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Production and characterization of biodegradable microparticles for the controlled delivery of proteinase inhibitors

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Abstract

An in-liquid drying process, based on the use of a water-in-oil-in-water (w/o/w) emulsion, for the microencapsulation of soybean trypsin inhibitor (SBTI) and basic pancreatic trypsin inhibitor (BPTI) is described. For the microparticle production, three different polymers were used, namely: poly(L-lactide), L-PLA; poly(DL-lactide), DL-PLA and poly(DL-lactide-co-glycolide) 50:50, DL-PLG. Optical and scanning electron microscopy were employed in order to study the external and internal morphology of microparticles. Rather different structures were observed, depending on the polymer and peptides employed. Swelling kinetics of microparticles and polymer degradation were also determined. Swelling analysis was carried out by an in vitro method based on the use of a viscous medium enabling the study of changes of individual microparticles. Peptide encapsulation and release kinetics from microparticles were determined by evaluating their in vitro inhibitory activity on trypsin. These experiments demonstrated that both SBTI and BPTI are still active after microencapsulation. Taken together our results indicate that the microparticles produced can be proposed as a non-parenteral controlled release system for trypsin inhibitors.

Keywords: Biodegradable microparticles; Proteinase inhibitors; Soybean trypsin inhibitor (SBTI); Basic pancreatic trypsin inhibitor (BPTI)

1. Introduction

Recent advances in biotechnology have seen an increase in the use of proteins and peptides for therapeutic applications. These protein and peptide drugs are very potent and effective at low concentration but generally have the disadvantage of requiring parenteral administration. Therefore, the development of delivery systems enabling alternative administration routes has become necessary (Story, 1991). For instance oral, transdermal, nasal and buccal are only some of the possible routes for peptide efficient administration. Nevertheless, it should be remembered that the oral route, although offering the greatest ease of application, presents several obstacles to drug absorption and stability. In particular peptides orally administered are prone to hydrolysis and enzymatic digestion that occurs in the stomach, in the intestinal lumen and at brush border level (Amidon et al., 1991). The degradation leads to low concentrations of peptide at target tissue level,

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Table 1
Characteristics of SBTI and BPTI inhibitors.

Parameters	SBTI	BPTI
Mw	20 000 Da	6500 Da
Source	Soybean meal	Bovine pancreas and lung
Structure	181 amino residues two disulphide bridges	1 chain (58 amino residues) three disulphide bonds
Activity	block of conversion of prothrombin to thrombin	Formation of a β-trypsin-BPTI complex
Stability	Denatured by heating to 80°C digested by pepsin at pH 2 or 1.5	Stable to high temperature, acid, alkali and enzymes
Kinetic properties	Stoichiometric inhibition of trypsin	Stoichiometric inhibition of trypsin

(Kassel, 1970a,b)

thus requiring repeated administrations. Hence the primary need for successful oral delivery of peptides is protection against proteolysis. Moreover, proteins and peptides have poor stability in solution as well as lyophilized preparations (Mrsny, 1991). Microparticles can well be proposed as a delivery system for peptides, having the advantage of providing protection against proteolytic degradation, offering a physical protection to the encapsulated compounds (Story, 1991). In addition, microparticles can modulate drug release, achieving optimal therapeutic drug profiles, and offering more comfort for the patient (Langer, 1990; Kissel et al., 1991).

The objective of this study was to produce and to characterize microparticles made of biodegradable polymers as a controlled delivery system for proteinase inhibitors, namely soybean trypsin inhibitor (SBTI) and basic pancreatic trypsin inhibitor (BPTI).

In particular this report describes: (a) the production of biodegradable microparticles by using different polymers (poly(L-lactide), L-PLA; poly(DL-lactide), DL-PLA; poly(DL-lactide-co-glycolide) 50:50, DL-PLG) and in-liquid drying preparation processes, (b) the influence of polymers and preparation procedures on microparticle characteristics (i.e., morphology and encapsulation yield) (c) the influence of polymers on swellability and degradability of microparticles and finally (d) the release profiles of trypsin inhibitors from the different microparticles.

With respect to the therapeutic value of the proteinase inhibitors studied here, it should be remembered that proteinases play a central role in several biological processes, such as digestion, hormone release, coagulation and complement activation. Moreover proteinases are involved in many pathological states including tumour progression and metastasis, inflammatory processes, pulmonary emphysema, acute pancreatitis and muscular dystrophy (Schnebli and Braun, 1986). In this view proteinase inhibitors, due to their potential biological properties have been and will be more and more utilized as drugs. In addition, such inhibitors have been efficiently used to protect peptide drugs from enzymatic degradation, promoting passage through the stomach and the intestinal tract, thus proteinase inhibitors are also recommended as coadjutants in formulations for oral delivery (Story, 1991).

