ELSEVIER

International Journal of Pharmaceutics 181 (1999) 159–172

international journal of pharmaceutics

Protein encapsulation in biodegradable amphiphilic microspheres

Philippe Bouillot [a,*], Nathalie Ubrich [b], Françoise Sommer [c], Tran Minh Duc [d], Jean-Philippe Loeffler [e], Edith Dellacherie [a]

[a] *Laboratoire de Chimie-Physique Macromoléculaire, UMR CNRS-INPL 7568, groupe ENSIC, BP 451, 54 001 Nancy cedex, France*

[b] *Laboratoire de Pharmacie Galénique et Biopharmacie, Faculté des Sciences Pharmaceutiques et Biologiques, 5 rue Albert Lebrun, BP 403, 54 001 Nancy cedex, France*

[c] *Biophy, 6 rue Anne Gacon, Village des Entreprises de Saint Henri, 13016 Marseilles, France*

[d] *Université Claude Bernard, 69 630 Villeurbanne cedex, France*

[e] *Laboratoire de neurophysiologie cellulaire et intégrée, Université Louis Pasteur, 21 rue Descartes, 67 084 Strasbourg cedex, France*

Received 28 October 1998; accepted 8 January 1999

Abstract

MPOE–PLA microspheres containing bovine serum albumin (BSA) were prepared by the double emulsion method with high encapsulation efficiency ($\approx 93\%$). Confocal scanning microscopic analysis using MPOE–PLA labelled with 1-pyrenemethanol showed the MPOE coating of the microsphere surface. This coating improves the performance of the release system compared with PLA microspheres; the hydrophilic chains reduce the BSA adsorption onto the microspheres and increase the amount of BSA released in the supernatant. Microsphere analysis using atomic force microscopy showed that the presence of the MPOE chains also leads to surface roughness. Studies of the diffusion of 1% rhodamine aqueous solution into the microspheres by means of confocal microscopy showed a fast diffusion of water through the matrices containing high molecular weight MPOE chains ($> 10\,000$ g mol^{-1}) and could explain the fast release of BSA from these microspheres.

Keywords: Diblock copolymers; MPOE–PLA; Microspheres; Adsorption; Release profiles

* Corresponding author. Present address: School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, UK; Tel.: + 44-117-9289000, ext.: 4275; fax: + 44-117-9250612.

E-mail address: p.bouillot@bris.ac.uk (P. Bouillot)

0378-5173/99/$ - see front matter

PII: S0378-5173(99)00023-X

1. Introduction

Until the early 1980s, biologically active agents, such as peptides, viral and bacterial antigens received attention for the design of drug delivery systems because of their great clinical potential. Fragmentation, unfolding, aggregation and adsorption were serious problems resulting in loss of activity of such macromolecules (Johnson et al., 1991; Tabata et al., 1993a). At present, the retention of the three-dimensional structure appears to be a new critical parameter.

Controlled release systems are widely based on biocompatible, biodegradable polyesters: poly(lactic acid) PLA or poly(lactic-co-glycolic acid) PLGA (Yolles et al., 1975; Williams et al., 1984; Alonso et al., 1994). However, Tabata et al. (1993b) reported that proteins such as BSA and trypsin can adsorb on PLA homopolymer because of hydrophobic interactions, leading to loss in activity. To prevent the protein adsorption, Jeon et al. (1991) covered the hydrophobic PLA surface with hydrophilic polyoxyethylene (POE) chains. Indeed, POE is accepted by FDA as an additive in tablet coatings. Therefore, block copolymers consisting of hydrophobic PLA segments associated with hydrophilic POE blocks are currently being investigated for the conception of drug delivery systems. Preliminary results showed their good biocompatibility, similar to the PLA homopolymer (Ronneberger et al., 1996).

Moreover, one of the problems with poly(lactic acid) is the extended time required for its degradation. It is well-known that the molecular weight (Ogawa et al., 1988), the stereochemistry (Vert et al., 1991), and the ratio of GA/LA (Ogawa et al., 1988; Vert et al., 1991) are a few of the parameters that can be changed to reduce the degradation time of the polymer. Several authors have recently reported that the presence of POE hydrophilic segments increased the degradation kinetics of the PLA block in the case of PLA–POE–PLA films (Shah et al., 1994) and even PLA–POE–PLA microparticles (Penco et al., 1996).

Microspheres based on poly(oxyethylene)–poly(lactic acid) triblock copolymers (PLA–POE–PLA) or monomethoxypoly(oxyethylene)–poly(lactic acid) diblock copolymers (MPOE–PLA), were prepared by the double emulsion method to form a MPOE coating. The presence of MPOE or POE segments at the surface of the microspheres and around the cavities (Fig. 1) should reduce the adsorption and the denaturation of proteins, and so improve the stability of entrapped drug and increase the release yield. The hydrophilic segments in the matrix should increase the degradation kinetics of the PLA chains and also increase the kinetics of protein release.

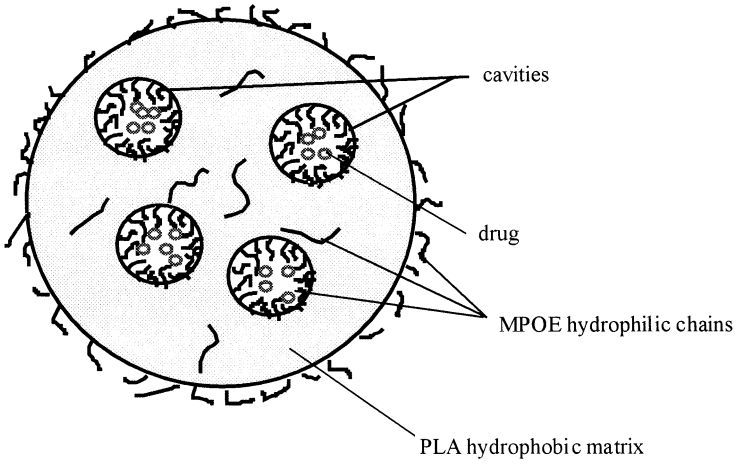


Fig. 1. Expected structure of a microsphere made from a MPOE–PLA diblock copolymer and prepared by the double emulsion method.

Table 1
Characterization of MPOE–PLA diblock copolymers prepared by polymerization in xylene

Polymers	$\overline{M_n}$ (g mol^{-1})[a]	$\overline{M_w}$ (g mol^{-1})[a]	I[a]	Yield (%)[b]
PLA*	37 800	49 500	1.30	–
MPOE2 K–PLA45 K	43 600	57 500	1.30	94
MPOE5 K–PLA45 K	46 300	58 800	1.25	94
MPOE10 K–PLA45 K	50 700	71 500	1.40	90
MPOE15 K–PLA45 K	54 200	79 100	1.45	91
MPOE20 K–PLA45 K	59 800	86 100	1.45	90

[a] Measured by HPSEC-MALLS (High performance size exclusion chromatography-multiangle laser light scattering).
[b] Amount of copolymer after purification on total amount of MPOE + lactide.
* Commercial PLA used as the reference.

