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Research paper

Protein encapsulation and release from poly(lactide-co-glycolide) microspheres: effect of the protein and polymer properties and of the co-encapsulation of surfactants

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Abstract

Despite the recognised role of the poly(lactide-co-glycolide) (PLGA) in the encapsulation and release of proteins from PLGA microspheres, the importance that the characteristics of the protein have in these processes has not yet been sufficiently investigated. The aim of this work was to study the simultaneous effect of the protein and PLGA properties and of the microencapsulation process on the physicochemical and in vitro release characteristics of protein-loaded PLGA microspheres. Two model proteins of different isoelectric points (p*I*), bovine serum albumin (BSA) (p*I* = 4.6) and lysozyme (LZM) (p*I* = 11.2), and two different molecular weights (M_w) of PLGA were selected. Microspheres were prepared using the water-in-oil-in-water (w/o/w) solvent extraction and the oil-in-oil (o/o) solvent evaporation techniques. Results showed that BSA was efficiently encapsulated independent of the PLGA M_w, whereas the encapsulation of LZM was favoured with the low M_w PLGA. The co-encapsulation of a surfactant (poloxamer 188 or 331) reduced the protein encapsulation efficiency, especially of BSA. These results suggested that the tensoactive properties of the protein and its affinity for the PLGA are major determinants of the protein encapsulation. Both proteins released faster from the microspheres prepared by the o/o solvent evaporation procedure, with respect to those prepared by the w/o/w solvent extraction technique. In addition, both polymer M_w and protein type had an effect on the protein release rate. The release rate of both proteins, in the absence of a surfactant, was faster from the low M_w PLGA microspheres. However, the release rate constant was higher for BSA than for LZM irrespective of the PLGA M_w. In addition, the co-encapsulation of a surfactant led, in most cases, to a faster release of the encapsulated protein. To conclude, these results suggest that protein release from PLGA microspheres is not only governed by the PLGA erosion rate and protein diffusion through the water-filled channels, but is highly affected by the protein properties and its possible interaction with PLGA and its degradation products. © 1998 Elsevier Science B.V.

Keywords: Protein delivery; Protein encapsulation; Poly(DL-lactide-co-glycolide); Microsphere; Protein–polymer interaction; Stabilising excipient; Poloxamer

1. Introduction

A wide range of proteins such as vaccines, cytokines, enzymes, hormones and growth factors are now commercially available in large quantity due to the recent advent of DNA technology. However, there are several problems associated with therapeutic trials of protein drugs. Among them are the short in vivo half-lives and the side effects attributable to the multiple and high-dose injections required to achieve desirable therapy. An interesting approach to prolong their therapeutic levels is to deliver these proteins via biodegradable polymers. In this sense, interest has been especially focused on the use of poly(DL-

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lactide-co-glycolide) (PLGA) microspheres as protein delivery systems [1,2].

In order to successfully develop protein-loaded PLGA microspheres, it is essential that the biological activity of the protein is retained, not only throughout encapsulation but also during its prolonged release. However, the complex physical and chemical instabilities inherent to protein molecules, are obstacles for achieving this goal. In this sense, many peptides and proteins show a pronounced tendency to the self-association and adsorption process [3–7] by electrostatic and hydrophobic mechanisms, forming high molecular weight aggregates of non-covalent or covalent nature [8]. These aggregates must be avoided, because they are frequently associated with a loss of protein activity.

Among the microencapsulation techniques, the o/o solvent evaporation [9] and the w/o/w solvent extraction techniques [10–15] are two of the most convenient ways for the encapsulation of proteins within PLGA microspheres. In both cases there is a minimal diffusion of the protein to the external phase. However, some authors have reported phenomena of protein aggregation and loss of activity following encapsulation by a w/o/w technique [16–18]. To overcome this problem, one solution could be to use stabilisers, such as poloxamers, which have been shown to stabilise the primary emulsion and to reduce protein–polymer interactions [19].

Another important limitation still associated to PLGA microspheres as protein delivery systems is that protein release kinetics is often unpredictable. They commonly exhibit an initial burst of release followed by a very slow and incomplete release [20]. In this sense, it is surprising that, despite the high number of protein release studies performed until now, the effect of the protein properties and its interaction with the PLGA matrix on the in vitro protein release have scarcely been considered [21]. Previous studies have shown, however, that basic drugs can interact with acidic degradation products, during the PLGA degradation [22]. This fact suggests that proteins charged positively could interact with the PLGA degradation products thus hindering its release. Furthermore, the protein entrapped within the microspheres may experience a low environmental pH [23] due to the high local concentration of trapped degradation products, thus forming water insoluble non-covalently bound protein aggregates [24].