2. Materials and methods

2.1. Chemicals

L-PLA, DL-PLA and DL-PLG, were supplied by BPI Birmingham Polymers, Inc. (Birmingham, Alabama, USA). SBTI was from Fluka Chemicals, Switzerland and BPTI (Trasylol® NK) was from Bayer AG, Germany. The main characteristic features of the two inhibitors are reported in Table 1.

2.2. Microparticle production

Microparticles were produced by an in-liquid drying process. The method was based on a water-in-oil-in-water (w/o/w) double emulsion (Esposito et al., in press). Briefly, an aqueous solution containing 10 mg of trypsin inhibitor was emulsified in 10 ml of CH_2Cl_2 containing 500 mg of polymer, by using an ultra-turrax. The primary emulsion was stabilised by adding to the aqueous phase 3% gelatin (w/w). The emulsion was then dispersed into 100 ml of an aqueous solution containing 0.25% (w/v) of 88% hydrolysed polyvynilalcohol (PVA) and 5% (w/v) NaCl, stirred at 300 rpm by a four blade turbine impeller. After 3 h, microparticles were isolated by filtration.

2.3. Microparticle morphology

Microparticle morphology, size and size distribution were evaluated by observation on optical and electron microscopy.

In order to study the internal morphology, dried microparticles were sectioned under a binocular microscope. Sectioned particles were gold coated (Edwards Sputter coating S150). Internal and external morphology was analysed at 15–20 kV by a scanning electron microscope (Cambridge S 360).

2.4. Determination of proteinase inhibitors

Encapsulation yield and release kinetics of SBTI and BPTI were determined by evaluating in vitro their inhibitory activity on trypsin.

Inhibitor calibration curves were made by preparing solutions in borate buffer containing increasing amounts of the polypeptides (50–300 mg/ml) and plotting their respective inhibitory activity against concentration.

The assay used for determining the activity of both peptide drugs was based on the inhibition of tryptic hydrolysis in 0.2 M triethanolamine buffer pH 7.8 at 25°C on *N*-α-benzoyl-D,L-arginine *p*-nitroanilide, measured by following the change in absorbance at 405 nm. SBTI and BPTI concentrations were calculated according to the method of Menegatti et al. (1985).

2.5. Release kinetics

Each microparticle sample (20 mg) was suspended in 40 ml of isotonic borate buffer (pH 7.5) and the resulting suspension was placed in sealed vials of appropriate volume, afterwards vials were placed in a horizontal shaker at 100 rpm. The release medium was periodically pipetted out from the vials and the same volume of fresh medium was replaced. 1 ml of release medium was analysed for trypsin inhibitory activity as above reported. Release profiles were determined three times in independent experiments.

2.6. Swelling and degradation tests

Microparticle swelling and polymer degradation were determined by pouring the particles in a hydrophilic gel (4%, w/v carboxymethyl cellulose sodium salt, having a viscosity of 13600 cps). Microparticles containing gels were incubated at 37°C. To study the kinetics of the increase of the initial diameter of particles and morphology changes, optical photomicrographs were taken at different times for up to 90 days. The swelling ratio q was calculated according to Eq. (1) by measuring the diameter of the microparticles, assuming a spherical geometry

$$q = \frac{V_s}{V_d} \tag{1}$$

where V_s and V_d are the volume of swollen and dried microparticles respectively.

3. Results and discussion

3.1. Microparticle production

With the aim of studying an adequate delivery system for non-parenteral administration of proteinase inhibitors, biodegradable microparticles were produced. Microparticles were prepared by using different polymers in order to investigate their influence on microparticle morphology, encapsulation yield, swelling, degradability and release kinetics.

Table 2
Recovery efficiency and encapsulation yield of biodegradable microparticles

Parameter	SBTI Microparticles			BPTI Microparticles		
	DL-PLG	L-PLA	DL-PLA	DL-PLG	L-PLA	DL-PLA
Microparticle recovery (%) [a]	91	92	95	92	90	93
Theoretical drug content (%) [b]	2.15	1.8	2.16	1.21	0.89	1.12
Actual drug content (%) [b]	0.83	1.57	0.76	0.55	0.12	0.23
Incorporation efficacy (%) [c]	38.84	18.92	35.5	45.53	13.66	20.82

[a] Percentage (w/w) of isolated microparticles with respect to the starting amount of polymer utilized for microparticle preparation.
[b] Percentage (w/w) of peptide content with respect to microparticles.
[c] Percentage (w/w) of encapsulated peptide with respect to the total amount used.

