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PLA-microparticles formulated by means a thermoreversible gel able to modify protein encapsulation and release without being co-encapsulated

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Abstract

The aim of this work was to develop a novel strategy for the formulation of biodegradable PLA microspheres as delivery systems for proteins or peptides. The strategy is based on the exploitation of the gel–sol transition of the thermoreversible Pluronic F127 gel. The gel allows the formation of the particles without be co-entrapped in the matrix. The microspheres prepared using the novel technique (TG-Ms, or thermoreversible gel-method microspheres) were characterized *in vitro* (as concerns the size, the morphology, the protein encapsulation, the release and the protein distribution in the polymer matrix), in comparison with microspheres prepared using the classical double emulsion/solvent evaporation method (w/o/w-Ms). Two types of bovine serum albumin (BSA), with different water solubility, were used as model proteins. TG-Ms exhibited small size (7–50 m) and high protein content (8.6%, w/w) regardless of the BSA water solubility, in contrast with w/o/w-Ms, which revealed a size range of 100–130 μm and a protein content related to the BSA water solubility. TG-Ms, in spite of their smaller size respect of the w/o/w-Ms, displayed a reduced initial burst effect and a higher rate in the second release phase that resulted in a quasi-constant profile. The release behavior of the TG-Ms may be attributable to both the localization of the protein in the particle core, as shown by the confocal laser scanning microscopy analysis on labeled-BSA loaded microspheres, and the few pores in the matrix, as shown by the scanning electron microscopy. A working hypothesis about the mechanism of the particle formation was also discussed.

Keywords: Microparticles; Pluronic gel; Protein microencapsulation; Polylactic acid

1. Introduction

The short biological half-life and the *in vivo* instability are inherent problems with most of biologically active proteins and peptides, hence, their development in controlled delivery systems is a great challenge.

Over the last two decades the development of microspheres based on biodegradable polymers as protein delivery systems has been investigated extensively (Kawaguchi, 2000; Edlund and Albertsson, 2002; Vasir et al., 2003; Freiberg and Zhu, 2004). Among the biodegradable polymers suitable for preparing protein-loaded microparticles, most experience has been gained with poly(D,L-lactic acid) (PLA) and its copolymer with glycolic acid (poly-D,L-lactic-*co*-glycolic acid or PLGA) (Smith et al., 1990; Okada, 1997; Puney and Burke, 1998). The popularity of these biocompatible polymers can be ascribed to their non-toxicity, non-immunogenicity and to their approval by the FDA for use in injections in humans.

Generally, the microencapsulation methods for proteins in PLA or PLGA polymers are modifications of two basic techniques, i.e. solvent extraction/solvent evaporation and spray drying (Murillo et al., 2002; Freitas et al., 2005). Amongst these techniques, the double emulsion/solvent evaporation method is considered as the most convenient for the water soluble proteins in terms of protein stability and encapsulation efficiency (Ogawa et al., 1988; Yang et al., 2000). Despite these advantages, double emulsion/solvent evaporation method suffers from two major drawbacks. First, the required use of hydrophobic and halogenated solvents (i.e. dichloromethane) that are toxic and not easy to be removed completely (Bodmeir and McGinity, 1988). Second, inadequate protein release that is often characterized by an initial burst release phase followed by little and incomplete release that does not match the polymer degradation (Park et al., 1998; Perez et al., 2002). Moreover, the production of acid degradation products, such as lactic and glycolic acids, is the primary instability source of the encapsulated acid-labile bio-

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macromolecules during their release process (van de Weert et al., 2000). To improve the release modalities and the stability of the protein during the second release phase, several approaches have been investigated including blends of structurally different polymers for microencapsulation (Sah et al., 1994) or the co-entrapment of the release modifying agents (Péan et al., 1999; Zhu et al., 2000). On the other hand, initial burst release phase, due to the large amount of protein located in the surface layer, is one of the major challenges in protein-encapsulated microparticle systems (Costantino et al., 2004). Since protein release during the initial stage depends mostly on the diffusional escape of the protein, a rational approach to prevent the initial burst have to be focused on efficient encapsulation of the protein within the microparticles (Leo et al., 1998; Yeo and Park, 2004).