In this work, we aimed at preparing microspheres made of PLA homopolymer and a series of MPOE–PLA diblock copolymers, by the double emulsion method. The PLA chain length was kept constant (45 000 g mol^{-1}) and the molar mass of the MPOE segment varied (2000, 5000, 10 000, 15 000, and 20 000 g mol^{-1}). A model protein, bovine serum albumin (BSA) was entrapped within the particles.

We were interested in the morphology and the structure of the particles, particularly the location of BSA inside the matrix and the position of the MPOE chains at the microsphere surface. Indeed, the expected properties of the microspheres are related to the MPOE coating. Finally, we studied the performance of our drug delivery systems (encapsulation yield, release profile, BSA adsorption rate) and determined the influence of the MPOE segment length on the various properties.

2. Materials and methods

2.1. Polymer and copolymers for microsphere preparation

D,L-PLA was purchased from Phusis (France). A series of MPOE–D,L-PLA was synthesized by ring opening polymerisation of D,L-lactide on the hydroxyl end group of MPOE with stannous octoate as catalyst, in solvent (xylene) (Bouillot et al., 1998). The PLA block length was kept constant at 45 000 g mol^{-1} and the MPOE molar mass varied between 2000 and 5000, 10 000, 15 000, and 20 000 g mol^{-1}. The corresponding copolymers were named MPOE2 K–PLA45 K, MPOE5 K–PLA45 K, MPOE10 K–PLA45 K, MPOE15 K–PLA45 K and MPOE20 K–PLA45 K, respectively. 2 K, 5 K, 10 K, 15 K, 20 K are the molecular weight given by the supplier (Shearwater Polymers Inc, USA) for MPOE; 45 K is the molecular weight of the PLA block. The copolymer characteristics are listed in Table 1.

We also synthesized a diblock copolymer MPOE5 K–PLA45 K labelled with 1-pyrenemethanol to study the position of the MPOE blocks inside the microspheres. This copolymer was synthesized by condensation ($T \approx 60$°C) of 1-pyrenemethanol, after deprotonation with BuLi, on MPOE epoxyde (5000 g mol^{-1}, Shearwater Polymers Inc, USA) in THF. The amount of unreacted 1-pyrenemethanol was determined by high performance size exclusion chromatography (HPSEC). Two columns (Lichrogel PS20 and PS400, Merck, Germany) were connected in series, with THF as eluent (1 ml min^{-1}), and calibrated with solutions of 1-pyrenemethanol with concentration varying from 10^{-2} to 5×10^{-2} mol l^{-1}. An UV detector L4200 (Merck, Germany) was used at $\lambda = 280$ nm. Then, the PLA chain was synthesized as mentioned previously by polymerisation of lactide on labelled MPOE. A 20% mole fraction of the final copolymer was labelled with 1-pyrenemethanol.

2.2. Microsphere preparation

Microspheres were prepared by the double emulsion method. A water-in-oil emulsion was obtained by sonication (60 s, 40 W) of 2 ml dichloromethane containing 400 mg of polymer with 100 μl of distilled water, or 100 μL of BSA solution (18 wt.%). Aqueous 1% poly(vinyl alcohol) (PVA) (1 ml, 88% hydrolysed, 13–23 000 g mol^{-1}, Sigma, Germany) or of aqueous 1% sodium cholate (Sigma, Germany) was added and the solution was stirred with a homogenizer (30 s) to make a (W/O)/W emulsion. The mixture was poured into 100 ml of distilled water and stirred for 15 min. During this period, the dichloromethane evaporates and a thin layer of polymer is formed. The remaining solvent was removed using a rotary evaporator, the microspheres were collected by centrifugation (10 000 × *g* for 9 min) and redispersed in distilled water. This cleaning procedure was repeated three times to remove free surfactant. Finally, the microspheres were freeze-dried.

2.3. Microsphere characterization

2.3.1. Morphology and particle size determination

The microspheres were viewed with a Scanning Electron Microscope (SEM) (Jeol JMS-T 330 A) after coating with a mixture of gold and palladium. They were also analyzed by means of an Atomic Force Microscope (Nanoscope III, Digital Instruments Inc., USA) in the tapping mode in order to determine the roughness parameters of the particles (R_a (nm), R_{diff} (%)).

R_a is the mean roughness and is calculated using:

$$R_a = \frac{1}{L_x L_y} \int_0^{L_y} \int_0^{L_x} [f(x, y)]\, dx\, dy$$

Where L_x and L_y are the surface dimensions and $f(x, y)$ is the surface related to the centred plane. The centred plane is a plane parallel to the mean roughness plane so that the volumes on both sides are equal. R_{diff} is the percentage increase in the surface area between that developed by the triangulation method and the scanned one. It gives an idea of the peak density at the surface.

A Coulter Multisizer II (Coultronics, USA) was used to measure the volume average diameter of the microspheres.

2.3.2. Internal organisation of MPOE and BSA

Microspheres made with a blend PLA45 K/MPOE5 K–PLA45 K (70/30 wt.%) labelled with 1-pyrenemethanol were observed by means of a Confocal Microscope (MRC 1024, Bio-Rad, USA) to study the position of MPOE inside the microspheres.

Microspheres containing 4.5 wt.% of a mixture of BSA/FITC–BSA (50/1 wt.%) (Sigma, Germany) were used, along with a confocal microscope to show the distribution of BSA inside the matrix.

2.3.3. Chemical analysis of the microspheres

A total of 80 mg of microspheres were digested in 5 ml of 0.1 N sodium hydroxide overnight. The solution was neutralised and the PVA concentration was determined according to the procedure described by Zwick (1966). Electron Spectroscopy for Chemical Analysis (ESCA) (Hewlett Packard HP 5950, USA) was used to show whether the surfactant (PVA or sodium cholate) remains adsorbed at the microsphere surface throughout the cleaning procedure.

The amount of BSA entrapped within the microspheres was measured by two methods:

1. Directly: about 70 mg of microspheres were digested in 0.5 ml sodium hydroxide solution containing 5% sodium dodecylsulphate overnight. Then, the solution was neutralised and the BSA concentration determined by the Lowry–Peterson procedure (Peterson, 1977).
2. Indirectly: the free BSA, recovered in the final external aqueous phase, was measured according to the blue coomassie G250 protein assay (Sedmak and Grossberg, 1977).

