



Critical attributes of formulation and of elaboration process of PLGA-protein microparticles



C. Martín-Sabroso^a, A.I. Fraguas-Sánchez^a, J. Aparicio-Blanco^a, M.F. Cano-Abad^{c,d,e},
A.I. Torres-Suárez^{a,b,*}

^a Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Complutense University of Madrid, Spain

^b Institute of Industrial Pharmacy, Complutense University of Madrid, Spain

^c Clinical Pharmacology, Health Research Institute, La Princesa University Hospital, Madrid, Spain

^d Teófilo Hernando Institute, Madrid, Spain

^e Department of Pharmacology and Therapeutics, Faculty of Medicine, Autonomous University of Madrid, Spain

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ABSTRACT

Low drug loading, burst effect during release and drug inactivation account for the main drawbacks of protein microencapsulation in poly(D,L-lactic-co-glycolic) acid (PLGA) matrix by the water-in oil-in water (W/O/W) solvent evaporation method. Thus, the current study was set to invest the critical attributes of formulation and of elaboration process which determine protein loading into microparticles as well as its further release, using albumin as protein model.

NaCl concentration in the external aqueous phase, poly(vinyl alcohol) (PVA) concentration and mostly viscosity of both the internal aqueous phase and the organic phase were critical attributes for improving drug loading, with polymer molecular weight and hydrophobicity likewise directly related to albumin loading. In such a way, when using 0.5% PVA as internal aqueous phase the highest albumin loading was achieved. Optimized microparticles exhibited a sustained *in vitro* release of albumin over 130 days. The influence of the microencapsulation process on albumin stability and biological activity was evaluated by carrying out cell proliferation assays on PC12 cells with albumin released from microparticles. Such assay demonstrated that the microencapsulation procedure optimized in this study did not affect the biological stability of the microencapsulated protein.

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1. Introduction

A large variety of protein drugs such as hormones, growth factors and vaccines with interesting pharmacological activities have been developed but their incorporation into therapeutic finds the difficulties of their low oral bioavailability and their short half-lives, as a consequence of the action of plasmatic and tissular proteases, that make necessary repeated injections for parenteral route. An interesting approach aimed to reduce the number of

injections in these biological therapies consists of the microencapsulation of the proteins using biodegradable polymers (Andreas et al., 2011; Formiga et al., 2010; Li et al., 2011; Ma, 2014; Tran et al., 2012; Zhang et al., 2013). These microparticles allow a slow protein release, and more importantly, they provide protection for the proteins against proteases in the administration site (Han et al., 2014; Pisal et al., 2010; Yeh et al., 2007).

Poly(D,L-lactic-co-glycolic) acid (PLGA) has been widely studied to develop controlled release systems for proteins and vaccines, and water-in oil-in water (W/O/W) solvent evaporation technique has been commonly used to entrap protein into PLGA microparticles (Bodmeier et al., 1992; Mundargi et al., 2008; Reinhold and Schwendeman, 2013; Sophocleous et al., 2013). In this process, the drug is dissolved in an inner aqueous phase, which is emulsified in the organic phase containing the polymer dissolved in an organic solvent, usually dichloromethane (DCM). This primary W/O emulsion is subsequently emulsified in an external aqueous phase containing an emulsifier such as poly(vinyl alcohol) (PVA). Polymer precipitation and as consequence microparticle formation are induced due to solvent removal from the organic phase by extraction

Abbreviations: ALB, albumin; BCA, bichoninic acid; BSA, bovine serum albumin; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; MP, microparticles; OD, optical density; PAO, phenylarsine oxide; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PLGA, poly(D,L-lactic-co-glycolic) acid; PVA, poly(vinyl alcohol); SEM, scanning electron microscopy; SPARC, secreted protein acidic and rich in cysteine; W/O/W, water-in oil-in water.

*Corresponding author at: Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense de Madrid, E-28040 Madrid, Spain. Tel.: +34 913941735; fax: +34 913941736.

toward the external aqueous phase and by evaporation. This technique presents **three important limitations** to microencapsulate proteins: the low loading capacity; the burst effect during protein release; and the protein inactivation during microencapsulation, mainly by denaturalization and formation of aggregates.