Taking into account these considerations, a novel technique to produce PLA microspheres by means the application of a thermosensible gel of Pluronic F127 has been developed. Pluronics or Poloxamers are poly(ethylene oxide)–(polypropylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymers able to form, in water, micelles at low concentrations and clear, thermoreversible gels above a concentration of 20% (w/w) (Vadnere et al., 1984). Pluronic F127 water solutions more concentrated than 20% (w/w) are liquid when refrigerated below 10 °C but gel upon warming. The gel–sol transition temperature of gels as well as the gel formation process have been widely reviewed (Gilbert et al., 1987; Chung Tung, 1994; Pandit and Kisaka, 1996). These studies show that the sol–gel transition temperature depends on the Pluronic F127 concentration and the gel formation occurs with the progressive dehydration of the polymer micelles as temperature increases, leading to increase the chain entanglement. Over the years the gels have received great attention as drug delivery system for topical transdermal, ophthalmic and implantable applications (Gilbert et al., 1986; Suh and Jun, 1996; Bochot et al., 1998). Moreover, Pluronic F127 was frequently used as excipient in the formulation of polymeric microparticles (Yeh et al., 1996; Carrasquillo et al., 2001; Giunchedi et al., 2001; Lagarce et al., 2005) but, to our knowledge, the application of its gel–sol transition in the microsphere formulation was not yet investigated.

Therefore, the aim of this study was to evaluate the potentiality as protein delivery systems of PLA microspheres prepared using the thermoreversible Pluronic gel comparing their characteristics to those of microspheres obtained by the classical method of the double emulsion/solvent evaporation. Two types of bovine serum albumin (BSA), with different water solubility, were used as model proteins throughout this investigation.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic acid) (PLA) (Resomer® 203; inherent viscosity: 0.25–0.35 dL/g 0.1% chloroform, 25 °C) was supplied from Boehringer Ingelheim (Ingelheim, Germany). Bovine serum albumin (BSA) fraction V, essentially γ-globulin free, initial fractionation by cold alcool precipitation (BSA type A, solubility of 70 mg/mL, determined in water at 25 °C), BSA fraction V, initial fractionation by heat shock (BSA type B, solubility of 40 mg/mL, determined in water at 25 °C) and FITC-BSA (fluorescein isothiocyanate-labeled bovine serum albumin) were purchased from Sigma (Milan, Italy). Pluronic F127 (Lutrol F127) was offered by BASF (Bergamo, Italy). Bio-Rad DC microassay kit was supplied from Bio-Rad Laboratories (Milan, Italy). All other chemicals and solvents were of reagent grade and used as received.

3. Methods

3.1. Preparation of PLA microspheres by means of the thermoreversible gel (TG-method)

BSA (type A or B) (20 mg) was dissolved in 2 mL of an aqueous solution (25%, w/w) of Pluronic F127 at 4 °C; then, the solution was kept at 37 °C until gelification (5 min). PLA (250 mg) was dissolved in 3 mL of acetone and then dispersed in the Pluronic gel containing the protein at 37 °C by an Ultra Turrax® (8000 rpm, Janke and Kunkel-Ika-Laboratortechnich, Staufen, Germany) during 3 min. After this time period, the dispersed system obtained was cooled at 4 °C in order to allow the gel–sol transition of the Pluronic gel. Following this transition, the diffusion of the acetone into the aqueous solution occurred; after 5 min, 40 mL of deionised water was added gradually (within 10 min) in order to complete the extraction of the acetone. Then, the microspheres were washed three times in deionised water, recovered by centrifugation (5100 × *g*; model 4235, A.L.C., Milan, Italy) and freeze-dried (Lyovac GT2, Leybold-Heraeus, Hanau, Germany) during 24 h.

3.2. Preparation of PLA microspheres by double emulsion/solvent evaporation method (w/o/w-method)

BSA (type A or B) (20 mg) was dissolved in deionised water (0.4 mL) and then emulsified by Ultra Turrax® (8000 rpm) with 3 mL of dichloromethane containing the PLA polymer (250 mg). This primary emulsion (w_1/o) was added to the external aqueous phase (w_2, 30 mL; 0.5%, w/v of Pluronic F127) and emulsified again by Ultra Turrax® at 8000 rpm. After the solvent evaporation (3 h at room temperature), the microspheres were washed three times in deionised water, recovered by centrifugation and freeze-dried as described above.