2.3.4. BSA adsorption studies

A total of 20 mg of empty microspheres prepared with PVA or sodium cholate as the surfactant were suspended in 5 ml of BSA solution overnight at 37°C. These solution concentrations vary from 10, 20, 40, 60, 80 to 100 μg ml^{-1}. The amount of BSA adsorbed was determined indi-

rectly by measuring the decrease in the BSA concentration of the medium according to the Lowry–Peterson assay.

2.3.5. Water uptake

Empty microspheres made of PLA45 K and MPOE–PLA45 K were suspended in a 1 wt.% rhodamine solution for 10 min, then collected by centrifugation and observed using a Confocal Microscope.

2.3.6. BSA release studies

About 70 mg of microspheres were suspended in 15 ml of phosphate-buffered saline (PBS, 0.05 M, pH 7.4) containing 0.03% sodium azide, then the samples were placed in incubator at 37°C and shaken under gentle stirring for at least 2 months. The amount of BSA released was determined by the Lowry–Peterson procedure. For each measurement, 3 ml of supernatant was removed and replaced by fresh buffer to ensure the pH was constant during the experiment. At the end of the experiment, the microspheres were digested in sodium hydroxide solution and the BSA concentration measured to check the mass balance.

3. Results and discussion

3.1. Microsphere morphology

Microspheres made with a series of synthesized MPOE–PLA45 K copolymers and commercial PLA45 K were prepared with PVA as surfactant. All the particles were spherical in shape when observed by SEM (Fig. 2). The PLA microspheres possessed a smooth surface, whereas those made from the copolymers had a rough surface. The level of roughness increased with the molecular weight of MPOE.

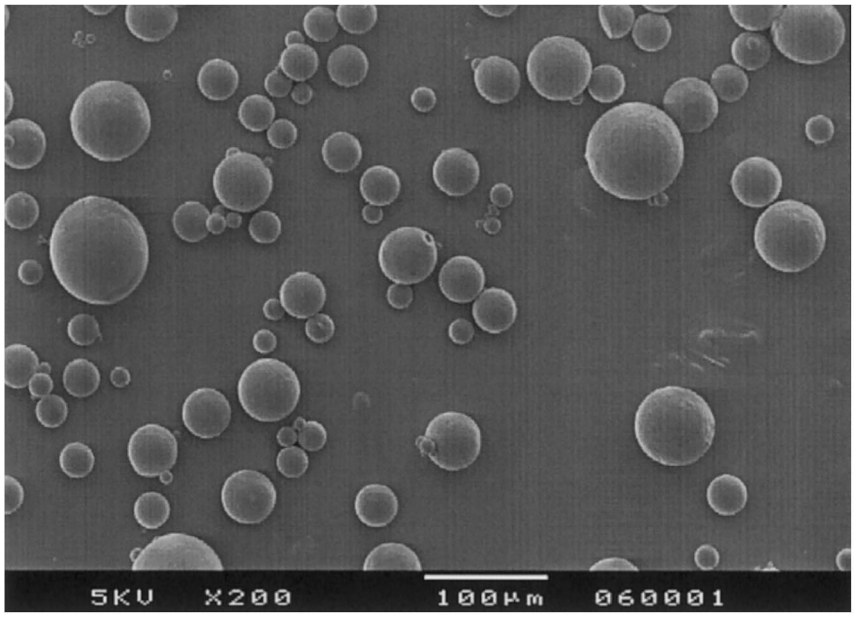
The samples analyzed by SEM were coated with a mixture of gold and palladium, which may smooth the microsphere surface. Therefore, the microspheres were also studied using atomic force microscopy. We determined the roughness parameters of the microspheres (R_a and R_{diff}). The results listed in Table 2 are in good agreement with the SEM observations. In the case of PLA microspheres, the roughness parameters were very low ($R_a = 35.9$ nm, $R_{diff} = 4.2\%$) and these values increased with increasing MPOE chain length. AFM analysis confirmed that the surface irregularities increased with the molecular weight of MPOE.

Irregular surface structures were observed similarly in the case of microspheres prepared with multiblock copolymers PLGA–POE–PLGA (Kissel et al., 1996).

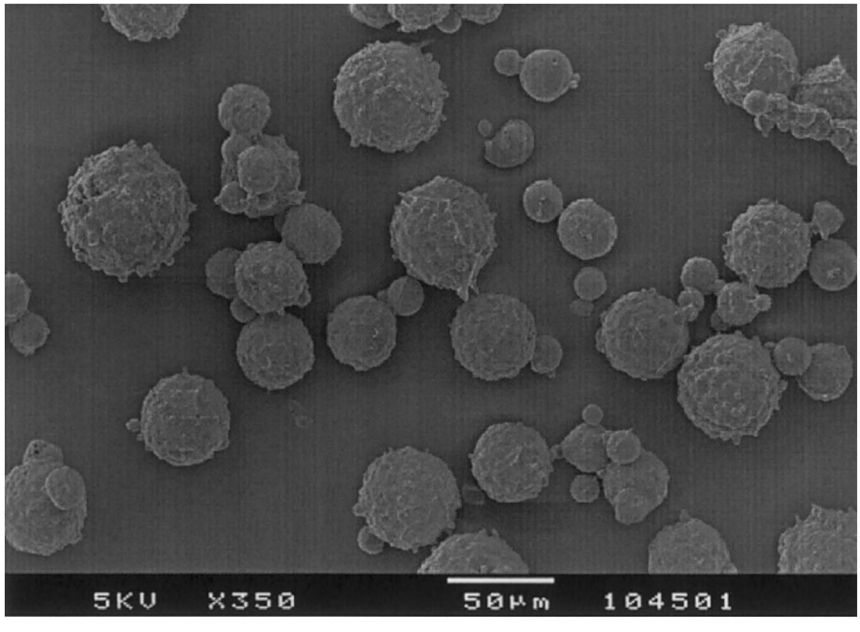
3.2. Microsphere size distribution

The volume size distribution of the microspheres was determined before and after freeze-drying. The results are listed in Table 3. Before freeze-drying, the size of microspheres decreased according to MPOE chain length up to 5 K. However, the microspheres made from copolymers with longer MPOE chains (10, 15 and 20 K) all had the same mean diameter. The diameter was slightly greater than that of the MPOE5 K–PLA45 K microspheres. This observation can be explained by the effect of several parameters including the amphiphilic properties of the copolymers, the viscosity of the polymer solution and the hydrophilicity of the copolymers, which have antagonistic effects. The increase in the MPOE chain length (i.e. the copolymer molecular weight) leads to an increase in the polymer solution viscosity (Yan et al., 1994) this should then result in an increase in particle size. Moreover, the copolymers with long MPOE blocks (10, 15, 20 K) strongly absorb water, leading to matrix expansion (Penco et al., 1996; Bouillot et al., 1998). As a consequence, the size of the microspheres (measured after solvent removal, just before freeze-drying) might also depend on the water uptake. However, the amphiphilic properties of MPOE–PLA copolymers must also be taken into account. By lowering the interfacial tension of the W/O emulsion during micropshere preparation (Babak et al., 1998), copolymer MPOE–PLA solutions lead to finer emulsions than the PLA ones. Therefore, the mean size of the MPOE–PLA microspheres prepared by the W/O/W emulsion procedure is smaller than for pure PLA. This

effect is probably enhanced when the MPOE chain length in the copolymer increased, explaining the decrease of particle size between MPOE2 K–PLA45 K and MPOE5 K–PLA45 K.