Microparticle preparation was performed by a w/o/w multiple emulsion method based on the placement of the peptide drug in the internal phase of a double emulsion, along with a stabilizer able to minimize loss of drug towards external phase, during evaporation (Okada et al., 1987). In a preliminary study (Esposito et al., in press), we investigated the effect of various stabilizers of the primary emulsion on the encapsulation yield of a model drug. Different hydrophilic polymers were studied, namely gelatin, pectin and pemulen (acrylates/C10-30 alkyl acrylate crosspolymer) demonstrating that gelatin is the best stabilizer, both in term of microparticle morphology and encapsulation efficiency.

Moreover, with the aim of obtaining high incorporation efficacy, a concentrated salt solution as a continuous phase was employed (Joly et al., 1994). In particular, a 5% (w/v) NaCl solution allowed the increase of the incorporation efficacy of both the peptides, whilst higher salt concentrations, for instance 10%, result in particle-particle agglomeration and in significant changes of microparticle shape. Recovery efficiency and microparticle encapsulation yields are reported in Table 2.

3.2. Microparticle morphology

Microparticles with different polyesters (namely, L-PLA, DL-PLA and DL-PLG), were produced and characterized. Optical and scanning electron microscopy were employed in order to analyze the external and internal structure of microparticles, since it is well known that morphology of microparticle influences the release of the encapsulated drug (Schugens et al., 1994). Fig. 1 reports electron micrographs of SBTI and BPTI containing microparticles. As is clearly evident, the preparation procedure does not lead to the aggregation/agglomeration phenomena often encountered in microparticle production, moreover microparticles show good spherical geometry. In addition, in Fig. 1 (panels C, F and I) frequency distribution plots are reported. One can observe that SBTI and BPTI microparticles constituted of DL-PLG and L-PLA show almost superimposable size and size distribution. A rather different behaviour was observed in the case of DL-PLA, where SBTI containing microparticles present larger size and size distribution with respect to BPTI containing microparticles.

Table 3 reports the geometrical parameters of SBTI or BPTI containing DL-PLG, DL-PLA and L-PLA microparticles, showing that microparticle mean diameters vary from 145 to 279 μm for SBTI and from 129 to 247 μm for BPTI containing microparticles, as a function of the polymer used.

In Fig. 2 and Fig. 3 microphotographs of SBTI or BPTI containing DL-PLG, DL-PLA and L-PLA microparticles are reported, showing at a higher magnification both their external and internal morphologies. In the case of the DL-PLG microparticle, a matrix type structure can be observed characterized by a number of little spheres in its interior (Fig. 2A and Fig. 3A). This structure reflects the aqueous droplets present in the

