washing step. On the basis of these results, the conditions chosen for the entrapment studies were: 0.8 g of polymer dissolved in 8 ml of methylene chloride

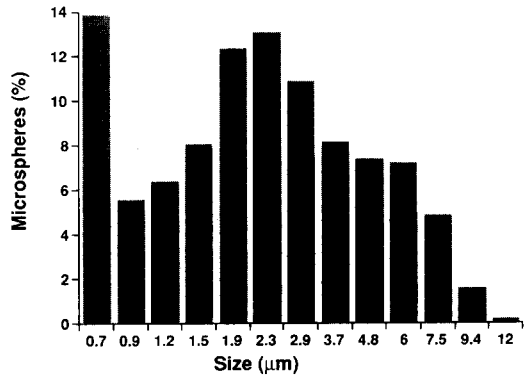

Fig. 1. Size distribution of microspheres prepared by the o/w solvent evaporation method, as determined with a Coulter counter.

Table 1
Influence of the volume of methylene chloride, and the amount of polymer and polyvinylic alcohol in the water phase on the diameter and aspect of the inner emulsion

w_1 PVA (%)	Viscosity of w_1 (mPa s)	CH_2Cl_2 (ml)	PLGA (g)	Viscosity of o (mPa s)	Globule diameter [a] (μm)
0		8	0.8	28.54	(1–10)
	4.20	8	0.8		100% < 10
1	7.64	8	0.8	28.54	(1–10)
					90% < 10
2				28.54	N.F.
0		8	1.6	284.50	N.F.
1	4.20	8	1.6	284.50	N.F.
2	7.64	8	1.6	284.50	N.F.
0		4	0.4	28.54	50% > 50
1	4.20	4	0.4	28.54	50% > 50
2	7.64	4	0.4	28.54	non-homogeneous
0		4	0.8	284.50	N.F.
1	4.20	4	0.8	284.50	N.F.
2	7.64	4	0.8	284.50	N.F.

N.F., not formed.
[a] Measured by light microscopy.

and emulsified with 0.5 ml of distilled water. This emulsion was dispersed in 80 ml of distilled water containing 2% of PVA. Under such conditions, 85% of the particles obtained were below 10 μm (Fig. 2). Classical solvent evaporation is an usual method for obtaining microspheres of the desired size (Jeffery et al., 1991), and does not allow high encapsulation efficiency with hydrophilic compounds such as peptides (Bodmeier and McGinity, 1987). Therefore, we have modified the $(w_1/o)w_2$ technique so that the original method producing microspheres with a diameter greater than 50 μm (Cohen et al., 1991; Iwata and McGinity, 1992) could lead to the preparation of smaller microspheres. Previously, Morris and Warburton (1982) also succeeded in preparing microcapsules with a mean diameter of 10 μm by the $(w_1/o)w_2$ solvent evaporation method. However, these particles were made of ethyl acetate and acacia which polymers are clearly not suitable for oral administration of vaccines. Indeed, such particles are not biodegradable and are too hydrophilic. This latter property is not consistent with one of the required conditions for the up-

Table 2
Influence of polyvinylic alcohol concentration in the outer water phase on microsphere diameter

w_2 PVA (%)	Viscosity (mPa s)	Diameter (μm)
1	4.20	1–50
		70% < 30
2	7.64	1–20
		90% < 10
5	84.71	1–10
		90% < 5

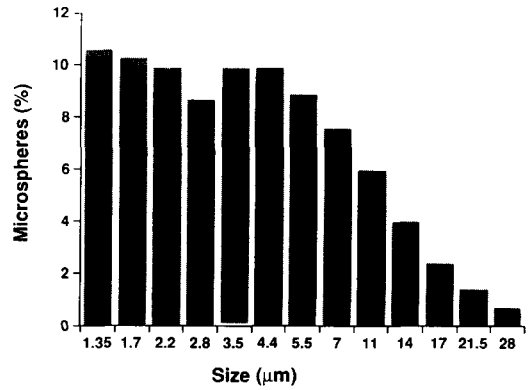

Fig. 2. Size distribution of microspheres prepared by the $(w_1/o)w_2$ solvent evaporation method.

take of Peyer patches, namely, that of the particles being hydrophobic (Eldridge et al., 1990).