3.3. Microsphere characterization

The preparation methods described above provided two kinds of BSA-loaded PLA microspheres: (i) TG-Ms by means of the thermoreversible gel-method; (ii) w/o/w-Ms obtained by the double emulsion/solvent evaporation method. For each kind of BSA-loaded PLA microspheres, sample-A or sample-B according to the use of BSA type A or B, respectively, were obtained.

The recovery percentage was calculated as the ratio of the amount of recovered Ms to the total amount of PLA and BSA added.

Protein loading and entrapment efficiency were calculated as following equations:

$$\text{BSA loading (\%, w/w)} = \frac{\text{mass of BSA in Ms}}{\text{mass of Ms recovered}} \times 100 \quad (1)$$

$$\text{entrapment efficiency (\%)} = \frac{\text{mass of BSA in Ms}}{\text{starting mass of BSA}} \times 100 \quad (2)$$

3.4. Scanning electron microscopy

Shape and size of the microspheres were analysed by scanning electron microscope (SEM) (XL-40 Philips, The Netherlands). Microspheres were mounted onto aluminum stubs (TAAB Laboratories Equipment Ltd., Berks, UK) using a double side sticky tab (TAAB Laboratories Equipment) and vacuum coated with gold–palladium in an argon atmosphere for 60 s (Sputter Coater Emitech K550, 2M Strumenti, Rome, Italy) and observed by SEM. To study the inner structure, microspheres were embedded in a epoxy resin (Taab epocure, TAAB Laboratories Equipment Ltd.), cross-sectioned using an ultra-microtome (Jang SM 2000R, Leizt, Wetzlar, Germany), coated with gold–palladium and then observed by SEM. To determine the size distribution of the particles, at least 500 particles for each preparation were sized from electron photomicrographs by an image analysis (Image Proplus, Media Cybernetics, USA).

3.5. Confocal laser scanning microscopy

A confocal laser scanning microscope (CLSM, Leica IRBE, Germany) was employed to observe FITC-BSA distribution within FITC-BSA loaded-microspheres formulated according the preparation methods above described (thermoreversible gel-method and double emulsion/solvent evaporation method). The CLSM was equipped with 25M_w Ar–Kr laser and associated filters for 488, 568, and 647 nm excitation wavelengths.

3.6. Determination of BSA content in the microspheres

The amount of BSA encapsulated in microspheres (%, w/w) was determined completely dissolving an exactly weigh amount of microspheres (about 10 mg) in 2 mL of SDS/NaOH (0.3 M NaOH containing 1% (w/v) of sodium dodecyl sulphate) during 48 h at room temperature. In this solution the amount of encapsulated BSA was determined using the Bio-Rad DC microassay kit.

3.7. Determination of Pluronic F127 residual

The amount of Pluronic F127 residual in the lyophilised microspheres was determined by a colorimetric method based on the formation of a colored complex between two hydroxyl groups of Pluronic F127, Ba^{2+} and an iodine molecule (Childs, 1975). Briefly, 10 mg of lyophilised microspheres were dissolved in 2 mL of dichloromethane. Then, 10 mL of deionised water were added and stirred until complete evaporation of the solvent (2 h). After the centrifugation of the water phase at 4200 rpm for 10 min, 1 mL of a 5% (w/v) $BaCl_2$ solution in HCl 0.1 M and 1 mL of a solution of I_2/KI (0.05 M/0.15 M) were added to 4 mL of supernatant. Finally, the absorbance of the samples was measured spectrophotometrically at 540 nm (UV/Vis Spectrophotometer V-530, Jasco, Tokyo, Japan) after 15 min incubation at room temperature and the amounts of Pluronic F127 was calculated using a calibration curve realized in the same conditions. No absorption was observed when only PLA polymer was used under the identical condition.