A



B

Fig. 2. Photographs of microspheres made of PLA45 K (A) and from MPOE10 K–PLA45 K (B) obtained with a scanning electron microscope.

Table 2
Roughness parameters R_a, R_{diff} of the microspheres measured using AFM[a]

Polymer	R_a (nm)	R_{diff} (%)
PLA45 K	27.6	4.2
MPOE2 K–PLA45 K	57.9	45.0
MPOE5 K–PLA45 K	85.6	56.1
MPOE10 K–PLA45 K	153.1	76.5
MPOE15 K–PLA45 K	161.9	83.7
MPOE20 K–PLA45 K	292.0	84.8

[a] These analyses were performed on a 5 μm square.

The mean size of microspheres made from PLA45 K, MPOE2 K–PLA45 K and MPOE5 K–PLA45 K was lower after freeze-drying probably due to shrinkage of the matrix. The microspheres made of MPOE–PLA with longer chains (10, 15 and 20 K) had a tendency to form aggregates after freeze-drying, probably due to the crystallisation of high molecular weight MPOE chains on their surface during the process. This assumption is based on work realised by Izutsu et al. (1996) describing the behaviour of POE in frozen solutions. Thus, the size of these particles, measured immediately after freeze-drying, was greatly increased (Table 3). However, after vigorous stirring (vortex, 15 min), the microspheres eventually recovered the same mean diameter as before freeze-drying. The matrices of the microspheres made with MPOE10 K–PLA45 K, MPOE15 K–PLA45 K, MPOE20 K–PLA45 K are more hydrophilic and flexible, therefore they quickly reabsorb the water lost during freeze-drying after redispersion in aqueous solution.

3.3. Microsphere structure

3.3.1. BSA distribution inside the matrix

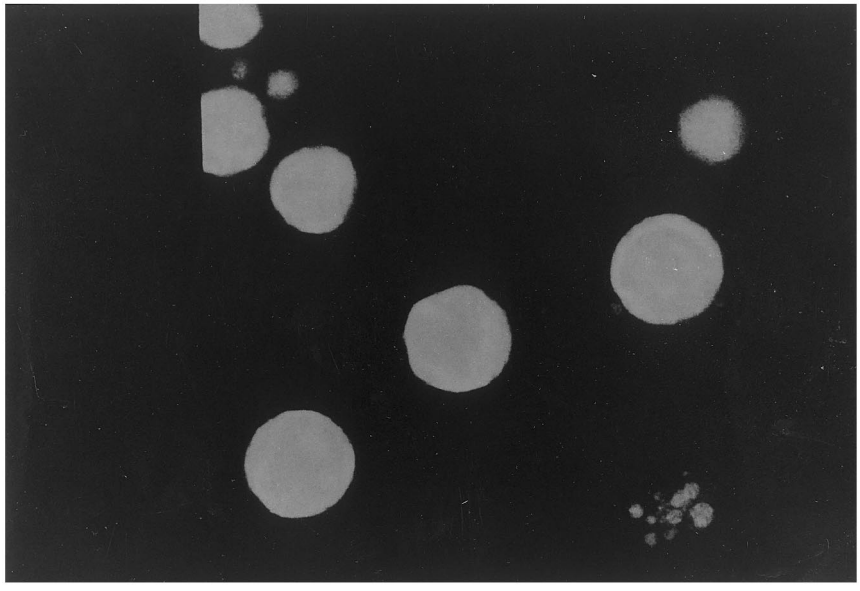
A mixture of BSA/FITC–BSA (1/50 in wt.) was entrapped in the microspheres by the double emulsion method. The confocal analysis showed a homogeneous distribution of the protein in the observed plane in the case of the microspheres made with MPOE–PLA copolymers (Fig. 3a). A microfine inner emulsion was formed when the inner W/O emulsion was prepared by sonication. Eventually, very small cavities were homogeneously distributed within the matrix after solvent evaporation in which the protein was located. Similar results have been observed by Yan et al. (1994) in the case of PLGA microspheres. The presence of these cavities was also shown by Sah et al. (1995) on fractured microspheres.

However, in the case of PLA microspheres, we observed a weaker fluorescent signal in the middle of the matrix (Fig. 3b) which means that the BSA loading was lower in the microsphere core. It seems that the internal droplets tend to migrate towards the particle surface during the evaporation phase. This phenomenon did not occur in the case of microspheres made of MPOE–PLA

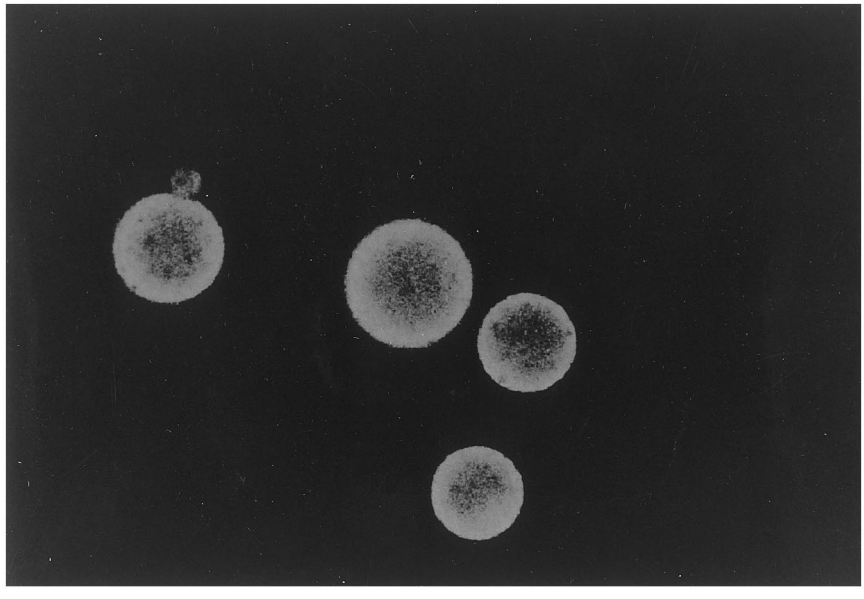
Table 3
Mean size of microspheres made from PLA45 K and from synthesized copolymers before and after freeze-drying[a]