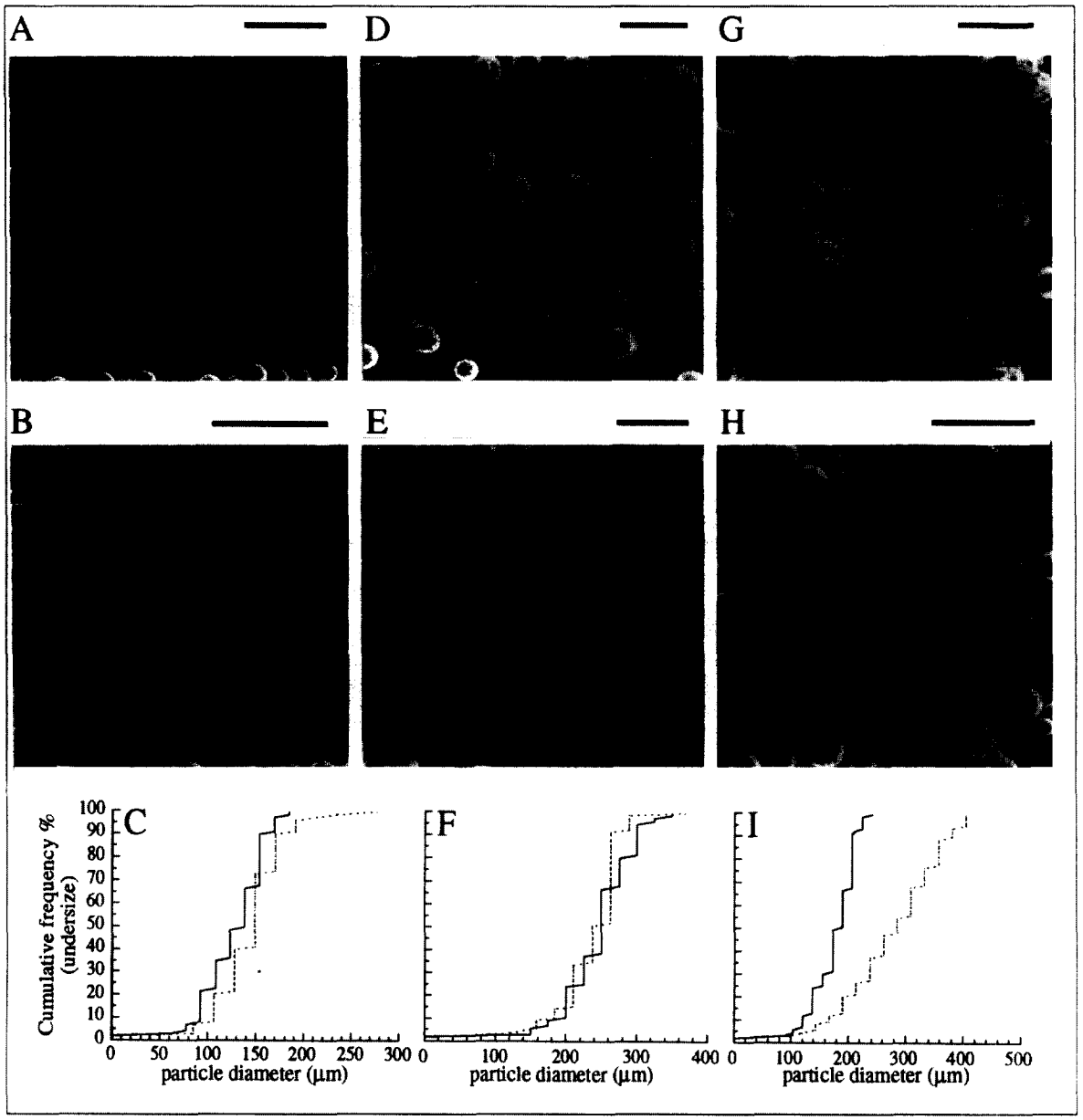

Fig. 1. Morphological and dimensional analysis of biodegradable microparticles. Scanning electron micrographs of SBTI (A, D, G) and BPTI (B, E, H) containing microparticles, prepared with DL-PLG (A, B), L-PLA (D, E) and DL-PLA (G, H). Bars correspond to 500 μm. Frequency distribution plots of microparticles produced with DL-PLG (C), L-PLA (F) and DL-PLA (I); solid line: SBTI; dotted line: BPTI.

primary w/o emulsion utilized for particle production. DL-PLG microparticles display a smooth surface both in the case of SBTI (Fig. 2A) and BPTI (Fig. 3A).

Also L-PLA microparticles show a matrix type structure, even if the internal spheres appear slightly larger and the surface more wavy, as shown in Fig. 2B and Fig. 3B.