These previous results show the necessity of further investigating the simultaneous effect of the protein and polymer characteristics in protein encapsulation, as well as the need of searching for stabilisers which would preserve the protein stability during its encapsulation and further release. Taking this into account, in the present work we studied the influence of the following variables: type of microencapsulation procedure (w/o/w solvent extraction vs. o/o solvent evaporation), type of protein (BSA or LZM), PLGA molecular weight (M_w) (10 kDa or 34 kDa), and the incorporation or not of the stabilisers (poloxamer 188 or poloxamer 331) on the protein encapsulation efficiency and release from PLGA microspheres.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, fraction V), fluorescein isothiocyanate labelled BSA (FITC-BSA) and lysozyme (LZM) were purchased from Sigma Chemical (Madrid, Spain). Poly(DL-lactide-co-glycolide) 50:50 (PLGA) copolymers with inherent viscosity (i.v.) of 0.16 and 0.4 dl/g were purchased from Birmingham Polymer (Birmingham, AL, USA) and Boehringer Ingelheim (Germany), respectively. Cottonseed oil was supplied by Sigma. The solvents ethyl acetate (EA), isopropyl alcohol and petroleum ether were purchased from Panreac (Madrid, Spain) and acetonitrile from Teknokroma (Barcelona, Spain). The emulsifying agents polyvinyl alcohol (PVA) (M_w 30 000–70 000 Da) and Poloxamer 188 and 331 were obtained from Sigma (Madrid, Spain) and BASF (Ludwigshafen, Germany), respectively.

2.2. Preparation of protein-loaded microspheres

Protein-loaded microspheres were prepared using emulsification processes followed by the extraction or the evaporation of the solvent. The solvent evaporation process involving an oil-in-oil emulsion was the described by Wada et al. [9]. Briefly, 200 mg of PLGA were dissolved in 5 ml of acetonitrile. In some preparations, 2 mg of Poloxamer 188 or 331 were also incorporated in this organic phase. A predetermined solution of protein (2 mg/50 μl) was added to the polymer solution leading to the formation of a very fine suspension. This phase, containing the polymer and the protein, was then added to 100 ml of cottonseed oil containing soybean lecithin (0.05% w/v) as an emulsifier. The resulting mixture was emulsified under agitation with a propeller rotating at 700 rpm for 2 h to allow the evaporation of the solvent. Finally, the microspheres were washed with petroleum ether and finally freeze-dried.

The solvent extraction process involved the formation of a multiple emulsion (w/o/w), as previously described by Alonso et al. [13]. Briefly, 50 μl of protein (2 mg) were emulsified in 1 ml of PLGA in ethyl acetate (200 mg/ml) by sonication (Branson 250, Sonifier®) for 15 s (15 W) in an ice bath to form a primary w/o emulsion. In some preparations, either 2 mg Poloxamer 188 or Poloxamer 331 were incorporated in the water or the organic phase respectively. Afterwards, 2 ml of an external aqueous phase, containing 1% w/v PVA as a stabiliser, was added and the mixture agitated by vortexing for 10 s. The resulting w/o/w emulsion was then poured into 100 ml of a 0.3% w/v aqueous PVA solution under magnetic stirring. After 5 min agitation, the solvent was rapidly eliminated by extraction with 100 ml of

an aqueous isopropyl alcohol solution (2% v/v). Finally, the microspheres were collected by filtration, washed with distilled water and freeze-dried. The dried particles obtained by both microencapsulation procedures were stored at 4°C in the presence of silica gel.

2.3. Physicochemical properties of microspheres

The surface appearance and inner structure of the different PLGA microparticles was analysed by scanning electron microscopy (SEM) (JEOL JSM-T220A, Kyoto, Japan). For the surface analysis, freeze-dried microspheres were mounted onto metal stubs using double-sided adhesive tape, dried under vacuum and coated with gold-palladium (metallisation) under an Argon atmosphere (Sputter Coater SCD004 BAL-TEC, Liechtenstein). For the analysis of the inner structure, microspheres were suspended in water, frozen and cross-sectioned using an ultra-microtome (Ultracut, Reichert-Jung, Austria). Finally, they were coated with gold-palladium and viewed by electron microscopy.

Particles were sized using Multisizer Coulter® Counter (Electronics, Northwell, UK). The freeze-dried microspheres were dispersed by bath-sonication in saline medium (Isoton® II, Coultronics) in the presence of surfactant to prevent aggregation. The particle size was expressed as the mean volume diameter in μm.

2.4. Protein encapsulation efficiency

The protein content of microspheres was analysed by a previously reported method [15]. Briefly, 20 mg of lyophilised microspheres were digested in 5 ml of a 5% sodium dodecyl sulfate (SDS), 0.1 N NaOH solution for 15 h at room temperature until a clear solution was obtained. The content of at least three samples was spectrophotometrically measured at 494 nm or by a microBCA protein assay (Pierce, IL, USA) for FITC-BSA and LZM, respectively. Results are presented as 'protein encapsulation efficiency' values, which indicate the percentage of protein encapsulated in the microspheres with respect to the total amount of protein used in the process.

2.5. Protein release studies

Twenty mg of dried microspheres were placed in test tubes and resuspended in 4 ml of phosphate buffer, pH 7.4. The microsphere suspensions were placed in a 37°C incubator for 4 weeks. At appropriate intervals, the samples were collected and centrifuged for 15 min at 4000 × g (Centrifuge 2–15, Sigma). Two ml of supernatant was assayed for protein release and replaced by 2 ml of fresh medium. Protein concentration in the release medium was determined by the microBCA protein assay.

Some control experiments were also performed in order to assess the interaction of the proteins with the PLGA microspheres. In these experiments, 20 mg of dried microspheres were incubated in 4 ml of phosphate-buffered solution containing 80 μg of protein (BSA or LZM), pH 7.4, at 37°C. At day 1 and day 5 samples were collected and centrifuged, and the supernatant assayed for protein content as specified above. The amount of protein which interacted with the microspheres was determined by the difference between the total amount of protein incorporated in the incubation medium and that remaining in the supernatant after the incubation process.