The implementation of the quality by design in the development of protein loaded microparticles leads to the definition of those critical attributes related both with the formulation as with the manufacture process, which will determine in greatest extent the characteristics of the microparticles obtained, taking into consideration their intended usage and the route of administration. By applying quality by design, optimized as well as robust protein microencapsulation procedures could be developed, understanding as “robustness” the ability of a procedure to tolerate changes of the formulation and of the technological process without negative impact on quality of the product.

The stage of formation of the primary W/O emulsion is considered the main cause of **protein denaturalization and formation of aggregates, due to both the contact of the proteins with the organic solvent**, favored by the dispersion of the small droplets of the aqueous phase into the organic phase, as well as to the mechanical agitation (Checa-Casalengua et al., 2012; Chen et al., 2004; Dorati et al., 2008; Jiskoot et al., 2012; Van de Weert et al., 2000). Other problems of protein microencapsulation are **the low loading and the initial burst release effect**. During microencapsulation process, the partition of hydrophilic proteins from the internal phase to the external aqueous one, leads to a reduction of the protein entrapment into the microparticles (usually less than 10% w/w) and to an increase of their burst initial release. Besides, the release of the proteins from microparticles is commonly low and non-uniform. To solve these problems, different excipients have been incorporated during microencapsulation process, as poly(ethylene glycol) (PEG) and sodium chloride (NaCl) (Bock et al., 2014; Buske et al., 2012; Chen et al., 2002; Nugraha et al., 2014; Van de Weert et al., 2000; Ye et al., 2010). These both excipients also improve proteins stability.

The aim of this work was to investigate the critical attributes of formulation and of elaboration process of PLGA-protein microparticles that influence on drug loading, protein release kinetic and protein stability throughout the microencapsulation process. Protein-loaded microparticles were obtained by the water-in oil-in water (W/O/W) solvent evaporation technique, and different strategies leading to an improvement of the loading, the release kinetic and the stability of microencapsulated proteins, were evaluated.

Albumin (ALB) was used as protein model, because of its good water solubility, its high molecular weight and its thermal stability (Dorati et al., 2008). It is the most plentiful human plasma protein with a major role in extracellular and intracellular transport of nutrients and with an interesting use as drug delivery carrier, chiefly chemotherapeutics agents, facilitating drug targets at tumor level, since albumin is the main energy and nutrition source for tumor growth (Martín Sabroso and Torres-Suárez, 2014; Elzoghby et al., 2012).

In this work, microparticles loaded with ALB were developed and characterized in terms of particle size, surface morphology, protein entrapment efficiency and release properties. In addition, to determine the effect of the microencapsulation procedure on the stability of the protein, the activity of the microencapsulated ALB on the proliferation of PC12 cells was evaluated.

2. Materials and methods

2.1. Materials

Albumin grade V (ALB) and polyvinyl alcohol (PVA, Mw = 30,000–70,000) were obtained from Sigma–Aldrich (Madrid,

Spain), poly(D,L-lactic-co-glycolic acid) (PLGA) with a ratio of 50:50 (Resomer RG504, RG504H, RG502) and with a ratio of 75:25 (Resomer RG756S) were purchased from Boehringer Ingelheim (Barcelona, Spain). Dichloromethane (HPLC grade), acetone, hydrochloric acid, sodium hydroxide, potassium dihydrogen phosphate, disodium hydrogen phosphate dehydrate, sodium chloride, poly(ethylene glycol) 400 and poly(ethylene glycol) 4000 were provided by Panreac (Barcelona, Spain). Distilled demineralised Milli-Q[®] water (Millipore, Madrid, Spain) was used.

2.2. Methods

2.2.1. Preparation of ALB microparticles

The microparticles were prepared by the water-in oil-in water (W/O/W) solvent evaporation technique, on the basis of the procedure described by Chen et al. (2002). Briefly, a primary emulsion is obtained by adding 1 ml of ALB aqueous solution (30 mg/ml) to 5 ml of dichloromethane (DCM) containing 300 mg of PLGA. An homogenization step was performed afterwards (8000 rpm for 2 min). The resulting W/O emulsion was subsequently mixed at 1000 rpm with 30 ml of continuous phase solution containing 5% (w/v) polyvinyl alcohol (PVA) as emulsion stabilizer. The resulting W/O/W multiple emulsion was magnetically stirred for 18 h at room temperature to allow DCM removal. Microparticles were separated by centrifugation (2000 rpm for 5 min), and resuspended in distilled water to remove PVA. This process was repeated three times. Finally the microparticles were freeze-dried for 12 h at -60°C , 200 mT (Flexi-Dry MP, FTS[®] Systems, NY, USA) and stored at 4°C .