Polymers	Mean size before freeze-drying (μm)	Mean size after freeze-drying (μm)	Mean size after freeze-drying and 15 min of stirring (μm)
PLA45 K	44 ± 1	40 ± 1	40 ± 1
MPOE2 K–PLA45 K	39 ± 1	33 ± 1	33 ± 1
MPOE5 K–PLA45 K	30 ± 1	26 ± 1	26 ± 1
MPOE10 K–PLA45 K	33 ± 1	56 ± 1	34 ± 1
MPOE15 K–PLA45 K	34 ± 1	52 ± 1	35 ± 1
MPOE20 K–PLA45 K	33 ± 1	53 ± 1	33 ± 1

[a] Sizes measured by Coulter Multisizer II and expressed as the average of measurements with three batches.



a



b

Fig. 3. Photographs of MPOE5 K–PLA45 K (a) and PLA (b) microspheres containing FITC–BSA obtained by means of confocal microscope.

diblock copolymers. Indeed, their amphiphilic properties take part in the stabilisation of the internal emulsion. This steric stabilisation of nanoemulsion by MPOE–PLA diblock copolymers for the preparation of nano- and microparticles has been investigated by Babak et al. (1998).

3.3.2. Location of the MPOE chains.

We prepared microspheres made of a blend of PLA45 K/MPOE5 K–PLA45 K labelled with 1-pyrenemethanol (70/30 wt.%) by the double emulsion method.

The confocal analysis showed that the fluorescent signal formed a ring around the microsphere surface (Fig. 4). The diblock copolymer migrated towards the surface (the outer aqueous phase) during the process because of its amphiphilic properties and led to a coating of MPOE at the surface.

A weak fluorescent signal was measured in the rest of the microspheres. As described previously, cavities arising from the first emulsion were homogeneously dispersed in the matrix. Therefore, we suppose this signal corresponds to a fraction of diblock copolymer that was also capable of migrating towards the inner aqueous phase (the cavities).

The preparation of microspheres made of copolymers MPOE–PLA by the double emulsion method leads to a preferential location of the MPOE chains at the microsphere surface and around the cavities as described in the introduction (Fig. 1).

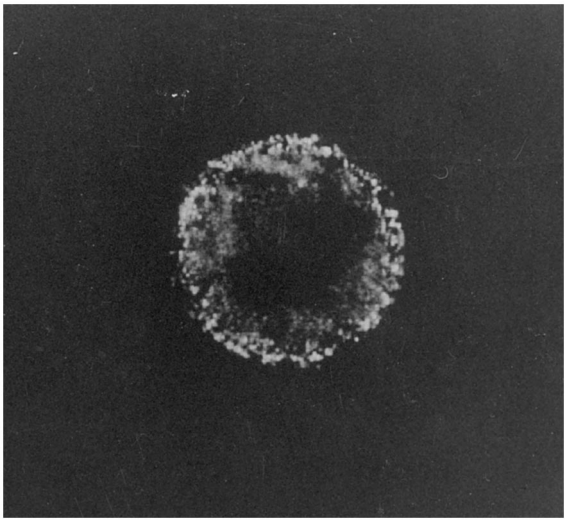


Fig. 4. Photograph of a microsphere made from the MPOE5 K–PLA45 K diblock copolymer labelled with 1-pyrenemethanol, obtained with a confocal microscope.

Table 4
Elementary chemical analysis performed by means of ESCA on PLA microspheres using PVA or sodium cholate as the surfactant

Surfactant	O/C[a]	CH_x (%)[b]	C–O (%)[b]	COO (%)[b]
PVA	0.59	41	37	22
Sodium cholate	0.64	36	33	31

[a] Molar ratio O/C.
[b] Percentage of carbon with respect to the total carbons atoms.

3.4. Chemical analysis

3.4.1. ESCA

PLA microspheres prepared with PVA and sodium cholate as surfactant were analysed by means of ESCA.

The results are reported in Table 4. According to the PLA homopolymer formula, chemical surface analysis should have theoretically shown a O/C ratio (mole of atoms) of 0.66 (Davies et al., 1989) and the presence of three different carbon types: CH_x, C–O, COO in equal proportions (33%). However, the surface of PLA microspheres obtained with PVA as surfactant had a higher carbon content than the theoretical value. In fact the calculated ratio was 0.59. Furthermore, the three carbon types were not present in equal proportions (41% CH_x, 37% C–O, 22% COO). The amounts of CH_x and C–O were higher than that of COO. This enrichment of carbon and of CH_x and C–O functions may be related to the presence of PVA adsorbed at the microsphere surface. This assumption was confirmed by the residual PVA assay, which showed that the microspheres contain 0.6 wt.% of PVA adsorbed on their surface despite the cleaning procedure. The presence of residual amounts of PVA adsorbed onto the surface of PLA or PLGA particles has already been described (Zambaux et al., 1998). On the other hand the analysis of the PLA microspheres prepared with sodium cholate as the surfactant showed an O/C ratio of 0.64, almost equal to the theoretical value. The three carbon types were also detected in nearly equal proportions (CH_x: 36%, C–O: 33%, COO: 31%). Sodium

cholate was a poor stabiliser for microspheres compared with PVA, indeed only 25% in weight of the initial amount of polymer led to spherical microspheres, but it had the advantage that it could be removed completely during the cleaning procedure.

3.4.2. BSA loading

BSA was entrapped in the microspheres using the double emulsion method and the theoretical content was 4.5 wt.%. The encapsulation efficiency was determined using two assay procedures and the results are in good agreement. The encapsulation yield decreased slightly from 99 to 93% when the molecular weight of the MPOE block increased from 2000 to 20 000 g mol^{-1}. In a previous paper (Bouillot et al., 1998), we showed that the longer the MPOE segment length, the higher the mobility and the flexibility of the copolymers, and the greater the water uptake. Therefore, we suppose that at high OE content i.e. for more hydrophilic matrices, BSA can diffuse through the matrix of the microspheres during the solvent evaporation phase; the BSA release started during microsphere preparation and led to the slight decrease of the encapsulation yield. It is noticeable that although no surfactant was used to stabilise the first emulsion, high encapsulation yields were obtained. Indeed, BSA is known to be a good stabiliser of water in dichloromethane emulsions (Shugens et al., 1994; Nihant et al., 1995). It is also described in the literature that the encapsulation of peptides or proteins in PLA or PLGA microspheres by the double emulsion method leads to high loading efficiency ($\geq 90\%$) (Couvreur and Puisieux, 1990; Cohen et al., 1991; Yan et al., 1994).

3.5. BSA adsorption

The first adsorption experiment was performed with empty microspheres made of PLA and MPOE–PLA and prepared with PVA as the surfactant. No adsorption was observed, even onto PLA microspheres, although Hu et al. (1993) and Crotts et al. (1997) have shown the adsorption of BSA on PLA films and PLGA microspheres, respectively. Previously, we showed that the PLA particles contained 0.6% of PVA on the surface, we suppose that the presence of surfactant may prevent BSA adsorption onto the microspheres.