2.6. Determination of the average molecular weight

The PLGA molecular weight of the microspheres during the in vitro release study was determined by gel permeation chromatography (GPC). The conditions were: lineal column (Phenomenex, Pharmacia, Madrid, Spain); column temperature: 35°C; mobile phase: chloroform; flow rate: 1 ml/min; detection: refractive index (Shodex RI SE-31, Spectra Physics, Madrid, Spain). At the appropriate intervals, after centrifugation and lyophilisation, microspheres were dissolved in chloroform and injected into the GPC equipment. The average molecular weight was calculated with reference to polystyrene standards (Teknokroma, Barcelona, Spain) using computer software developed by Spectra Physics. The molecular weight was expressed as the weight average molecular weight.

2.7. Stability and integrity of protein in PLGA microspheres

The integrity of the protein that remained microencapsulated following the in vitro release study was analysed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Twenty mg of microspheres were incubated in 20 ml of 0.1 N NaOH containing 5% SDS until the complete dissolution of the microspheres. This solution was then neutralised at pH 7 with 1 N HCl. The protein samples and several standards were solubilised in Laemmli buffer, boiled for 30 min and loaded into 12.5 or 20% SDS–polyacrylamide gels (Phast System, Pharmacia, Madrid, Spain) for BSA or LZM samples, respectively. After electrophoresis, gels were stained with silver to visualise the protein.

3. Results and discussion

The aim of this study was to investigate the mechanism of encapsulation of two model proteins, using two different microencapsulation procedures, and to elucidate the role of the protein properties on its encapsulation and release from PLGA microspheres. For this purpose two model proteins, BSA and LZM, which differ in their isoelectric point (4.6 and 11.2, respectively) and molecular weight (66 200 and 14 000 Da, respectively) were selected. They were encapsulated in two kinds of PLGA of low and high intrinsic viscosity (0.16 and 0.4) and thus, low and high M_w. Two

surfactants with different hydrophilia/lipophilia balance (HLB) (poloxamer 188, HLB = 29 and poloxamer 331, HLB = 1) which were expected to affect polymer–protein interactions were also incorporated in some microspheres at the ratio of 1% (w/w) with respect to the PLGA.

In an attempt to investigate the above mentioned factors we first analysed the surface morphology and inner structure of the microspheres; this information together with the encapsulation efficiency values allowed us, in a second step, to elucidate the encapsulation mechanism. Finally, this information was used for the interpretation of the protein release mechanisms.

3.1. Microstructure and particle size of protein-loaded microspheres

As shown in Fig. 1, the BSA-loaded PLGA microspheres were spherical and had a smooth surface without pores or cavities which could affect the release of the encapsulated protein. The same appearance was observed for all formulations, independent of the microencapsulation procedure, type of protein or incorporation of poloxamer agents.

With respect to the inner microstructure of the microspheres, the SEM analysis of cross-sectioned microspheres revealed that those prepared by the o/o solvent evaporation process had a matrix-type structure irrespective of the type of protein, polymer and of the co-encapsulation of the poloxamers (Fig. 2A). However, the inner structure of BSA-loaded microspheres prepared by the w/o/w solvent evaporation procedure was greatly affected by the incorporation of the poloxamers. The microspheres which did not contain poloxamer had a compact matrix-type structure whereas those containing poloxamer 188 displayed small holes and cavities (Fig. 2B). A more important change was observed when poloxamer 331 was used as a surfactant; in this case, the inner structure changed from a matrix system to a capsular system (Fig. 2C). These changes could be attributed to the effect of the surfactant on the stability of the primary emulsion. It has already been reported that, in the absence of a stabiliser, an interfacial film is formed by the interaction between the protein and the polymer which stabilises the microdroplets of the w/o primary emulsion [25, 26]. However, the incorporation of poloxamer 188 hinders the formation of the stabilising film because of the competition phenomenon between the poloxamer and the protein in

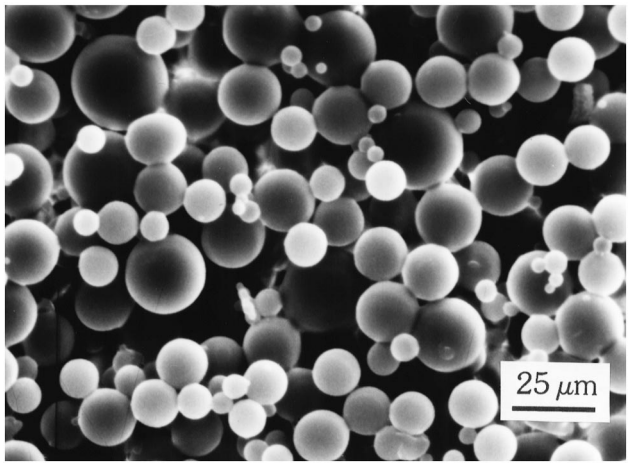

Fig. 1. Scanning electron micrograph of BSA-loaded PLGA microspheres (PLGA i.v.: 0.16) prepared by the w/o/w solvent extraction process.