In order to overcome some of the traditional drawbacks concerning protein microencapsulation associated with this initial procedure, several changes were introduced in the formulation so as to assess the role played by each phase composition on protein loading, release and preservation of biological activity. Table 1 compiles all changes performed.

The yield of the process was calculated for all microparticles batches by dividing the amount of microparticles collected by the initial amount of polymer and ALB in the formulation.

2.2.2. Characterization of the microparticles

2.2.2.1. Morphology and shape. The surface and shape of the microparticles were examined by scanning electron microscopy (SEM) (Jeol-JSM-6400 Electron Microscope Tokyo, Japan). For SEM

Table 1
Experimental conditions evaluated in the optimization of the albumin polymeric microparticles elaboration procedure.

Batch	Polymer	Organic phase solvent	External aqueous phase	Inner aqueous phase
1	RG504	DCM	2% PVA	1 ml Water
2	RG504	DCM	2% PVA 5% NaCl	1 ml Water
3	RG504H	DCM	2% PVA 5% NaCl	1 ml Water
4	RG502	DCM	2% PVA 5% NaCl	1 ml Water
5	RG756S	DCM	2% PVA 5% NaCl	1 ml Water
6	RG504	DCM	5% PVA 5% NaCl	1 ml Water
7	RG504	Ethyl acetate	2% PVA 5% NaCl	1 ml Water
8	RG504	DCM	2% PVA 5% NaCl	1 ml PBS pH 7.4
9	RG504	DCM	2% PVA 5% NaCl	1 ml Water 0.5% PVA
10	RG504	DCM	2% PVA 5% NaCl	1 ml Water + 500 μl PEG 400
11	RG504	DCM	2% PVA 5% NaCl	1 ml Water + 100 mg PEG 4000
12	RG504	DCM + 100 mg PEG 4000	2% PVA 5% NaCl	1 ml Water
13	RG504	DCM + 100 mg PEG 4000	2% PVA 5% NaCl	1 ml Water + 500 μl PEG 400

examination the samples were placed onto aluminum tubs with a carbon tape and they were coated with gold under vacuum (Emitech K550X, Eitech Ltd., UK).

2.2.2.2. Size. The particle size was measured by laser diffraction (Microtrac[®] SRA 150 Particle Size Analyser, Leeds & Northrup Instruments, Ireland). Volume diameter distribution was obtained and mean particle size, expressed as volume mean diameter, and standard deviation were determined. Each sample was run in triplicate.

2.2.2.3. Drug loading and encapsulation efficiency. The content of encapsulated ALB was determined by using a bicinchoninic acid (BCA) protein determination assay. 3 ml of 0.1 N sodium hydroxide (NaOH) were added on 20 mg of microparticles in a thermostated bath at 37 °C in order to dissolve the microparticles and left overnight. Afterwards, once neutralized with 1 ml of 0.3 N hydrochloric acid (HCl), the solution was centrifuged (10,000 rpm for 15 min) and the supernatant analyzed by spectrophotometry at 562 nm to determine ALB concentration. Protein content was determined from a standard curve of known concentrations of ALB over the range 25–250 µg/ml ($r=0.998$) contained in an equal volume of 0.1 N NaOH and 0.3 N HCl. All measurements were conducted in triplicate. ALB content in the microparticles was expressed as µg of ALB in 1 mg of microparticles. The entrapment efficiency was calculated using Eq. (1).

$$EE(\%) = \frac{(\text{ALB : polymer})_{\text{actual}}}{(\text{ALB : polymer})_{\text{initial}}} \times 100 \quad (1)$$

2.2.2.4. In vitro release assay. 20 mg of microparticles were suspended in 5 ml of 0.01 M phosphate buffered saline (PBS) pH 7.4, in closed vials and immersed in a thermostatic shaking water bath (Clifton[®] NE5-28, United Kingdom) at 37 °C under continuous agitation (50 strokes/min). At specific time intervals, 4 ml of the supernatant were withdrawn and fresh medium was added. Sink conditions were maintained during the entire assay.