3.8. In vitro release study

Microsphere samples (25 mg) were incubated in 20 mL of phosphate buffer (20 mM, pH 7.4) at 37 °C under magnetic stirring. At fixed time intervals an aliquot (0.5 mL) was withdrawn and replaced with 0.5 mL of fresh solvent to maintain constant volume. The sample was centrifuged (10,000 rpm for 10 min) and BSA content was determined in the supernatant by the kit of Bio-Rad DC microassay. In order to assay the integrity of the BSA released from the TG-Ms a SDS-PAGE analysis was performed on the protein released after 72 h.

The analysis was performed using a Bio-Rad model 1000/500 system (Hercules, CA, USA). The samples of native BSA and the BSA released from the microparticles after 72 h were diluted with Tris-buffer (pH 6.8) with 2% SDS. Electrophoresis of samples was performed at a constant voltage of 200 V in a Tris/glycine/SDS buffer. After migration, the gel was stained with Coomassie bright blue (R-250, Bio-Rad Laboratories, Milan, Italy) in methanol–acetic acid–water (2.5:1:6.4) to reveal protein, destained and dried.

Table 1
Recovery percentage, Pluronic F127 residual, protein loading and encapsulation efficiency of the microspheres prepared with the double emulsion (w/o/w-Ms) or thermoreversible gel (TG-Ms) techniques

Sample	Preparation technique	BSA type	Recovery percentage (%, w/w)	Pluronic F127 residual (%, w/w)	BSA loading (%, w/w)	Encapsulation efficiency (%)
W/o/w-A	Double emulsion	A	83.5 (±3.2)	1.15 (±0.07)	6.4 (±0.3)	72.4 (±0.7)
W/o/w-B	Double emulsion	B	85.3 (±4.3)	1.22 (±0.10)	8.0 (±0.4)	92.5 (±0.8)
TG-A	Thermo-reversible gel	A	80.2 (±4.7)	0.21 (±0.04)	8.6 (±0.5)	93.0 (±0.5)
TG-B	Thermo-reversible gel	B	82.3 (±3.1)	0.11 (±0.02)	8.5 (±0.4)	94.5 (±0.8)

All samples were prepared in triplicate (±S.D.).

4. Results

4.1. Microsphere characterization

According to the described preparation methods, two microsphere samples were prepared using BSA type A (w/o/w-A and TG-A samples) or B (w/o/w-B and TG-B samples). Recovery percentage, size, Pluronic F127 residual, BSA loading and encapsulation efficiency of the microspheres are summarized in Table 1.

The recovery percentage for all the sample was in the range of 80–85%.

The residual of Pluronic F127 was high for the w/o/w-Ms (1.15% (w/w) and 1.22% (w/w) for the samples w/o/w-A and w/o/w-B, respectively) and very small for the TG-Ms (0.21% (w/w) for the sample TG-A and 0.11% (w/w) for the sample TG-B).

The BSA content and the encapsulation efficiency for the w/o/w-Ms was dependent on the BSA type; on the contrary for TG-Ms the BSA content was about 8.6% (w/w) regardless of which type of BSA was used.

Finally, the microspheres prepared with the two methods showed very different dimensions. The sizes of w/o/w-Ms were quite uniform and in the range of 100–130 μm; on the contrary, TG-Ms resulted much more smaller and non-homogeneous being in the range of 7–50 μm (see Table 2).

4.2. Scanning electron microscopy

The SEM microphotographs (Fig. 1) show representative images of the external (a) and cross-sectional (b) morphologies of the w/o/w-Ms. The surface morphology was smooth and regular; the inner cross-section appeared with a typical honeycomb structure with the holes distributed uniformly in the particle.

With regards to TG-Ms, the particles were spherical, had a broad size distribution and presented some pores on the surface (Fig. 2a); the cross-section shows an inner structure compact and few pores (Fig. 2b) are evident.

4.3. Confocal laser microscopy

The BSA distribution in the microspheres was studied by a confocal laser scanning microscopy (CLSM). Two batches containing FITC-BSA were prepared according both the w/o/w-method and the TG-method. Fig. 3 represents four images in sequence of the single plane in z thickness of the w/o/w-Ms and the TG-Ms, respectively. It was observed that the protein distribution within the w/o/w-Ms was practically uniform in the particle matrix. On the contrary, in the TG-Ms the protein was assembled in the core of the particle and a small amount of protein was observed near the particle surface.