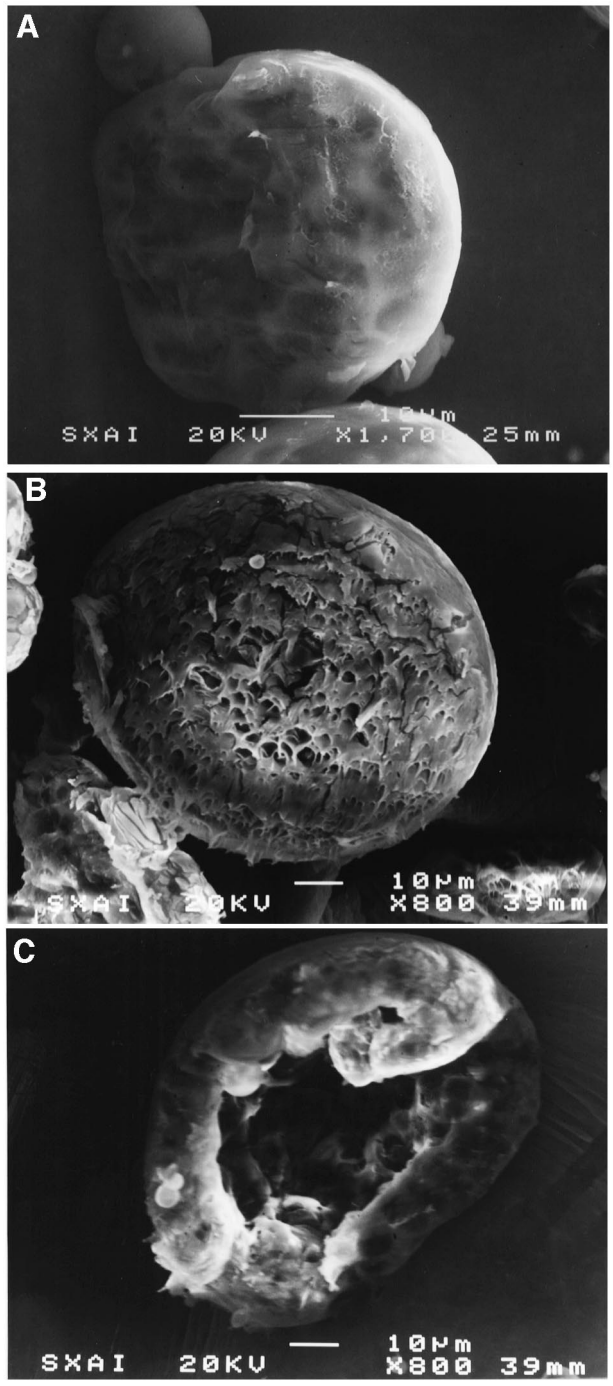

Fig. 2. Scanning electron micrographs of cross-sections of BSA-loaded PLGA microspheres: (A) prepared by the o/o solvent evaporation process (PLGA i.v.: 0.2); (B) prepared by the w/o/w solvent extraction process containing poloxamer 188; (C) prepared by the w/o/w solvent extraction process containing poloxamer 331.

Table 1

Influence of the microencapsulation method water-in-oil-in-water (w/o/w) solvent extraction and oil-in-oil (o/o) solvent evaporation on the microspheres size

Copolymer	Surfactant	Particle size (μm)		
		w/o/w		o/o
		BSA	LZM	BSA
DL-PLGA i.v.: 0.4 (M_w: 34 kDa)	Without	46 ± 1	48 ± 2	31 ± 2
	Poloxamer 188	45 ± 2	43 ± 2	30 ± 1
	Poloxamer 331	44 ± 2	45 ± 3	30 ± 3
DL-PLGA i.v.: 0.16 (M_w: 10 kDa)	Without	18 ± 1	16 ± 1	44 ± 1
	Poloxamer 188	20 ± 1	20 ± 3	46 ± 1
	Poloxamer 331	18 ± 1	20 ± 1	48 ± 2

their interaction with the polymer. This phenomenon, which was more pronounced for poloxamer 331 because of its lipophilia, led to the fusion of the inner aqueous droplets and, consequently, to the formation of capsular structures.

As shown in Table 1, the size of the microspheres, prepared by both microencapsulation procedures, was highly affected by the intrinsic viscosity of the polymer, but not by the type of protein and the co-encapsulation of the poloxamers. However, the polymer viscosity had an opposite effect in both microencapsulation procedures; the size of microspheres prepared by the w/o/w solvent extraction procedure increased with the intrinsic polymer viscosity, whereas the size of those prepared by the o/o solvent evaporation become larger by reducing the PLGA viscosity. The first situation has previously been observed [12] and was attributed to the difficulty for the emulsification of high viscosity solutions. The second situation could be explained by the coalescence of the organic droplets during the slow evaporation of the solvent. In that sense, it should be noted that the w/o/w extraction process causes the rapid precipitation of the polymer (15 min) thus avoiding the coalescence of the droplets.

3.2. Protein encapsulation efficiency: mechanisms of encapsulation

The BSA and LZM encapsulation efficiency of the microspheres prepared by the w/o/w solvent extraction procedure are shown in Table 2. Results indicate that, in the absence of poloxamer, BSA was very efficiently encapsulated in the low and high M_w PLGA; however, LZM was preferably encapsulated in the low M_w PLGA. On the other hand, the encapsulation of BSA in low and high M_w PLGA was significantly reduced ($\alpha < 0.01$) by the incorporation of the poloxamers. The same was found for the encapsulation of LZM in the low M_w PLGA; however, in the case of high M_w PLGA, the LZM encapsulation was not influenced by the incorporation of poloxamers. The high encapsulation of BSA within both PLGA types could be related to the tensoactive properties of this protein and its already known tendency to interact with PLGA forming an interfacial stabilising film at the w/o interface [25]. LZM do not exhibit as important tensoactive properties as BSA [27] and, thus, its entrapment within the microspheres could be predominantly determined by its interaction with PLGA. An explanation may be the possible ionic interaction between the LZM (positively charged amino groups) and the PLGA carboxyl-end groups (negatively charged). In this sense, it could be accepted that although the exact concentration of the final carboxyl groups in these copolymers is not available, these groups would normally be more numerous in the low M_w PLGA than in the high M_w PLGA [28].