3.2. Encapsulation efficiency of V3 BRU peptide

V3 BRU peptide is a 33 amino acid synthetic peptide immunogenic against HIV (Javaherian et al., 1989). The partition coefficient in the methylene chloride/water system gave a value of log K_p of -1. It was found that this highly water soluble V3 BRU was completely entrapped (100%) within microspheres obtained by the $(w_1/o)w_2$ solvent evaporation method whereas only 37% was encapsulated using the single solvent evaporation method. This was likely due to the leakage of the peptide from the organic phase (where it was dispersed) to the aqueous phase where, with regard to the partition coefficient, it was much more soluble. In contrast, the $(w_1/o)w_2$ solvent evaporation technique allowed the peptide to be entrapped in the inner aqueous phase surrounded by methylene chloride. Since the peptide had very low solubility in methylene chloride, its diffusion across this phase to reach the outer aqueous phase should be strongly reduced. In addition, the presence of the peptide appeared to affect neither the size nor the shape of the microspheres (data not shown). Increasing the amount of V3 BRU peptide in the initial emulsion to a value of up to 10 mg only slightly reduced the entrapment efficiency (Fig. 3). Finally, an encapsulation rate of 0.92 mg/100 mg of polymer was obtained when 10 mg of peptide was dissolved in the inner aqueous phase of the emulsion.

3.3. Electron microscopy

For electron microscopy observations, it was necessary to decrease the temperature of fracture as much as possible (-150°C) in order to obtain a cross fracture through the microspheres. After fracturing, the temperature was increased to -100°C and the samples were etched for 5 min before shadowing. Many microspheres were observed with a size ranging from 4 to 17 μm. Each microsphere contained several cavities that were clearly evident after etching. When the microspheres were prepared in the presence of the peptide, most of the cavities exhibited particles which were supposed to correspond to agglomerates of peptide molecules (Fig. 4). Within each microsphere, the cavities are separated by the polymer core exhibiting characteristic oriented striations due to plastic deformation occurring in the direction of the fracture.

3.4. Release kinetics

Fig. 5 shows the release profile of V3 BRU from PLGA microspheres in phosphate buffer. Only 25% was released in 48 h. The microspheres exhibited a faster release phase, which was followed by a slower phase. The faster release phase was probably due to a burst effect, resulting from the release of the fraction of peptide adsorbed onto the surface of the microspheres. The peptide fraction that was entrapped within the internal globules was released very slowly.

The release of V3 BRU peptide from microspheres and its degradation in gastric and intestinal medium are depicted in Fig. 6 and 7, respectively. In gastric medium, the peptide was not released from microspheres, whereas 60% of V3 BRU was released after 4 h in the intestinal medium. This indicates that V3 BRU was totally protected in simulated gastric medium. On the other hand, the degradation of peptide was more extensive in the intestinal medium (100% after 4 h) than in gastric medium (80% after 4 h) (Fig. 7). Microencapsulation is currently used to protect

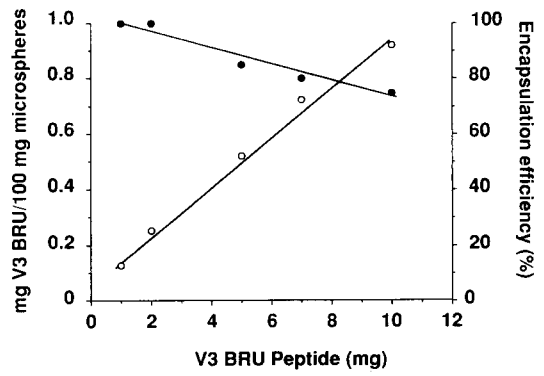

Fig. 3. Entrapment efficiency (●) and percentage of V3 BRU peptide entrapped (○) in microspheres by a $(w_1/o)w_2$ solvent evaporation method.

an active drug against degradation or to favor its delivery to a specific tissue of the body. Oral administration of peptide vaccines with the aid of microspheres required a particle size below 10 μm together with the protection of the peptide from gastrointestinal enzymatic hydrolysis (Eldridge et al., 1990). PLGA is a biodegradable polymer frequently used for this purpose. Although classical solvent evaporation is a usual method to obtain microspheres of the desired