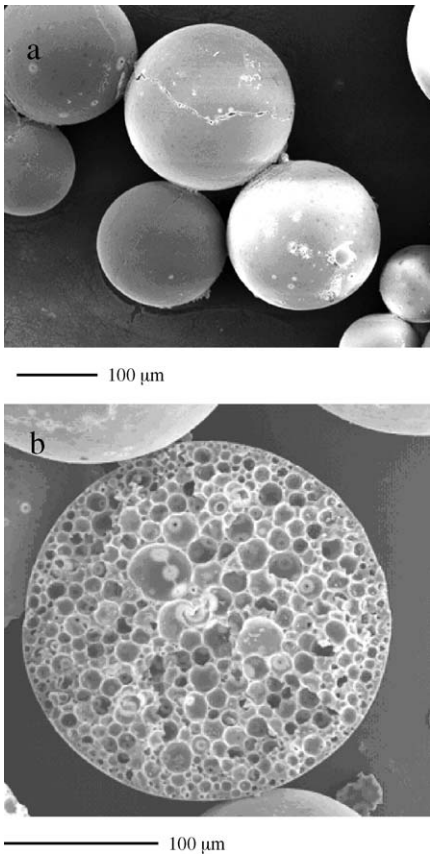

Fig. 1. Scanning electron micrographs of w/o/w-Ms: (a) external morphology; (b) inner structure.

Table 2
Particle size and burst release related to the w/o/w-Ms and TG-Ms

Sample	Particle size (μm)			Burst release within 4 h (%)
	D_{10}	D_{50}	D_{90}	
W/o/w-A	100	120	130	63.7
W/o/w-B	105	122	140	58.9
TG-A	8	20	40	40.0
TG-B	7	23	50	29.5

D_{10}, D_{50}, D_{90} refer to 10%, 50% and 90% of particles respectively having a size less than indicated values.

4.4. In vitro BSA release

The BSA release profile from PLA microspheres obtained in 20 mM phosphate buffer is shown in Fig. 4. During the time analysis period (72 h), the entrapped BSA was release completely from the TG-samples and partially for the w/o/w-samples. However, all the release patterns appeared biphasic: an initial burst effect followed by a more slowly release phase. The percentage of BSA released after the initial burst period (4 h) is reported in Table 2. The values indicate a low burst effect for the TG-Ms respect to the homologues w/o/w-samples. In addition, regardless the preparation method used, the burst effect was greater for the samples prepared using the BSA type A (i.e. w/o/w-A and TG-A samples).

During the second release phase, the TG-samples displayed a linear profile with a complete release of the protein within 72 h, while for the two w/o/w-Ms a slow release phase was observed and, after 72 h, the 80% of the drug release was accomplished. The integrity of the BSA released from the TG-samples was