Table 2

Encapsulation efficiency of protein-loaded microspheres prepared by the w/o/w solvent extraction technique

Copolymer	Surfactant	Encapsulation efficiency	
		BSA	LZM
DL-PLGA i.v.: 0.4 (M_w: 34 kDa)	Without	91.09 ± 1.57	60.39 ± 5.08
	Poloxamer 188	59.06 ± 4.18	55.68 ± 1.50
	Poloxamer 331	65.86 ± 1.80	56.51 ± 0.46
DL-PLGA i.v.: 0.16 (M_w: 10 kDa)	Without	91.53 ± 3.35	93.50 ± 3.02
	Poloxamer 188	68.24 ± 1.58	79.15 ± 1.68
	Poloxamer 331	74.94 ± 1.54	88.35 ± 2.09

Three main conclusions can be drawn from these data: (i) in the absence of poloxamer and for the high M_w copolymer, the encapsulation of BSA is more important than that of LZM; (ii) the encapsulation of BSA is independent of the PLGA M_w whereas that of LZM increases significantly for the low M_w copolymer; (iii) the presence of poloxamer leads, in all cases, to a reduction in the protein encapsulation efficiency. These results suggest that the predominant mechanisms of encapsulation of BSA and LZM are different; the high tensoactive character of BSA seems to be a major determinant in the formation of an interfacial BSA-PLGA film and, thus, in the encapsulation efficiency. In the case of LZM, its positive charge may play a role in its interaction with PLGA and, thereby, in the encapsulation efficiency [29].

On the other hand, the loading efficiency of the formulations prepared by the o/o solvent extraction technique was considered as 100%, since this procedure involves the use of an external oily phase to which the protein could not diffuse. We should, however, accept that some protein could be deficiently encapsulated or retained on the surface of the microspheres.

3.3. In vitro release of protein from PLGA microspheres

3.3.1. BSA release from PLGA microspheres: the effect of the microencapsulation technique

In a first step of this work we analysed the effect of microencapsulation technique on the in vitro release of BSA from microspheres made of PLGA (i.v. :0.4) with or without poloxamer (Fig. 3). The BSA release profiles were biphasic, characterised by an initial protein burst followed

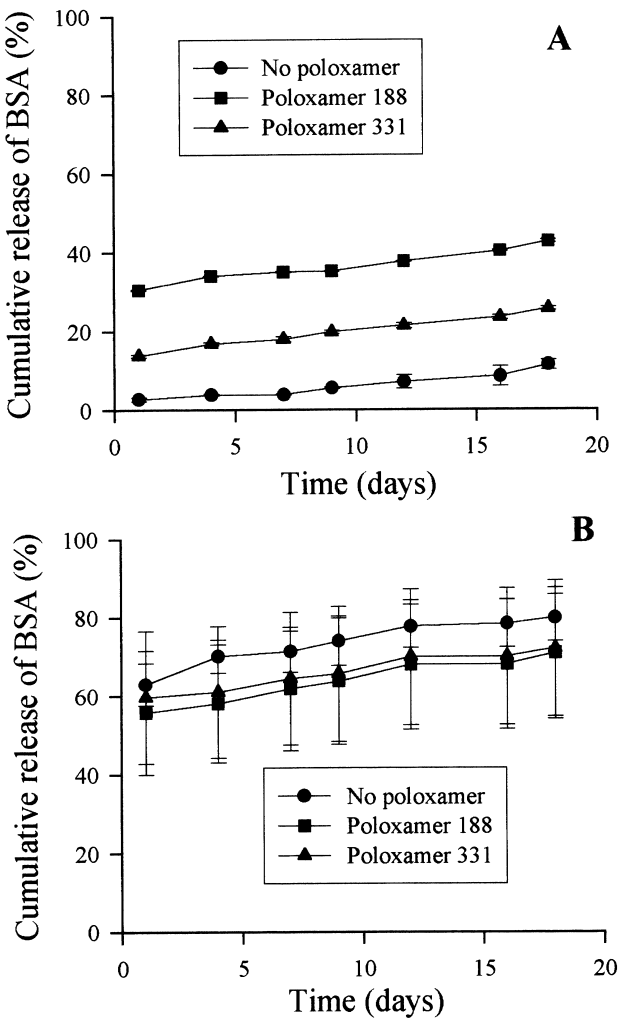

Fig. 3. The influence of the microencapsulation procedure and of the incorporation of poloxamers on the BSA in vitro release from PLGA microspheres (PLGA i.v.: 0.4). (A) w/o/w solvent extraction; (B) o/o solvent evaporation.