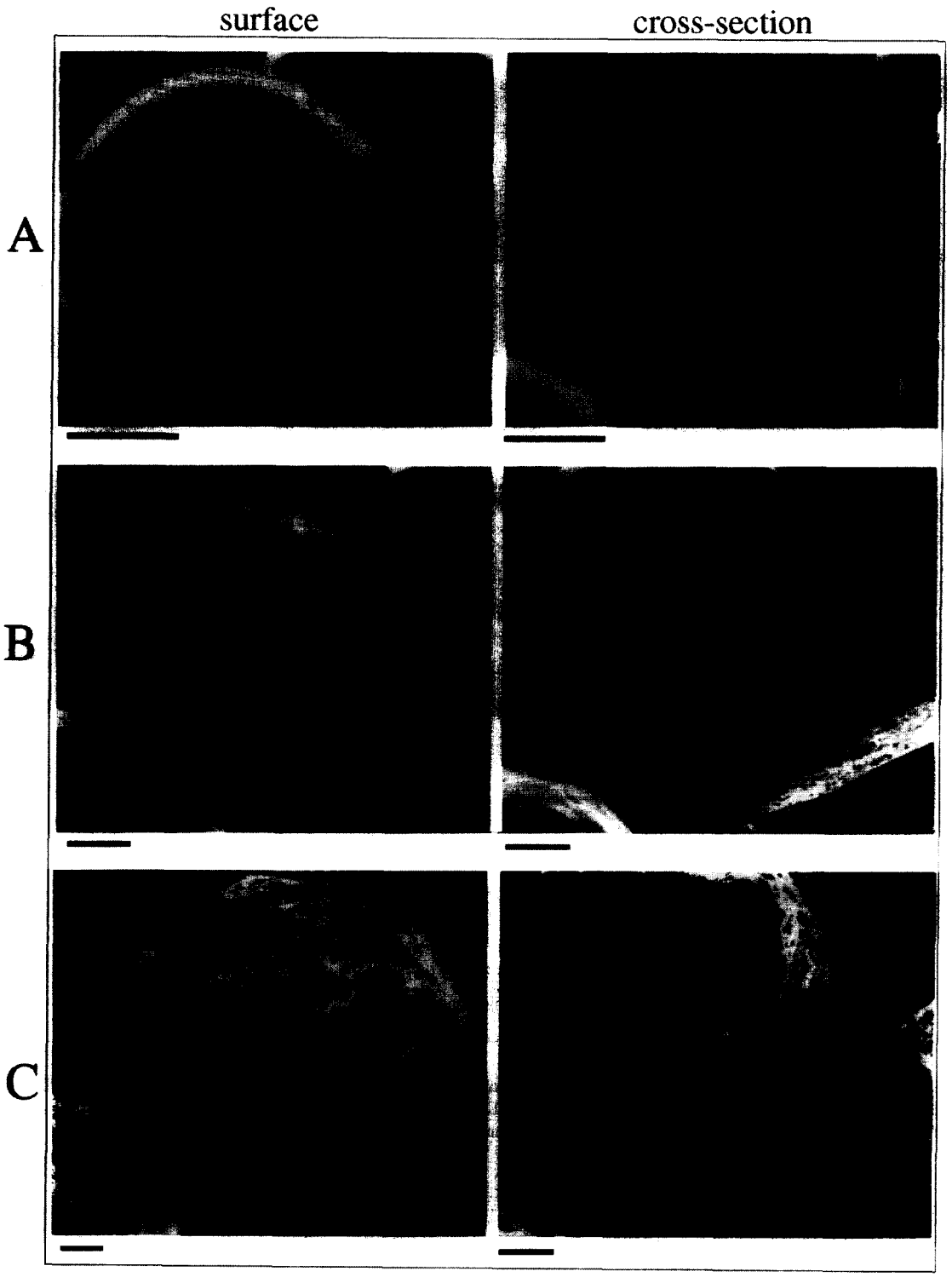

Fig. 2. Scanning electron micrographs showing the external and internal morphology of SBTI containing microparticles constituted of DL-PLG (A), L-PLA (B) and DL-PLA (C). Bars correspond to 50 μm.