The supernatant was analyzed for protein content using the micro BCA protein assay. The amount of ALB released was determined using a calibration curve ranging 0.5–20 µg/ml ($r=0.996$). Release profiles were expressed in terms of the cumulative ALB released at different incubation times.

Concurrently polymer degradation during the release assay was observed by SEM.

2.2.2.5. Cell proliferation assay. The assay was performed using PC12 cells. PC12 cells are derived from a pheochromocytoma of the rat adrenal medulla. Cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum, 7.5% horse serum, 1% L-glutamine and 0.1% penicillin–streptomycin; and were incubated at 37 °C in a humidified chamber in an atmosphere containing 5% CO₂ (Diaz-Prieto et al., 2008).

To evaluate the effect of ALB on cell proliferation, cells were seeded in 48-well plates and treated with ALB 0.5075 µM during 24 h. Likewise, the effect of unloaded and ALB loaded microparticles on cell proliferation was also evaluated.

To assess mitochondrial function of cells, bromide (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl) (MTT) reagent was added to each well and incubated for 1 h at 37 °C, and formazan crystals were formed. Then, dimethylsulfoxide (DMSO) was added to dissolve those crystals. After resuspending the content of the wells,

optical density (OD) was read using an Elisa reader at 550 nm (Berthold Detection Systems, Sirius). Phenylarsine oxide (PAO) 10 µM was used as positive control of cell death.

Cell viability, expressed as percentage of control, was calculated with the following Eq. (2). Each result described is based on 6 different determinations.

$$\text{Viability} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{control}}} \times 100 \quad (2)$$

3. Results and discussion

3.1. Preparation of ALB microparticles

The initial microencapsulation procedure (batch 1) rendered ALB microparticles adequate for subcutaneous or intramuscular administration with a mean diameter of 55.9 ± 19.12 µm, but the process yield was lower than 50% and the amount of protein microencapsulated was under the quantification limit of the analytical method. For this reason, different changes in the initial formula were made in order to improve microencapsulation yield and the characteristics of the microparticles obtained. Microencapsulation yields for all microcapsule batches prepared are represented in Fig. 1; microparticle sizes are depicted in Fig. 2; whereas protein loading and encapsulation efficiency of all batches are stated in Table 2.

Except with batches 5 and 12, microparticle sizes ranging from 10 to 100 µm were obtained with all the other batches. Protein microparticles are usually designed for their subcutaneous or

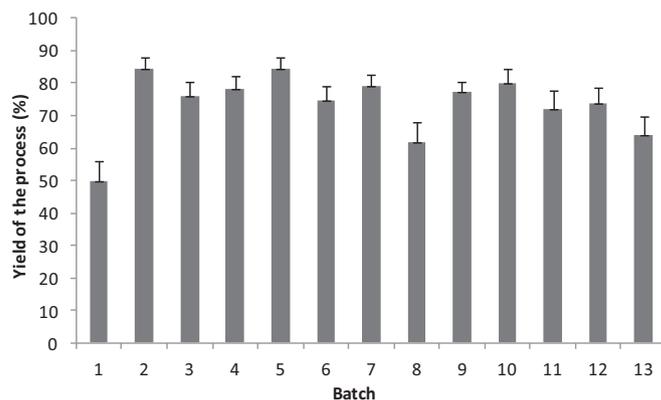


Fig. 1. Yield obtained in the different batches of ALB microparticles.

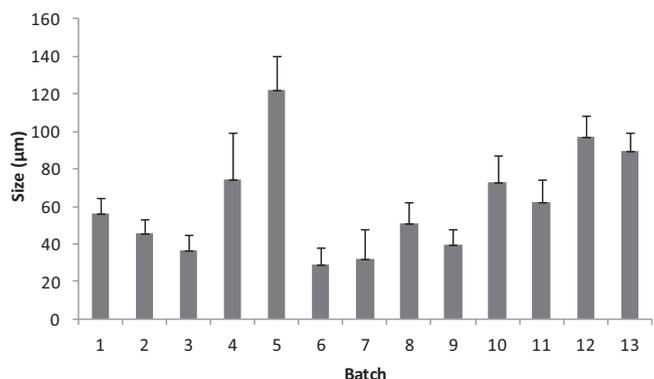


Fig. 2. Average particle size of the different batches of microparticles, expressed as volume mean diameter in µm.