by a near-constant release of protein. The values obtained for the initial burst and rate constants are presented in Table 3. The results showed that microspheres prepared by the o/o solvent evaporation procedure have a higher initial burst (60%) than those prepared by the w/o/w solvent extraction procedure. This could be explained by the accumulation of the protein at the acetonitrile/oil interface during the solvent evaporation process and, consequently, near to the surface of microspheres. The second phase of the release profiles corresponds to the release of the entrapped protein. It has been reported that the release of proteins from PLGA microspheres is determined by the polymer degradation and by the protein diffusion through the water-filled channels created during the process [10,30]. In this study we found that the BSA releases more rapidly from the microspheres prepared by the o/o solvent evaporation procedure with respect to those prepared by the w/o/w solvent extraction. This could be attributed to the leaching-out of the protein molecules located at the surface of the microspheres and, thereby, the formation of large pores and interconnecting cavities through which the encapsulated protein can diffuse. Another interesting observation is that the co-encapsulation of poloxamer leads to a faster BSA release from the microspheres prepared by the w/o/w solvent extraction technique. These results could be explained by the reduced BSA–PLGA interaction caused by the presence of poloxamer, as indicated above. However, the large dispersity in the data obtained for the microspheres prepared by the o/o technique do not allow us to conclude that the poloxamers affect their in vitro release behaviour.

3.3.2. Protein release from PLGA microspheres: the effect of the type of protein and PLGA M_w

In a second step of this work we analysed the influence of the type of protein and PLGA M_w on the in vitro release behaviour of microspheres prepared by the w/o/w solvent extraction technique. The analysis of the in vitro release profiles from microspheres prepared by the solvent extraction method gives complementary information on the possible interaction between the PLGA and the proteins. The protein profiles of BSA and LZM loaded microspheres are shown in Figs. 4 and 5, respectively, and the intensity of the burst effect and constant release rate are summarised in Table 4. The results indicate that the intensity of the burst effect is not influenced by the type of protein or polymer M_w but is significantly affected by the incorporation of poloxamers (ANOVA $\alpha < 0.01$). The higher BSA release from the formulations containing poloxamer could be related to their destabilising effect of the w/o primary emulsion, as indicated in Section 3.1, which leads to the coalescence of the aqueous microdroplets and, thus, to a more porous structure.

The BSA constant release rate values (Table 4) indicate that this parameter significantly increases by reducing the PLGA M_w and by the presence of poloxamer (Kruskal–Wallis, $\alpha < 0.01$). The highest constant rate was observed for the formulation with the lowest M_w in the presence of poloxamer 188 (1.01% per day). To investigate the mechanism of protein release from the microspheres, we followed the evolution of the polymer M_w during the in vitro release process. Results in Fig. 6 indicate that the evolution of the residual molecular weight (%) of the polymer depends on

Table 3

Results of burst effect (% BSA release) and release constant (% BSA release. day-1) from microspheres prepared with PLGA i.v. 0.4

Microencapsulation technique	Surfactant	% Initial release	Release constant
w/o/w Solvent extraction	Without	2.75 ± 0.50	0.48 ± 0.14
	Poloxamer 188	30.47 ± 0.09	0.65 ± 0.01
	Poloxamer 331	13.74 ± 0.34	0.66 ± 0.05
o/o Solvent evaporation	Without	62.98 ± 5.41	0.92 ± 0.10
	Poloxamer 188	55.79 ± 15.82	0.88 ± 0.11
	Poloxamer 331	59.71 ± 16.91	0.75 ± 0.04

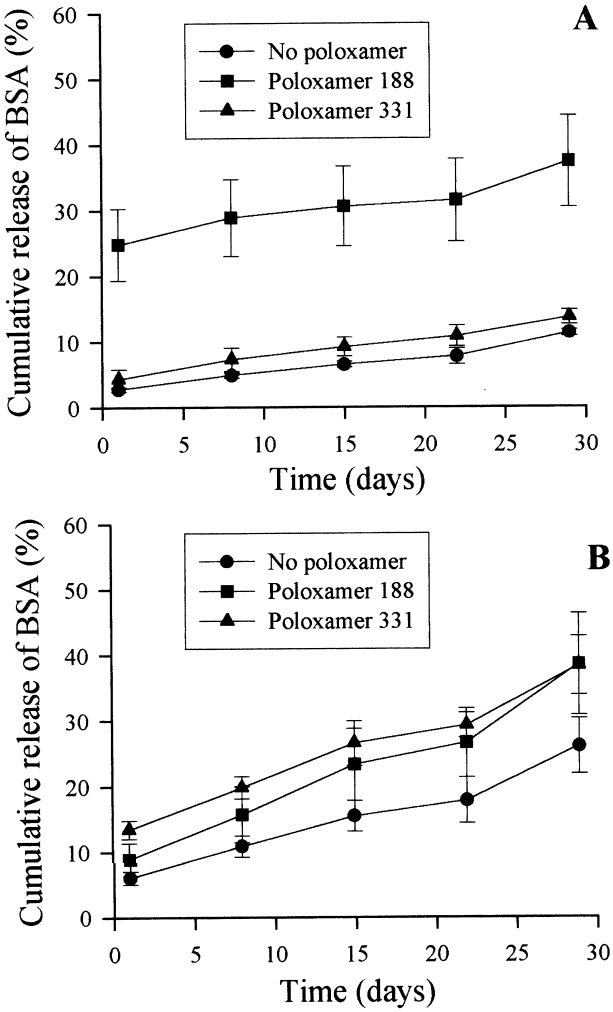

Fig. 4. The influence of the molecular weight of the polymer and of the incorporation of poloxamers on the BSA in vitro release from PLGA microspheres prepared by the w/o/w solvent extraction technique. (A) PLGA i.v.: 0.4; M_w: 34 kDa. (B) PLGA i.v.: 0.16; M_w: 10 kDa.