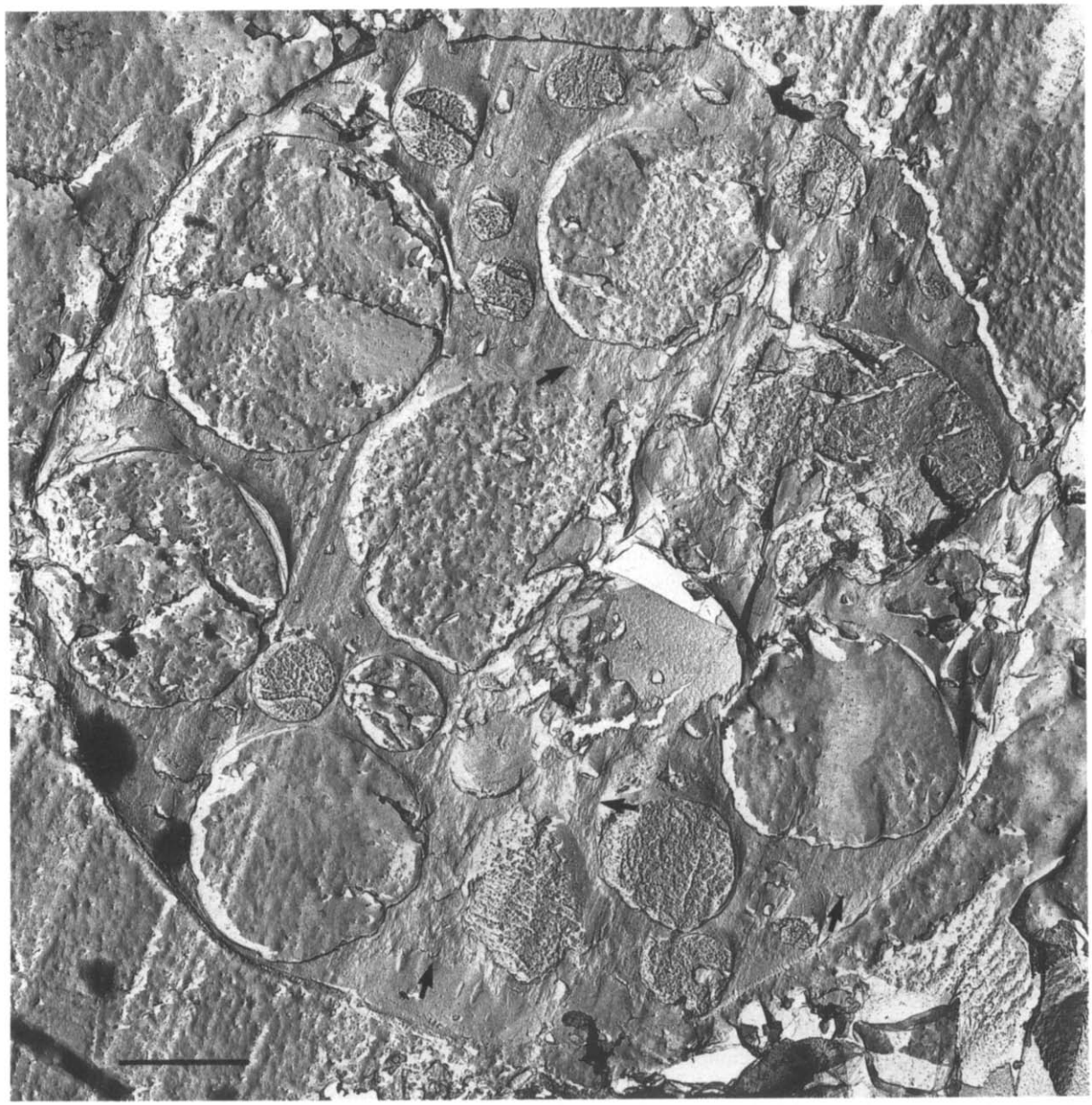

Fig. 4. Freeze-fracture electron microscopy of one microsphere. Many cavities, a few of which are smooth but most of which exhibit particles, are separated by the polymer core (arrow). See text. Bar = 2 μm.

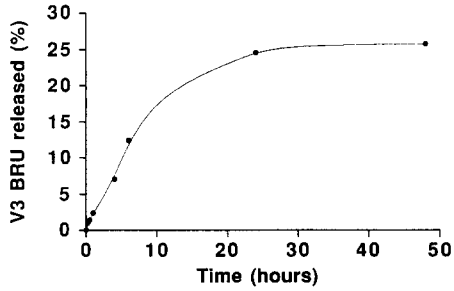

Fig. 5. Release kinetics of V3 BRU from PLGA microspheres in PBS at 37°C.