Therefore, a second experiment was performed with empty particles prepared with sodium cholate as the surfactant because of the complete removal of the latter during the cleaning procedure.

The microsphere size was measured by means of Coulter Multisizer and varied from 15 to 22 μm. The PLA particles were the largest and should possess the smallest surface area.

The results of BSA adsorption (Fig. 5) indicated that BSA adsorption on PLA microspheres increased when the concentration of the BSA solution increased and reached a plateau which should correspond to the formation of a protein layer on the microspheres. The same release profile was obtained when the PLA chain was associated with MPOE2 K segment. We conclude that in this case the MPOE coating thickness is not large enough to prevent adsorption. When the MPOE chain length increased, the amount of BSA adsorbed decreased. At low BSA concentration (< 60 μg ml^{-1}) there was a significant de-

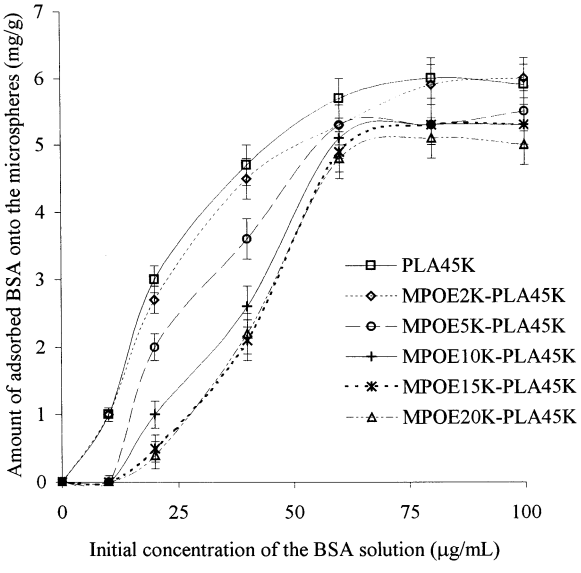


Fig. 5. Influence of the MPOE chain length on the BSA adsorption onto the microsphere surface. Each point represents mean ± S.D. for three determinations.

crease in the amount of BSA adsorbed onto the surface. The longest MPOE chains showed a greater efficiency, indeed, the amount of adsorbed BSA decreased from 3.8 to 1 mg g^{-1} of microspheres, when the MPOE chain length increased from 2 K to 20 K in the case of a 30 μg ml^{-1} BSA solution. The presence of MPOE chains at the microsphere surface reduced the yield of adsorbed BSA. However, at higher BSA concentrations (>60 μg ml^{-1}: i.e. at the adsorption plateau) the amount of adsorbed BSA was approximately the same when the MPOE length increased from 5 K to 20 K, even if it was lower than in the case of PLA and MPOE2 K–PLA45 K microspheres. A slight decrease of BSA adsorption from 6 to 5 mg g^{-1} was observed for 80 μg ml^{-1} BSA solution. A possible explanation is that the probability of contact between BSA and PLA increases with BSA concentration, while above a certain threshold, the steric protection provided by MPOE becomes ineffective.

3.6. Water uptake

Empty microspheres made of PLA homopolymer and MPOE–PLA diblock copolymers were suspended into a 1% rhodamine solution for 10 min and then observed using a confocal microscope.

We noticed that in the case of microspheres made of PLA45 K, MPOE2 K–PLA45 K and MPOE5 K–PLA45 K, the fluorescent signal was only observed at the microsphere surface after 10 min of contact (Fig. 6a). The water does not diffuse through the matrix that is too hydrophobic, and therefore, the water is only absorbed in the superficial level.

On the other hand, the fluorescent signal was observed throughout the microspheres made of MPOE10 K–PLA45 K, MPOE15 K–PLA45 K, MPOE20 K–PLA45 K (Fig. 6b). When the OE content increases in the microspheres, the matrix becomes more and more hydrophilic and the water diffuses quickly through the matrix to the core of the particles. These results are in good agreement with those obtained in the case of MPOE–PLA film (Bouillot et al., 1998).

This behaviour should have an effect on the release profiles; BSA is expected to diffuse faster throughout the matrix that absorbs a high amount of water.

3.7. BSA release profiles

Fig. 7 shows the amount of free BSA in the supernatant as a function of time. In the case of PLA microspheres, the release profile consisted of an initial release burst (3%) for the first day, then, no release was observed for the 10 following days. Afterwards, BSA was slowly released. Over a period of 44 s, 7.5% BSA was released.

The release profile obtained is characteristic of the protein release from PLA (Crotts and Park, 1997) and PLGA (Soriano et al., 1995) microspheres. Indeed, the initial release burst corresponds to the diffusion of the protein located near the microsphere surface through the matrix pores (Boury et al., 1997). A second peak of release is observed when the polymer starts to be hydrolysed and channels are formed from the microsphere core to the surface. However, the amount of BSA released is very low and drawn out, because the hydrolysis of the PLA homopolymer is very slow (the degradation usually requires more than 4 months) (Yan et al., 1994). Moreover, Crotts et al. (1997) showed that during polymer degradation, the specific surface of the microsphere increased and led to a high BSA adsorption and to a low amount of free BSA in the supernatant.

At this point, it must be noted that in Fig. 7 the amount of BSA detected in the supernatant corresponds to the combined release and readsorption of BSA onto the microsphere surface as shown above. It was also ensured that the amount of BSA loaded was equal to the amount of BSA released from the particles and adsorbed to their surface.

Similar release profiles were obtained from microspheres made of PLA45 K, MPOE2 K–PLA45 K, and MPOE5 K–PLA45 K. The association of the PLA segment with a short MPOE hydrophilic segment (2000 or 5000 g mol^{-1}) did not change the release kinetics, whereas, in the case of microspheres made of