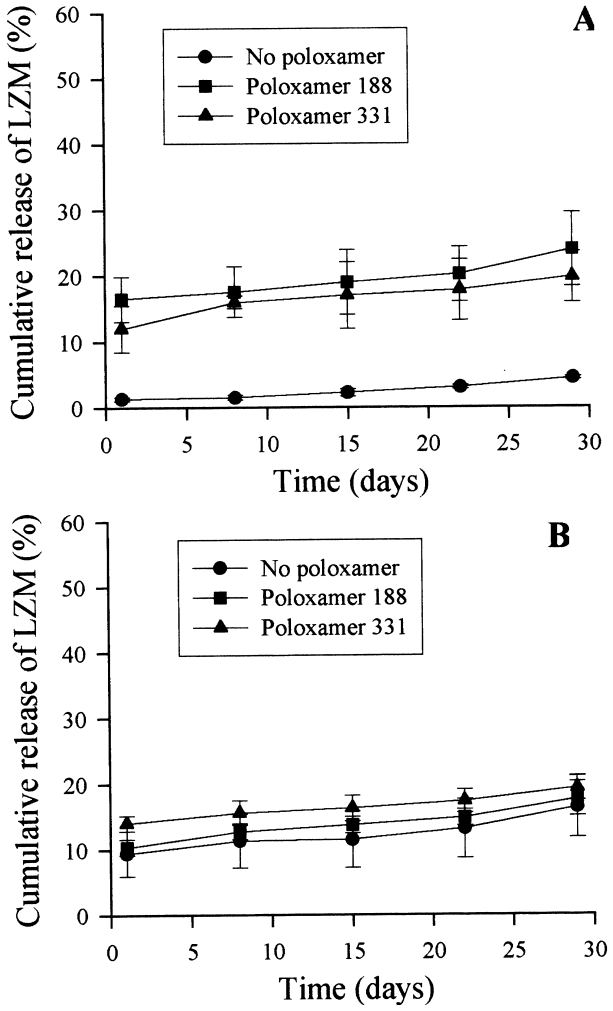

Fig. 5. The influence of the PLGA M_w and of the incorporation of poloxamers on the LZM in vitro release from PLGA microspheres prepared by the w/o/w solvent extraction technique. (A) PLGA i.v.: 0.4; M_w: 34 kDa. (B) PLGA i.v.: 0.16; M_w: 10 kDa.

the initial copolymer M_w. The higher the initial M_w, the greater the reduction of the residual M_w. It should be added that for the high M_w PLGA the M_w distribution of the residual polymer becomes broader over the time due to the accumulation of low or intermediate M_w fragments. On the contrary, the low M_w PLGA degraded into small oligomers which are soluble in the aqueous release medium, thus leading to a faster erosion of the microspheres. During erosion, the matrix becomes more and more hydrophilic, allowing more water to penetrate, thereby enhancing polymer degradation and thus, protein release. This erosion process explains the faster BSA release for the microspheres

Table 4

Results of burst effect (% protein release) and release constant (% protein release day-1) from microspheres prepared by the w/o/w solvent extraction technique

Copolymer	Surfactant	% Initial release		Release constant	
		BSA	LZM	BSA	LZM
DL-PLGA i.v.: 0.4 (M_w: 34 kDa)	Without	2.80 ± 0.32	1.33 ± 0.29	0.29 ± 0.03	0.11 ± 0.01
	Poloxamer 188	24.80 ± 5.47	16.50 ± 3.41	0.40 ± 0.05	0.25 ± 0.08
	Poloxamer 331	4.38 ± 1.44	11.97 ± 3.50	0.32 ± 0.07	0.25 ± 0.07
DL-PLGA i.v.: 0.16 (M_w: 10 kDa)	Without	6.04 ± 0.98	9.38 ± 3.47	0.68 ± 0.15	0.23 ± 0.04
	Poloxamer 188	8.83 ± 2.52	10.37 ± 1.2	1.01 ± 0.19	0.24 ± 0.06
	Poloxamer 331	13.42 ± 1.36	14.00 ± 1.19	0.85 ± 0.11	0.18 ± 0.03

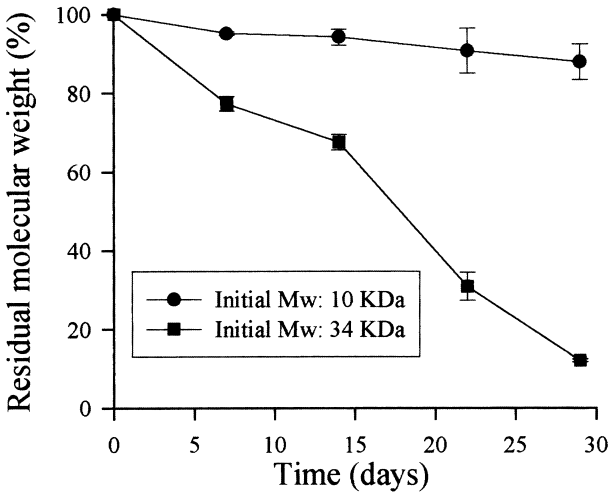

Fig. 6. The in vitro PLGA degradation profiles of different M_w PLGA microspheres.

prepared with the low M_w polymer. On the other hand, the higher porosity of the microspheres prepared with poloxamer and the reduced protein–polymer interactions, discussed above, could justify the higher release rate constant observed for these formulations.