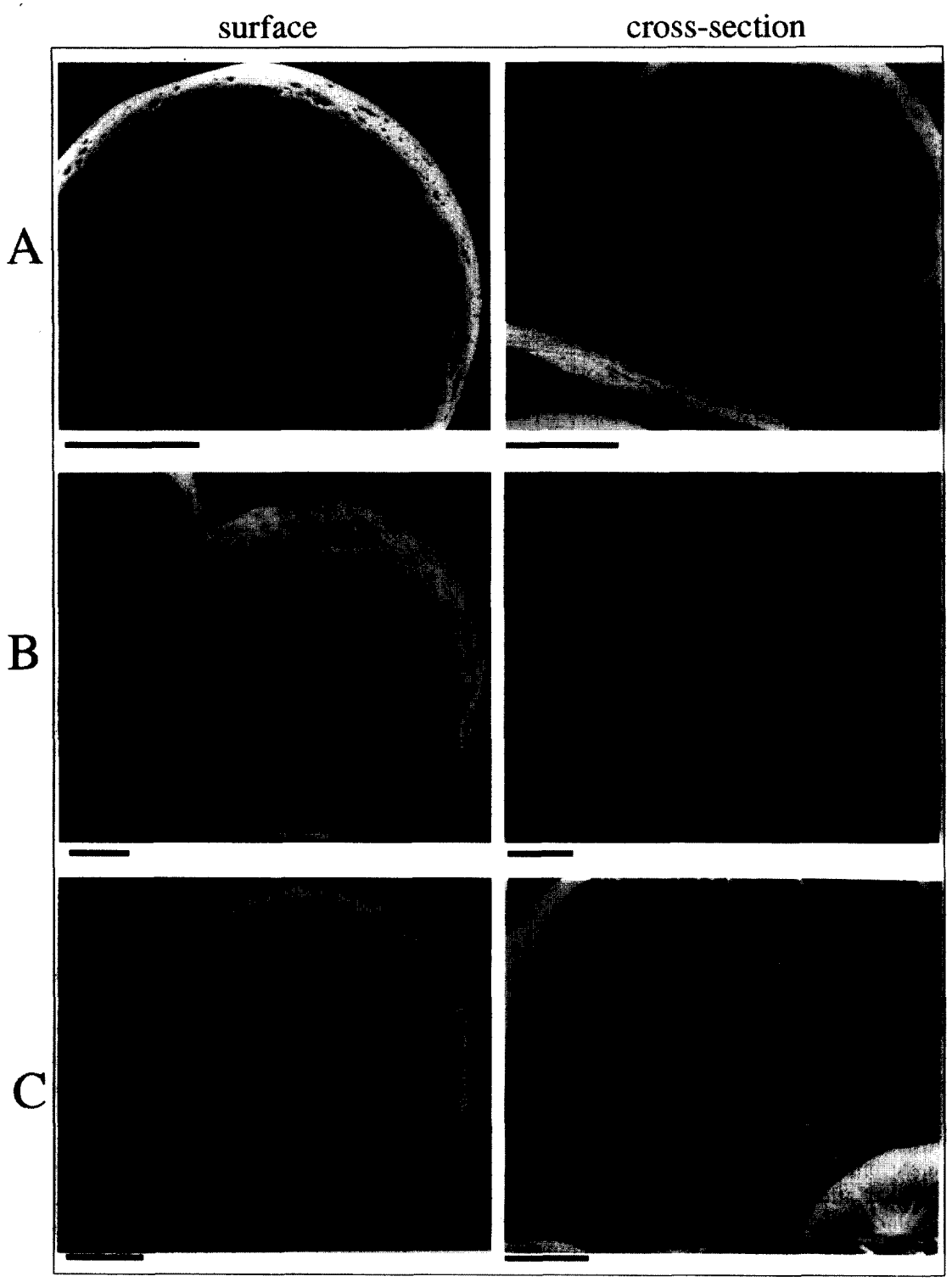

Fig. 3. Scanning electron micrographs showing the external and internal morphology of BPTI containing microparticles constituted of DL-PLG (A), L-PLA (B) and DL-PLA (C). Bars correspond to 50 μm.

Table 3
Geometrical parameters of biodegradable microparticles

Parameter	SBTI Microparticles			BPTI Microparticles		
	DL-PLG	L-PLA	DL-PLA	DL-PLG	L-PLA	DL-PLA
Mean diameter	145.47	238.48	279.46	129.03	247.81	177.22
SD	32.9	40.88	36.21	28.28	43.79	33.66
SE	3.68	4.57	8.52	3.16	4.9	376
Geometric mean	141.93	234.51	267.72	125.72	243.81	173.66
Kurtosis	2.31	1.57	−0.72	−0.81	−0.18	−0.38
Skewness	0.83	−0.59	−0.27	−0.23	−0.06	−0.58
Surface aspect	smooth	wavy	spongiform irregular	smooth	wavy	smooth

Different morphologies are appreciable in microparticles made of DL-PLA. In the case of SBTI containing particles (Fig. 2C) indeed, a multivesicular system is evident. The big pores within the inner structure of particles can be attributed to a partial coalescence of the internal aqueous droplets of the primary w/o emulsion (Schugens et al., 1994). BPTI containing microparticles, on the other hand, are double walled microcapsular systems (Fig. 3C), these microparticles are constituted by an external compact layer and an inner layer characterized by a porous matrix. Finally, the surface of DL-PLA particles appears spongiform, irregular and with several pores in the case of SBTI, whilst it is smooth and regular in the case of BPTI.

3.3. Determination of proteinase inhibitors

The encapsulation efficiencies and the release kinetics were determined by evaluating the in vitro biological activity of proteinase inhibitors on trypsin. In particular inhibitory activity and inhibitor concentration were measured by forming a trypsin-inhibitor complex and by determining the decrease in the trypsin hydrolysis of the substrate.

It should be noted that this method not only allows a quantitative determination of the proteinase inhibitors but it also enables demonstration that after microencapsulation both peptides maintain their biological function. On the contrary, the use of analytical methods commonly employed to study the release of peptides from microdevices (e.g., spectroscopic methods, gel electrophoresis and radioactive tracers) can fail in evidencing possible loss of peptide activity due to its denaturation during the microencapsulation procedure.

3.4. Microparticle swelling and degradation

In order to compare swelling and degradation characteristics of DL-PLG, DL-PLA and L-PLA microparticles, in vitro experiments were performed. In particular, the swelling kinetics were studied in a carboxymethyl cellulose sodium salt hydrophilic gel. This approach was chosen to investigate the behaviour of the produced microparticles when placed in hydrophilic media simulating biological fluids. The viscosity of the gel permits maintaining the microparticles in fixed positions during microscopic observation. For this reason the method was proposed to study the swelling of individual particles, allowing analysis of size influence on swelling and possible inter particle interactions, together with the average swelling of a microparticle population (Esposito et al., 1994). This method allows also the evaluation of the morphological changes of single microparticles accompanying the in vitro degradation of the polymeric matrix.