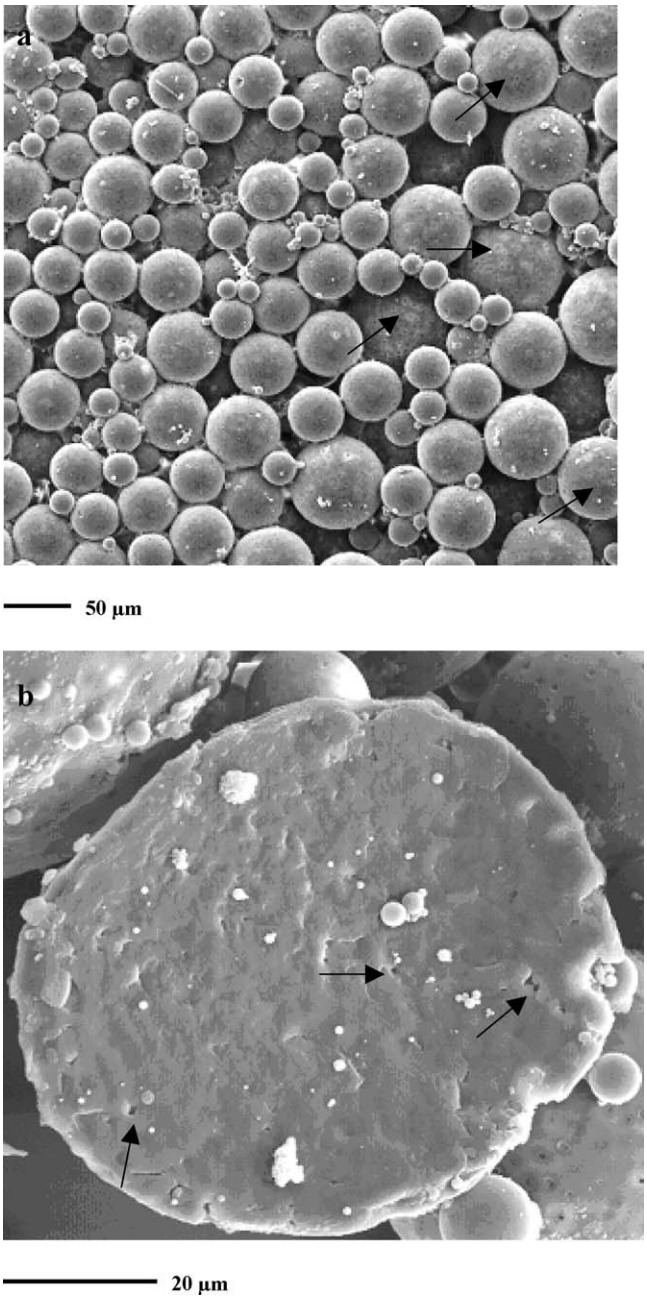

Fig. 2. Scanning electron micrographs of TG-Ms: (a) external morphology; (b) inner structure.

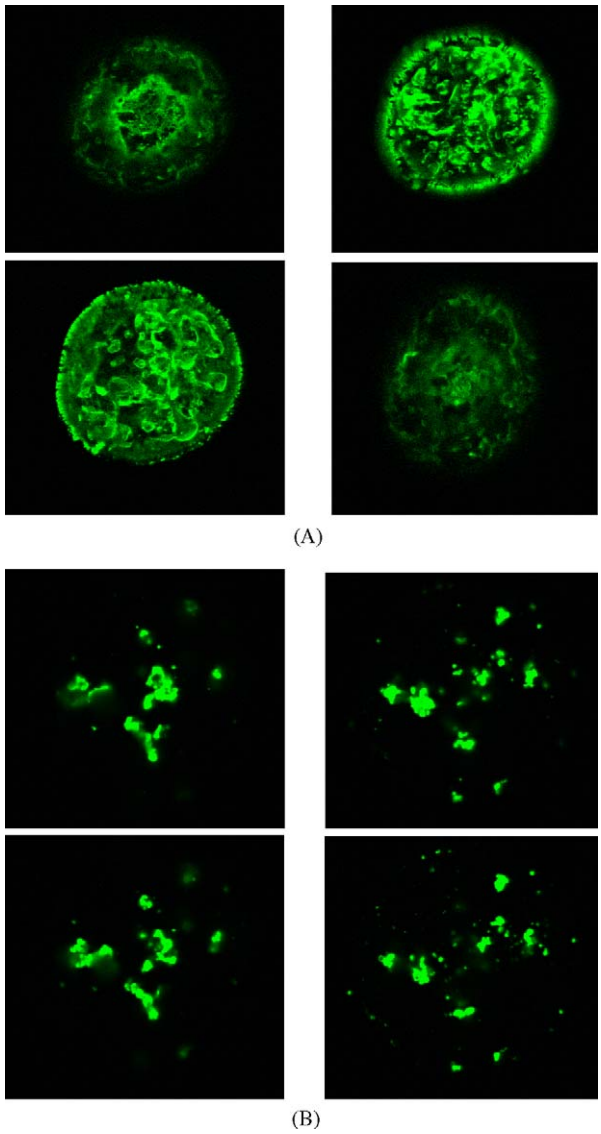

Fig. 3. (A) Four CLSM images in sequence of single plane in z thickness of FITC-BSA loaded PLA microspheres prepared by double emulsion solvent evaporation method (w/o/w-Ms). (B) Four CLSM images in sequence of single plane in z thickness of FITC-BSA loaded PLA microspheres prepared by the thermoreversible gel-method (TG-Ms).

demonstrated by the SDS-PAGE analysis being present in the gel only the band identical to the native protein without additional bands.