Table 2

Encapsulation efficiency obtained in the different batches of microparticles (Mp) elaborated. 3 µg ALB/mg Mp corresponds to the quantification limit of the analytical method.

	Mean (standard deviation)	
	Drug loading (µg ALB/mg Mp)	Encapsulation efficiency (%)
Batch 1	<3	<3
Batch 2	37.13 (1.03)	38.56 (1.11)
Batch 3	3.92 (1.19)	3.94 (1.20)
Batch 4	<3	<3
Batch 5	<3	<3
Batch 6	33.13 (3.04)	34.26 (3.25)
Batch 7	<3	<3
Batch 8	<3	<3
Batch 9	59.3 (4.83)	63.03 (5.46)
Batch 10	30.74 (2.99)	31.72 (3.19)
Batch 11	24.09 (5.06)	24.68 (5.32)
Batch 12	<3	<3
Batch 13	55.94 (3.56)	59.26 (4.00)

intramuscular administration, looking for an extended protein release avoiding their degradation by tissular proteases, which happens when the proteins are administered in solution by the same routes. For these administration routes, a microparticle size smaller than 100 µm but larger than 10 µm (to avoid the phagocytosis by macrophages) is desirable. It must be taken into account that the larger the microparticles, the greater the diameter of the needle and the administration will be more painful. With this size range, a standard 25 gauge needle (internal diameter of 0.3 mm) could be used for administration, because particles larger than one-third of the internal diameter of the needle should be avoided to prevent its clogging (Patel, 2010). Thus, the most of protein-PLGA microparticle preparations developed for i.m. administration, have microparticle sizes ranging 10–125 µm (Tran et al., 2012). Although, as it has been discussed, 25 gauge needles are commonly used for parenteral administration of suspensions, some protein microparticle preparations use needles with greater internal diameter, because biopharmaceutical criteria (intended drug release) must prevail over technological ones (particle size and syringeability) in the design of protein microparticles. For example, PLGA microparticles of gonadotropin analogues (as triptorelin or leuprolide) are administered by i.m. route using a 23 gauge needle (D'souza et al., 2014).

If an intravenous administration were desirable, microparticles smaller than 1 µm with no particles greater than 5 µm should be designed, in spite of the limits set by USP for particulate matter is much higher (not more than 3000 particles >10 µm and not more than 300 particles >25 µm). This is so because this smaller size not only aims to avoid embolism but also to warrant the long circulating of the systems by the bloodstream.

3.1.1. Effect of the addition of sodium chloride (NaCl) to the external aqueous phase

The first variable studied was the effect of the addition of NaCl to the external aqueous phase during microparticle preparation. When 5% (w/v) of NaCl was added to the external aqueous phase (batch 2) no significant changes in microparticle mean diameter or in size distribution were detected, and spherical microparticles

with smooth surfaces were observed by scanning electron microscopy. However, much higher process yield and encapsulation efficiency were reported (with ALB loading reaching 37.13 µg ALB/mg microparticles). These results are in accordance with the literature (Chen et al., 2002; Dorati et al., 2008, 2005; Jiang et al., 2002) where the addition of NaCl to the aqueous phase is claimed to lead to an increase in the osmotic pressure preventing thereby protein diffusion to this external aqueous phase and consequently increasing both encapsulation efficiency and protein loading. According to Chen et al. (2002), the inner structure of microparticles prepared using NaCl was very dense and compact regardless of the physical–chemical properties of the polymer used. Such structural feature limits the permeation of protein molecules across the polymeric chains during the solidification process (Dorati et al., 2008).

In fact, when Chen et al. (2004), evaluated the role played by the presence of NaCl to encapsulate proteins into PLA microparticles, the conclusion to be drawn was that the higher the amount of sodium chloride in the continuous phase, the higher both drug loading and encapsulation efficiency, the longer protein biological activity was kept unchanged and the less noticeable the initial burst effect, thanks to the higher osmotic pressure generating denser microparticle internal structures. This means that PVA with sodium chloride in the continuous phase not only increased protein loading and yield, but also retained protein deeper inside a layer of microparticles.