Details of optical photomicrographs showing individual microparticles after 10, 30, 60 and 90 days of incubation at 37°C (panel A) together with swelling kinetics of the different microparticles (panel B) are reported in Fig. 4.

In the case of DL-PLG microparticles, by 30 days of incubation significant degradation takes

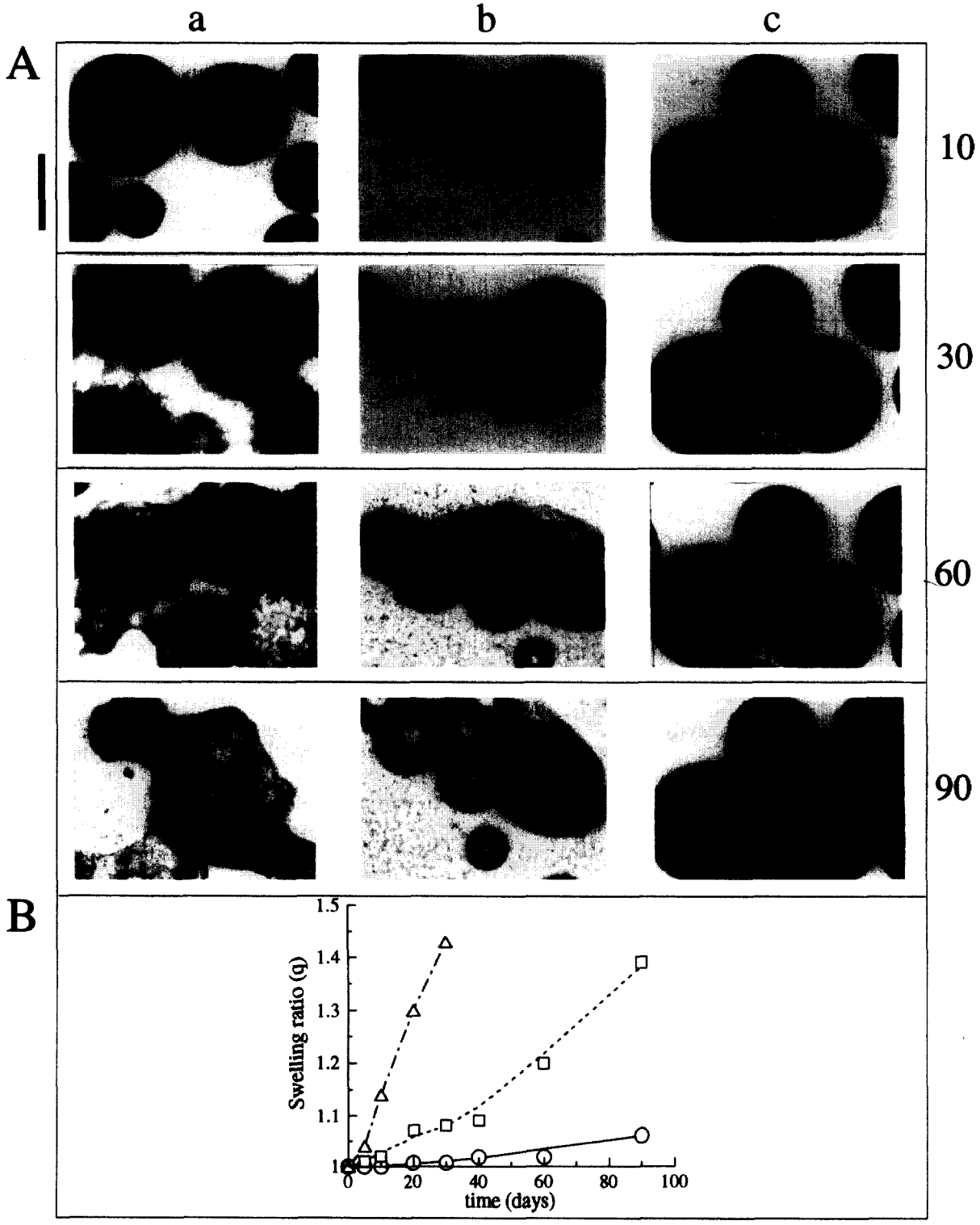

Fig. 4. Swelling kinetics of biodegradable microparticles. A: Microscopical analysis of DL-PLG (column a), DL-PLA (column b) and L-PLA (column c) microparticles; optical photomicrographs were taken after 10, 30, 60 and 90 days. The bar corresponds to 60, 100 and 48 μm for columns a, b and c, respectively. B: Swelling kinetics of DL-PLG (△), DL-PLA (○) and L-PLA (□) microparticles. In the case of DL-PLG microparticles swelling is plotted only up to 30 days, since after that period of time almost complete particle degradation occurs.The swelling test was carried out as described in the Materials and methods section.