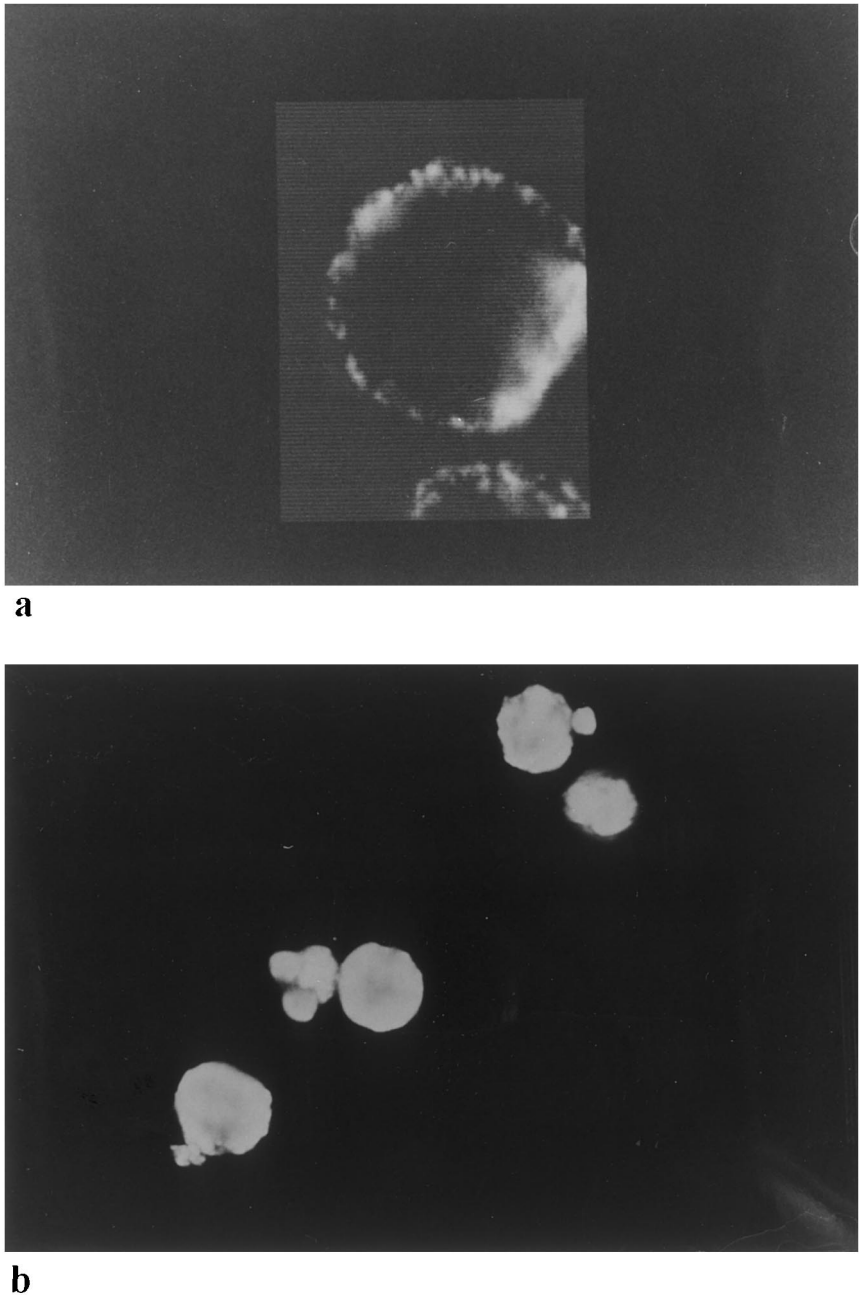


Fig. 6. Photographs of PLA (a) and MPOE15 K–PLA45 K (b) microspheres after 10 min of contact with a 1% rhodamine solution, obtained with a confocal microscope.

MPOE10 K–PLA45 K, MPOE15 K–PLA45 K, MPOE20 K–PLA45 K, the longer the MPOE chain, the higher the amount of free BSA. The release plateau was reached for the microspheres made of MPOE10 K–PLA45 K, MPOE15 K–PLA45 K, and MPOE20 K–PLA45 K, respec-

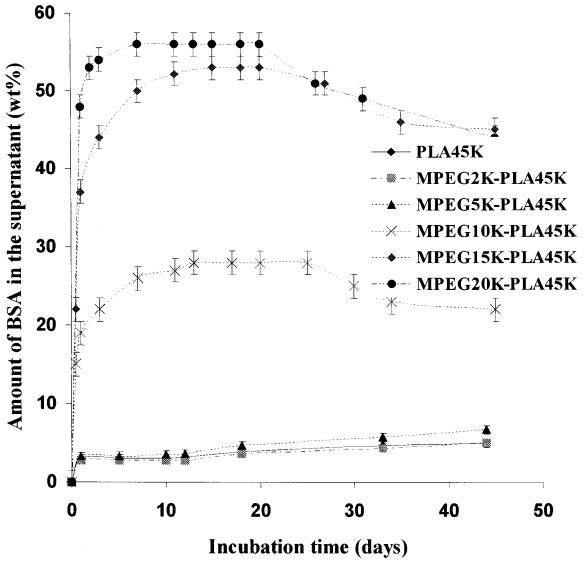


Fig. 7. In vitro BSA release from microspheres made of PLA and MPOE–PLA. Each point represents mean ± S.D. for three determinations.

tively when 28, 53 and 56% of the amount of entrapped BSA was detected in the supernatant. Indeed, despite the quick adsorption of some free BSA from the supernatant to the microsphere surface (as shown previously), the preferential location of the MPOE segments at the surface and around the cavities reduced the level of BSA adsorption and increased the amount of free BSA by the same extent. At the same time, as the MPOE segment increased the flexibility and the mobility of the copolymer chains increased (Shah et al., 1994). This leads to a higher water uptake (Penco et al., 1996) and therefore to a faster degradation of the PLA segment (Shah et al., 1994). All these parameters lead to a faster diffusion of BSA through the matrix. The release plateau was reached after approximately 10 days. Then the amount of free BSA decreased in the supernatant. The readsorption of free BSA can be explained by the hydrolysis of the MPOE–PLA copolymers (i.e. the release of MPOE segments) which decreases the protecting effect of the hydrophilic chains against BSA. The increase of the specific surface due to the microsphere degradation is also responsible for the increase of BSA adsorption; this phenomenon has been observed on PLGA microspheres by Crotts and Park (1997).

4. Conclusion

This study showed that BSA was entrapped homogeneously and with a high efficiency in MPOE–PLA microspheres, using the double emulsion method. The MPOE coating is obtained in the cavities of the matrix and on the microsphere surface, this arrangement leads to a decrease in BSA adsorption onto the matrix and to an increase in the amount of BSA released. BSA was the model protein chosen for this study. To fully establish the merits of these microspheres, bioactivity/antigenicity testing of released proteins would be necessary. Indeed, we would expect that the MPOE chains in the internal cavities would protect the proteins from inactivation because of interactions with the hydrophobic PLA regions. Recently, interest in microspheres containing POE blocks was shown for the entrapment of a therapeutically active protein, tetanus toxoid (Alonso et al., 1994). We believe that with further studies, MPOE–PLA microspheres will find applications for the controlled release of various macromolecules, such as vaccines.

References

Alonso, M.J., Gupta, R.K., Min, C., Siber, G.R., Langer, R., 1994. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccine 12, 299–306.

Babak, V.G., Gref, R., Dellacherie, E., 1998. The effect of hydrophile-lipophile balance of water-soluble poly(ethylene glycol)–poly(lactic acid) diblock copolymers on the stability of microscopic emulsion films and nanoemulsions. Mendeleev comm. 3, 105–107.