3.1.2. Effect of the polymer

In order to analyze the influence of polymer molecular weight and polarity, batches 2–5 were prepared using different poly (lactide-co-glycolide) copolymers (the characteristics of each are compiled in Table 3).

RG504 (batch 2), RG504H (batch 3) and RG502 (batch 4) are PLGA 50:50 copolymers; whereas RG756S (batch 5) is a PLGA 75:25 copolymer. Besides, they show differences in terms of molecular weight, and consequently viscosity, being RG502 < RG504 = RG504H < RG756S. Lastly, RG504H, unlike RG504, is capped with free carboxylic acid groups, being thus typically more hydrophilic and enabling faster hydration than non-H polymers.

Regardless of polymer properties, microencapsulation yield was always above 75%.

Fig. 3 shows that by using the lowest molecular weight polymer (RG502), broken microparticles were obtained; whereas the other polymers rendered spherical microparticles. It is also to be appreciated in the figure that the higher the polymer molecular weight or the more the polymer hydrophilicity, the higher the microparticle size.

Other issue related to the molecular weight of the polymer is the presence of pores on the microparticle surface, observed when RG756S polymer was used whereas microparticles prepared with the other polymers showed a smooth surface free of pores. The presence of pores in the surface of the microparticles could lead to a faster release of the protein.

Le Visage et al. (2001) obtained similar results as regards the morphology of the microparticles when they studied the effect of the molecular weight of poly(methylidene malonate 2.1.2) on the

Table 3

Main characteristics of the polymers used.

Polymer	Composition	Molecular weight range	Viscosity (dl/g)	Tg (°C)	End group
RG502	Poly(D,L-lactide-co-glycolide) 50:50	7000–17,000	0.19	44	Alkyl ester
RG504	Poly(D,L-lactide-co-glycolide) 50:50	38,000–54,000	0.52	48	Ester
RG504H	Poly(D,L-lactide-co-glycolide) 50:50	38,000–54,000	0.50	48	Free carboxylic acid
RG756S	Poly(D,L-lactide-co-glycolide) 75:25	190,000–240,000	1.5	52	Alkyl ether