5. Discussion

For the most part of the protein microencapsulation processes, the encapsulation efficiency closely depends on the water solubility of the drug. Indeed, in order to compare the novel technique to the classic one of the double emulsion, two types of BSA, with different water solubility, were employed: BSA type A, with a solubility in water at 25 °C of 70 mg/mL and BSA type B with a solubility in water at 25 °C of 40 mg/mL. Considering each method, the BSA-loaded microparticles were formulated using the same amounts of proteins, polymer, and the same Ultra Turrax® speed. Hence, for the w/o/w-Ms the difference in the BSA loading and the encapsulation efficiency observed between the two w/o/w-Ms (Table 1) may only depend on the solubility of the BSA: the higher was the BSA solubility (sample w/o/w-A) the lesser the protein content. On the other hand, the protein loading and the encapsulation efficiency of the two TG-Ms (TG-A and TG-B) were identical indicating that, in this case, the protein solubility did not affect the BSA content.

The information obtained by the CLSM analysis were very useful in order to explain these results. Indeed as far as TG-Ms, it is clearly visible that very poor protein is located close to the particle surface and the most part of the labeled protein is assembled in the core of the polymer matrix. This means that

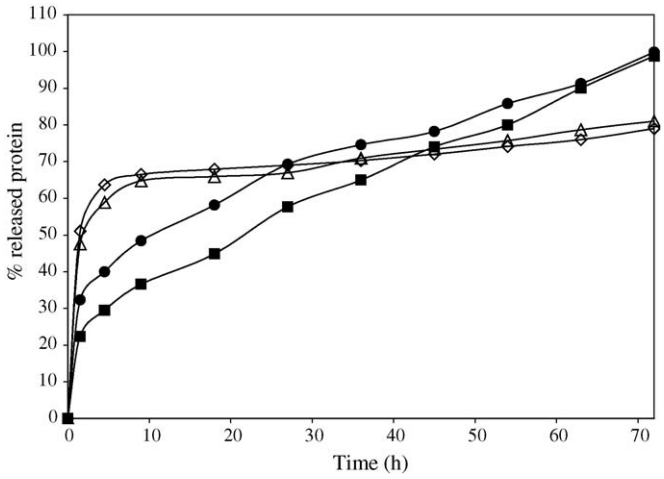

Fig. 4. In vitro release in 20 mM phosphate buffer at 37 °C of BSA type A or B from PLA-microspheres obtained by the w/o/w-method or TG-method. Results are the means of three independent experiments (S.D. less than 10%). w/o/w-A sample (◊); w/o/w-B sample (△); TG-A sample (●); TG-B sample (■).

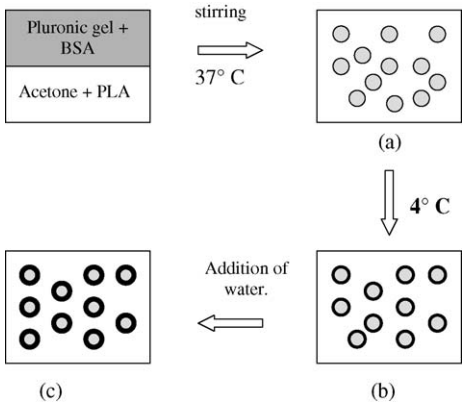

Fig. 5. Schematic description of the proposed mechanism of formation of PLA microspheres by the thermoreversible gel-method: (a) formation of the pseudo-emulsion between Pluronic-gel and acetone; (b) formation of the polymer crust upon the gel–sol transition; (c) formation of particles upon the solvent extraction.

during the particle formation only a little amount of protein was interfaced with the external water phase, thus, the BSA solubility did not affect the loading capacity of these microspheres.

W/o/w-samples exhibited a large presence of the labeled protein close the particle surface. As a consequence, it is possible to hypothesise that, during the microparticle formation, the protein located or close to the surface may be lost in the external aqueous phase as function of its water solubility. This finding agrees with a recent study about the mechanism in the microparticle formation by double emulsion/solvent evaporation (Rosca et al., 2004). This study highlights as, during the shrinkage due to the solvent evaporation, the encapsulated substance is continuously partitioning with the external aqueous phase.