The LZM release rate constants (Table 4) were much lower than those observed for BSA. Furthermore, no influence of the PLGA M_w, and thus of the erosion process, on the LZM release rate was observed. An explanation of this could be found in the physicochemical properties of LZM and its possible interaction with PLGA. To assess this hypothesis we incubated two sets of blank microspheres with free BSA and LZM (in the conditions used for the release studies as specified in the Section 2.5), and found that LZM interacted with the PLGA microspheres in a 26.19% and 35.65% at day 1 and 5, respectively, whereas the interaction of BSA was only 0.5% and 3.94% (day 1 and day 5) of the initial protein introduced in the incubation medium. These results led us to accept that there is an important interaction between LZM and PLGA, and that this interaction could be hypothetically mediated by hydrophobic forces and also by ionic forces between the positively charged LZM and the negatively charged carboxyl groups of PLGA and its degradation products. This interaction could explain the slow release of LZM from the PLGA microspheres. The same effect has recently been reported for other proteins [29,31]. Moreover, these results confirm the hypothesis about the reduction of protein–polymer interactions caused by the poloxamers [19,26] and agree well with those previously observed by our group when studying the interaction of proteins with polymers with carboxyl-end groups [32]. This information led us to the conclusion that the release of proteins from PLGA microspheres is not only governed by the degradation or erosion rate of the polymer, but that other factors such as the affinity of the protein versus the polymer may affect significantly the process.

Among the various protein inactivation sources, there is great concern that proteins incorporated into PLGA microspheres will undergo an aggregation process due to the decreasing pH inside of the microspheres, this drop in pH being caused by the accumulation of the polymer degradation products [1]. In order to investigate the possibility of protein aggregation due to its interaction with the polymer or with the polymer degradation products or by the acidic microenvironment, we analysed the integrity of the remaining encapsulated protein following the incubation of the microspheres for 1 month, at 37°C. The single lines in the

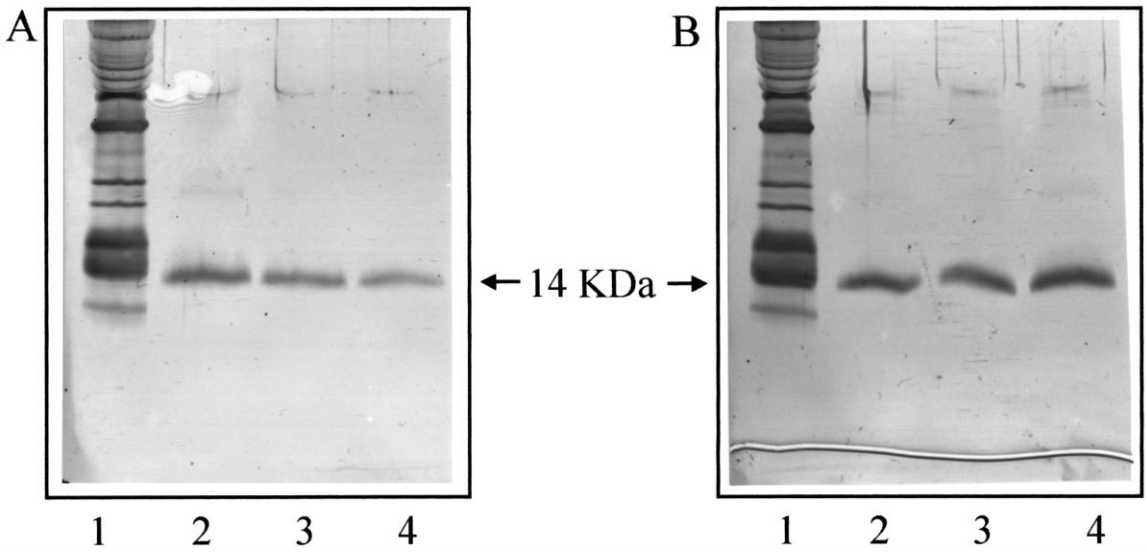

Fig. 7. The SDS-PAGE analysis of LZM-loaded microspheres following incubation for 1 month. Molecular weight markers (lane 1, gel A and B). Gel A: LZM in PLGA i.v. 0.4 without surfactant, with poloxamer 188 and with poloxamer 331 (lanes 2, 3 and 4, respectively). Gel B: LZM in PLGA i.v. 0.16 without surfactant, with poloxamer 188 and with poloxamer 331 (lanes 2, 3 and 4, respectively).

gels (Fig. 7) provided evidence that the entrapped proteins, in all formulations tested, did not suffer a significant covalent aggregation or fragmentation during the protein release process.

4. Conclusions

This study shows the effect of the polymer–protein interactions on the inner structure, encapsulation efficiency and protein release from PLGA microspheres. These interactions were found to be dependent not only on the specific properties of the protein and the polymer, but also on the microencapsulation process and the co-encapsulation of the tensoactives, i.e. poloxamers. In a general sense, the more important the interaction is, the higher the protein loading and the slower the protein release rate. However, the protein–polymer interaction may be driven by different phenomena; during encapsulation by the w/o/w technique, the tensoactive properties of the protein play an important role; whereas during release, the interaction can be mediated by hydrophobic and ionic forces between the protein and the polymer.

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