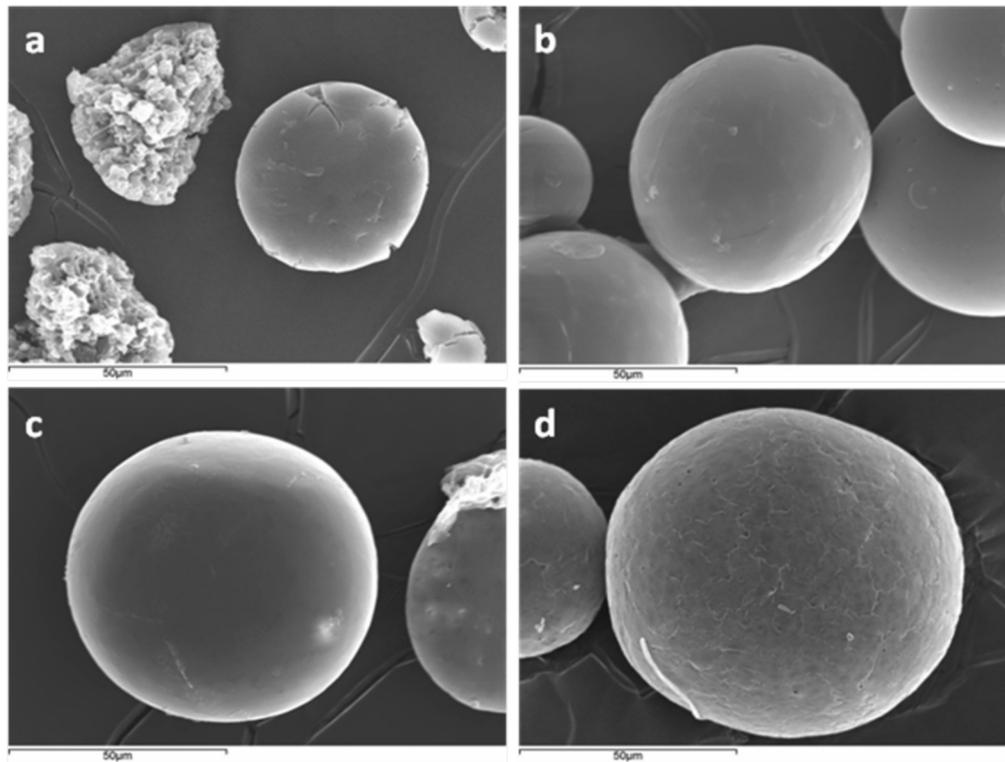


Fig. 3. SEM images of polymeric microparticles obtained with different polymer (scale bar = 50 µm). Microparticles obtained with RG502 (a), RG504 (b), RG504H (c) and RG756S (d). Representative microphotographs of the four types of ALB polymeric microparticles are shown.

characteristics of protein microparticles obtained by water-in oil-in water (W/O/W) solvent evaporation technique. The increase in the microparticle size and porosity were explained by the increase of the inherent viscosity of the polymer with higher molecular weight which hinders the dispersion of the organic phase into the external aqueous phase.

Besides, polymer characteristics highly influence protein loading into microparticles and thus encapsulation efficiency: **the higher the polymer hydrophilicity and the lower their molecular weight, the lower the encapsulation efficiency.** In fact, protein loading decreased from 37.13 µg ALB/mg of microparticles with RG504, to 3.92 µg ALB/mg of microparticles when increasing polymer hydrophilicity (RG504H); and to less of 3 µg ALB/mg microparticles when lowering polymer molecular weight (RG502).

3.1.3. Effect of the concentration of PVA in the external aqueous phase

PVA concentration in the external aqueous phase was increased in order to increase the stability of the emulsion and improve microparticles characteristics. To that aim, batch 6 was obtained using 5% PVA with a microencapsulation yield slightly above 75%. In comparison with batch 2, with 2% PVA, microparticles were smaller and showed a greater tendency to aggregate as evidenced by SEM (Fig. 4), with non-spherical microparticles forming clusters being observed. **By increasing the concentration of emulsion stabilizer, the interfacial tension between the aqueous and the organic phases drops, and the breakage of the internal phase in smaller droplets into the external phase is facilitated, thus obtaining smaller size microparticles,** as stated by Cho et al. (2000). Likewise, PVA enables faster removal of the organic solvent

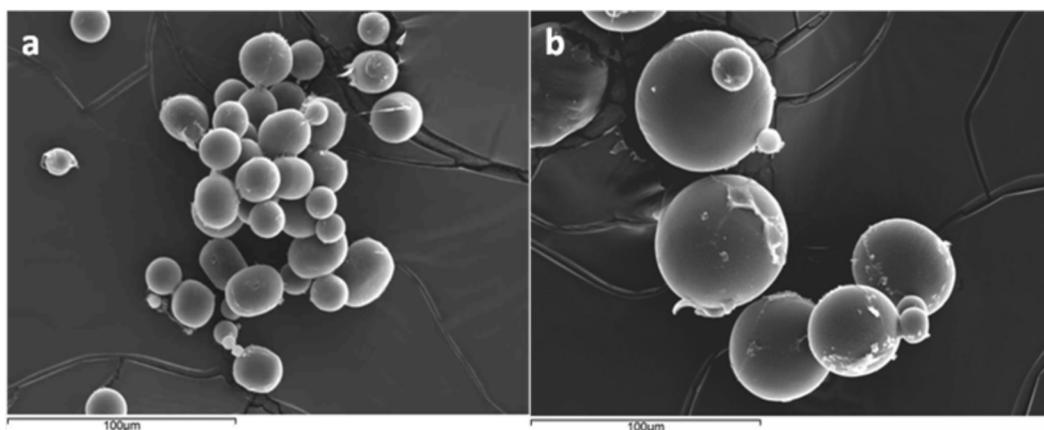


Fig. 4. SEM images of ALB polymeric microparticles obtained with 5% PVA (a) and 2% PVA (b) (scale bar = 100 µm). Representative microphotographs of the two types of ALB polymeric microparticles are shown.

to the external aqueous phase, accounting for the deformation of microparticles. Since only a slight decrease in protein loading (33.13 μg ALB/mg microparticles) and encapsulation efficiency was observed, protein diffusion to the external aqueous phase, facilitated by the higher concentration of PVA, seems to be partially compensated by the faster solidification of the polymer due to the extraction of the solvent.