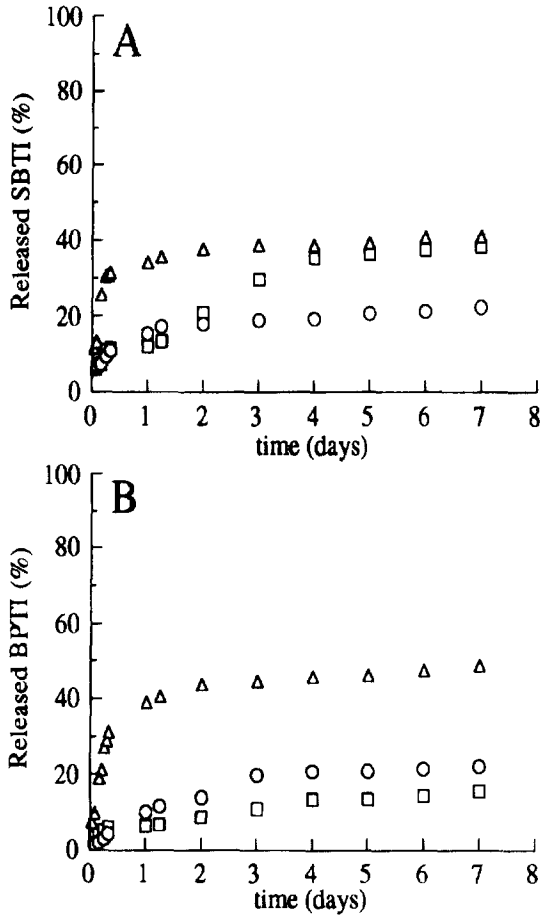

Fig. 5. Release profiles of SBTI (A) and BPTI (B). ○: DL-PLA, □: L-PLA, △: DL-PLG microparticles. Data represent the average of three independent experiments, standard deviations were within ± 8.

place. At this time particles show a pronounced porosity, no longer maintaining a well defined water/polymer interface. On the contrary, both DL-PLA and L-PLA microparticles, after 30 days, do not present appreciable variations in porosity. DL-PLA microparticles present minor changes in their texture only after 60 days, possibly reflecting an increased porosity. In the case of L-PLA microparticles, on the other hand, appreciable variations in structure occur after 90 days of incubation.

This different degradability is ascribed to both polymer type and molecular weight. It is well known that degradation of the polymer is related to presence and content of glycolide units (O'Hagan et al., 1994). Glycolide units in fact, being more hydrophilic than lactide units, promote water uptake into the polymer, thus favouring hydrolitic degradation which is consistent with swelling profile. In addition, one should consider that the poly(DL-lactide-co-glycolide) 50:50 copolymer has a lower molecular weight (59600) with respect to the other two polymers utilized. Poly(L-lactide) has the highest molecular weight (171000) and the lowest degradability, while poly(DL-lactide) is characterized by intermediate molecular weight (86400) and degradability. Our experimental data consistently confirm the expected degradability and swellability.

3.5. Release kinetics

In Fig. 5 SBTI (panel A) and BPTI (panel B) release from DL-PLG, DL-PLA and L-PLA microparticles are reported. The influence of the different polymers on in vitro release profiles of the two polypeptides was analyzed. Generally, the release profiles are constituted of two different phases: an initial relatively fast period, followed by a slower and linear release phase.

Both in the case of SBTI and BPTI one can observe that the release from DL-PLG microparticles is, as expected, faster than from DL- and L-PLA.

In particular BPTI is very slowly released from both L-PLA and DL-PLA microparticles. In the case of SBTI, release from DL-PLA microparticles is again slow, whilst a different behaviour can be noted for L-PLA microparticles, where the release is more rapid and constituted of two almost linear portions with different slope.

3.6. Concluding remarks

With the aim of finding a delivery system for non-parenteral administration of proteinase inhibitors, we designed and produced polymeric microparticles. Microparticles with adequate size, morphology and entrapment efficiency were produced by employing polyesters with different biodegradability rates. As function of the polymer used, microparticles display different morphology,

swellability and degradability, demonstrating the possibility of varying release kinetic of the encapsulated compounds according to the request therapeutic target. All together, our results indicate that microparticle encapsulating polypeptidic proteinase inhibitors could represent an interesting candidate for their sustained delivery after in vivo administration.

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