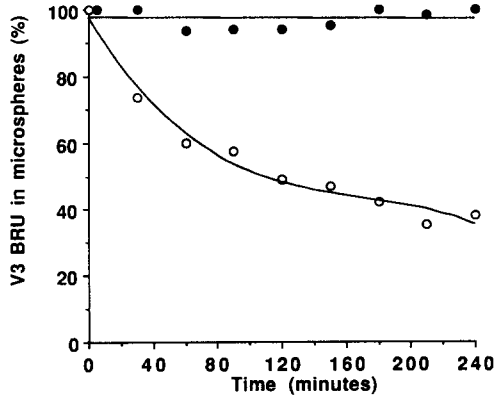

Fig. 6. Percentage of V3 BRU remaining in microspheres, after incubation in artificial gastric medium (•) and artificial intestinal medium (○).

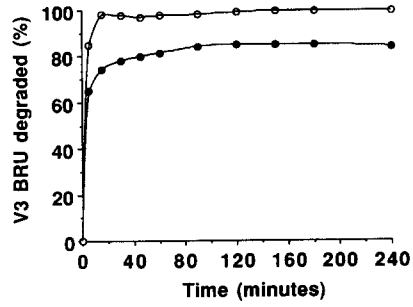

Fig. 7. Degradation of V3 BRU peptide in both, gastric (•) and intestinal medium (○).

size (Jeffery et al., 1991), this technique does not permit a high encapsulation efficiency to be attained with hydrophilic compounds such as peptides (Bodmeier and McGinity, 1987). Therefore, we have modified the $(w_1/o)w_2$ technique in which the internal phase was an aqueous solution of the V3 BRU peptide. Several parameters were studied in order to obtain small PLGA microspheres with a high encapsulation ratio.

Acknowledgements

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References

Bodmeier, R. and McGinity, J.W., The preparation and evaluation of drug-containing poly(dl-lactide) microspheres formed by the solvent evaporation method. *Pharm. Res.*, 4 (1987) 465–471.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R., Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm Res.*, 8 (1991) 713–720.

Eldridge, J.H., Hammond, C., J., Meulbroek, J.A., Staas, J.K., Gilley, R.M. and Tice, T.R., Controlled vaccine release in the gut-associated lymphoid tissues: I. Orally administered biodegradable microspheres target the Peyer's patches. *J. Controlled Release*, 11 (1990) 205–14.

Eldridge, J.H., Staas, J.A., Meulbroek, J.R., McGhee, J.R., Tice, T.R. and Gilley, R.M., Biodegradable microspheres as a vaccine delivery system. *Mol. Immunol.*, 28 (1991) 287–294.

Iwata, M. and McGinity, J.W., Preparation of multi-phase microspheres of DL-lactic acid and poly(DL-lactic-co-glycolic acid) containing a w/o emulsion by a multiple emulsion solvent evaporation technique. *J. Microencapsul.*, 9 (1992) 201–214.

Javaherian, K., Langlois, A.J., McDanal, C., Ross, K.L., Eckler, L.I., Jellis, C.L., Profy, A.T., Rusche, J.R., Bolognesi, D.P., Putney, S.D. and Matthews, T.J., Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci. USA*, 86 (1989) 6768–6772.

Jeffery, H., Davis, S.S. and O'Hagan, D.T., The preparation and characterisation of poly(lactide-co-glycolide) microparticles: I. Oil-in-water emulsion solvent evaporation. *Int. J. Pharm.*, 77 (1991) 169–175.

LeFevre, M.E., Hancock, D.C. and Joel, D.D., Intestinal barrier to large particulates in mice. *J. Toxicol. Environ. Health*, 6 (1980) 691–704.

Mestecky, J. and McGhee,.J.R., Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosyn-

thesis and immune response. *Adv. Immunol.*, 40 (1987) 153–245.

Morris, N.J. and Warburton, B., Three-ply w/o/w microcapsules formed by a multiple emulsion technique. *J. Pharm. Pharmacol.*, 34 (1982) 475–479.

O'Hagan, D.T., Palin, K., Davis, S.S., Artursson, P. and Sjoholm, I., Microparticles as potencially orally active immunological adjuvants. *Vaccine*, 7 (1989) 421–424.

O'Hagan, D.T., Rahman, D., McGee, J.P., Jeffery, H., Davies, M.C., Williams, P., Davies, S.S. and Challacombe, S.J., Biodegradable microparticles as controlled release antigen delivery systems. *Inmunology*, 73 (1991) 239–242.