Bouillot, P., Petit, A., Dellacherie, E., 1998. Protein encapsulation in biodegradable amphiphilic microspheres I. Polymer synthesis and characterization, and microsphere elaboration. J. Appl. Polym. Sci. 68, 1695–1702.

Boury, F., Marchais, H., Proust, J.E., Benoit, J.P., 1997. Bovine serum albumin release from poly(L-hydroxyacid) microspheres: effects of polymer molecular weight and surface properties. J. Control. Release 45, 75–86.

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H., Langer, R., 1991. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm. Res. 8, 713–719.

Couvreur, P., Puisieux, F., 1990. Nano- and microparticles for the delivery of peptides and proteins. Adv. Drug Del. Rev. 5, 265–285.

Crotts, G., Sah, H., Park, T.G., 1997. Adsorption determines in vitro protein release rate from biodegradable microspheres: quantitative analysis of surface area during degradation. J. Control. Release 41, 101–111.

Crotts, G., Park, T.G., 1997. Stability and release of bovine serum albumin encapsulated within poly(D,L-lactide-co-glycolide) microparticles. J. Control. Release 44, 123–134.

Davies, M.C., Short, R.D., Khan, M.A., Watts, J.F., Brown, A., Eccles, M.J., Humphrey, P., Vickerman, J.C., Vert, M., 1989. An XPS and SIMS analysis of biodegradable biomedical polyesters. Surf. Interface Anal. 14, 115–120.

Hu, D.S., Liu, H.J., Pan, I.L., 1993. Inhibition of bovine serum albumin adsorption by poly(ethylene glycol) soft segment in biodegradable poly(ethylene glycol)/poly(L-lactide) copolymers. J. Appl. Polym. Sci. 50, 1391–1396.

Izutsu, K.I., Yoshioka, S., Kojima, S., Randolph, T.W., Carpenter, J.F., 1996. Effects of sugars and polymers on crystallisation of poly(ethylene glycol) in frozen solutions: phase separation between incompatible polymers. Pharm. Res. 13, 1393–1400.

Jeon, I., Lee, J.H., Andrade, D., Degennes, P.G., 1991. Protein surface interactions in the presence of polyethylene oxide.I. Simplified theory. J. Colloid Interface Sci. 142, 149–158.

Johnson, R.E., Lanaski, L.A., Gupta, V., Griffin, M.J., Gaud, H.T., Needham, T.E., Zia, H., 1991. Stability of atriopeptin III in poly(D,L lactide-co-glycolide) microspheres. J. Control. Release 17, 61–67.

Kissel, T., Li, Y.X., Volland, C., Görich, S., Koneberg, R., 1996. Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-coglycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks. J. Control. Release 39, 315–326.

Nihant, N., Shugens, C., Grandfils, C., Jérome, R., Teyssié, P., 1995. Polylactide microparticles prepared by double emulsion-evaporation. II effect of the poly(lactide-co-glycolide) composition on the stability of the primary and secondary emulsions. J. Colloid Interface Sci. 173, 55–65.

Ogawa, Y., Yamamoto, M., Takada, S., Okada, H., Shimamoto, T., 1988. Controlled-release of leuprodile acetate from polylactic acid or copoly(lactic/glycolic) acid microcapsules: Influence of molecular weight and copolymer ratio of polymer. Chem. Pharm. Bull. 36, 1502–1507.

Penco, M., Marcioni, S., Ferruti, P., D'antone, S., Deghengi, R., 1996. Degradation behaviour of block copolymers containing poly(lactic-glycolic acid) and poly(ethylene glycol) segments. Biomaterials 17, 1583–1590.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.

Ronneberger, B., Kao, W.J., Anderson, J.M., Kissel, T., 1996. In vivo biocompatiblility studies of ABA triblock copolymers consisting of poly(L-lactic-co-glycolic acid) A. blocks attached to central poly(oxyethylene) B. blocks. J. Biomed. Mater. Res. 30, 31–40.

Sah, H.K., Toddywala, R., Chien, Y.W., 1995. Biodegradable microcapsules prepared by a W/O/W technique: effects of shear force to make a primary W/O emulsion on their morphology and protein release. J. Microencapsul. 12, 59–69.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive and versatile assay for protein using coomassie brilliant blue G250. Anal. Biochem. 79, 544–552.

Shah, S.S., Zhu, K.J., Pitt, C.G., 1994. Poly D.L lactic acid: Polyethylene glycol block copolymers. The influence of polyethylene glycol on the degradation of poly D,L lactic acid. J. Biomater. Sci. Polymer. Ed. 5, 421–431.

Shugens, C., Laruelle, N., Nihant, N., Grandfils, C., Jérome, R., Teyssié, P., 1994. Effect of the emulsion stability on the morphology and porosity of semicrystalline poly L-lactide microparticles prepared by W/O/W double emulsion-evaporation. J. Control. Release 32, 161–176.

Soriano, I., Delgado, A., Diaz, R.V., Evora, C., 1995. Use of surfactants in polylactic acid protein microspheres. Drug Dev. Ind. Pharm. 21, 549–558.

Tabata, Y., Takebayashi, Y., Ueda, T., Ikada, Y., 1993a. A formulation method using D,L-lactic acid oligomer for protein release with reduced initial burst. J. Control. Release 23, 55–64.

Tabata, Y., Gupta, S., Langer, R., 1993b. Controlled delivery systems for proteins using polyanhydride microspheres. Pharm. Res. 10, 487–496.

Vert, M., Li, S., Garreau, H., 1991. More about the degradation of LA/GA derived matrices in aqueous media. J. Control. Release 16, 15–26.

Williams, D.L., Nuiwayser, E.S., Creeden, D.E., Gay, M.H., 1984. Microencapsulated local anesthetics. Proc. Int. Symp. Control. Rel. Bioact. Mater. 11, 69–74.

Yan, C., Reseau, J.H., Hewetson, J., West, M., Rill, W.L., Kende, M., 1994. Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique. J. Control. Release 32, 231–241.

Yolles, S., Eldridge, J.E., Leafe, T.D., Woodland, J.H., Blake, D.R., Meyer, F.J., 1975. Long-acting delivery systems for narcotic antagonists II. Release rates of naltrexone from poly(lactic acid) composites. J. Pharm. Sci. 64, 348–349.

Zambaux, M.F., Bonneaux, F., Gref, R., Maincent, P., Dellacherie, E., Alonso, M.J., Labrude, P., Vigneron, C., 1998. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method. J. Control. Release 50, 31–40.

Zwick, M.M., 1966. The blue complexes of iodine with poly(vinyl alcohol) and amylose. J. Polym. Sci. 4, 1642–1644.