As concerns the protein release from the microspheres, considering the samples obtained by the same method (w/o/w-A versus w/o/w-B and TG-A versus TG-B), higher was the solubility of BSA higher the burst effect. Moreover, the burst release of the w/o/w-Ms was higher respect to the TG-Ms (notwithstanding w/o/w-Ms presented a bigger size) as a result of the massive presence of BSA near the particle surface, as the CLSM images show.

The second release phase from the samples was not affect by the protein solubility but strongly influenced by the formulation method. Indeed, it was more linear for the TG-Ms respect to the w/o/w-Ms probably owing to the different protein distribution in the particle matrix. It is known that the drug distribution in a sphere matrix could modify the diffusion path length of the drug and, thus, its release. For example, the localization of low molecular weigh drugs in the core of the particles was exploited in order to obtain a relatively constant drug release rate (Kakish et al., 2002). Thus, the quite rapid and complete release of protein displayed by the TG-Ms samples can be strictly related to the protein localization in the core of the particles. In all probability the diffusion of BSA in this case occurred by a preferential way, i.e. the pores visible on the particle surface and in the particle cross-section (Fig. 2). These pores may be produced by the migration of Pluronic in acetone toward the external aqueous phase, during the particle formation. Since the Pluronic residual in the freeze-dried samples was very low (Table 1) the pores were not filled by Pluronic. Owing to capillarity forces, the dissolution medium penetrated in the particles and, as a consequence, a rapid solubilization and diffusion of the protein occurred before polymer degradation increased, preserving the protein from the acidic degradation.

On the contrary, w/o/w-Ms, in agreement to the high number of protein release studies performed previously (Biwa et al., 1985; Crotts and Park, 1997; Blanco and Alonso, 1998), exhibited an initial burst of release followed by a slow and incomplete release. Several approaches have been used to modulate release of proteins from such as PLGA microspheres. Techniques include colyophilizing amphiphilic polymers with the protein to be released (Morita et al., 2001), addition of surfactants to the aqueous drug-containing phase (Crotts and Park, 1997; Rojas et al., 1999) copolymerizing with poly(ethylene glycol) (Péan et al., 1999; Bae et al., 2000), blending with polymers of different hydrophobicity/hydrophilicity (Yeh et al., 1996; Ravivarapu et al., 2000). These approaches cannot only change the release kinetics, but the bioactivity of encapsulated proteins also may be protected. Differently from the strategies described previously, the approach proposed here was based simply on the modification of the preparation procedure, i.e. on the use of a thermoreversible gel that, finally, was not co-encapsulated in the particle matrix.

In order to clarify the formation mechanism for the TG-Ms the following working hypothesis was formulated (Fig. 5). Afterward the dispersion of acetone (containing the polymer) in the Pluronic gel (containing protein) at 37 °C, an acetone/gel pseudo-emulsion was formed (Fig. 5a). Subsequently, the pseudo-emulsion was cooled and the gel–sol transition of Pluronic occurred (Fig. 5b). At this point the diffusion into the water of acetone and the simultaneous migration of Pluronic in acetone occurs. This is the key point for the microparticles formation: the mixing of water and acetone induces the precipitation of a polymer crust that prevent the depletion of protein. The passage of the Pluronic in the acetone may be responsible of the formation of pores that are well visible on the surface and on

the cross-section of the particles (Fig. 2). Then, the further dilution of acetone with water accomplished the PLA precipitation (Fig. 5c) and the stabilization of microparticles.

6. Conclusion

A new strategy based on the use of a thermoreversible gel for achieving BSA-loaded microspheres, characterized by a high encapsulation efficiency and a linear release profile, was described. The microsphere characteristics may be substantially attributable to the localization of the protein in the core of the particles and to the few pores in the matrix. The proposed mechanism of the particle formation should explain why BSA is located in the core of the particle and BSA encapsulation efficiency is independent of BSA water solubility.

Future studies will focus on the application of this encapsulation method to peptides or low molecular weight proteins.

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