In spite of the small size of the microparticles obtained, batch 6 was discarded because of both the great aggregation tendency and the slightly lower protein loading.

3.1.4. Effect of the nature of the organic solvent

With the purpose of evaluating the role played by the nature of the organic solvent, DCM was substituted by ethyl acetate as organic solvent in batch 7. However, the dissolution of the polymer in this new solvent proved to be more difficult. In spite of rendering a high microencapsulation yield, SEM images (Fig. 5) showed that highly porous microparticles, most of which were broken and free polymer could be observed. Mentioned facts stand for the low encapsulation efficiency and the protein loading under the quantification limit of the analytical method obtained for batch 7.

Different researchers (Cho et al., 2000; Le Visage et al., 2001; Kim and Park, 1999) employed ethyl acetate instead of dichlorometane to prepare protein microparticles using different polymers. Such solvent change is justified given the environmental and human toxicity problems of the halogenated alkanes including methylene chloride. Using RG504, Cho et al. (2000) obtained microparticles with smaller size and higher protein loading when DCM was substituted by ethyl acetate. The water miscibility of ethyl acetate is higher than that of DCM, showing thus microparticles elaborated with ethyl acetate a more porous structure, due to the tendency of the solvent to quickly diffuse to the external aqueous phase. These authors described the high porosity of the microparticles obtained with ethyl acetate, but this high porosity did not affect drug loading. In our study, on the contrary, the high porosity of the microparticles could facilitate protein diffusion to the external aqueous phase, thus obtaining lower protein loadings.

3.1.5. Effect of the composition of the inner aqueous phase

Different changes in the inner aqueous phase were aimed at increasing its viscosity or protein solubility, avoiding thereby protein migration from the internal to the external aqueous phase. To that aim, PBS was used as solvent of the internal phase (batch 8), being PVA (batch 9), PEG 400 (batch 10) or PEG 4000 (batch 11) added to the internal aqueous phase.

When PBS pH 7.4 was used as internal phase, the worst results were obtained, as the microencapsulation yield was the lowest (near 60%), and protein loading was so low that it could not be determined.

With the other batches microencapsulation yields above 70% were always obtained. The addition of PVA and PBS produced no changes in microparticles morphology, but the presence of PEG (batches 10 and 11) rendered more porous microparticles, mainly when PEG 400 was used (Fig. 6). Changes in microparticle size were also noticed: batches prepared by adding PEG in the internal phase exhibited significantly larger mean diameter. As regards encapsulation efficiency, the highest values were obtained with batch 9, with 0.5% PVA as internal aqueous phase, being the difference with the other batches very significant. Indeed, drug loading of batches 9–11 were 59.3, 30.7 and 24.1 μg ALB/mg microparticles, respectively.

The use of PEG to increase the viscosity of the internal aqueous phase and thus to avoid protein migration from the internal to the external phase has been extensively studied (Dorati et al., 2005, 2008; Yeh et al., 1995). Moreover, PEG limits the penetration of the protein into the interfacial film of the primary W/O emulsion and consequently, it stabilizes the protein by reducing the contact with the organic phase (Pean et al., 1999). Dorati et al. (2005) outlined that PEG addition in the formulations leads to obtain larger microparticles, as we have also observed in our study. This effect may be due to water retention caused by PEG, enhancing thereby hydration of the polymer matrix (Dorati et al., 2005). Dorati et al. (2005) also described that the simultaneous addition of PEG and NaCl avoids the pore formation due to the incorporation of PEG, obtaining microparticles with a smooth surface and compact internal structure. However, in our study porous microparticles were obtained when PEG was used in the internal aqueous phase in spite of the presence of NaCl in the external aqueous phase, and any improvement in protein loading was observed. However, the increase of the viscosity of the aqueous internal phase obtained with PVA proved to be an efficient tool to increase encapsulation efficiency without modifying microparticle morphology. Representative microphotographs of the four types of ALB polymeric microparticles are shown.

3.1.6. Effect of PEG incorporation in the organic phase

The last modification introduced in the microencapsulation procedure was the addition of PEG in the organic phase in order to increase its viscosity and therefore to avoid the diffusion of the protein from the internal to the external aqueous phase. To that aim, batch 12 was prepared. However, this modification did not

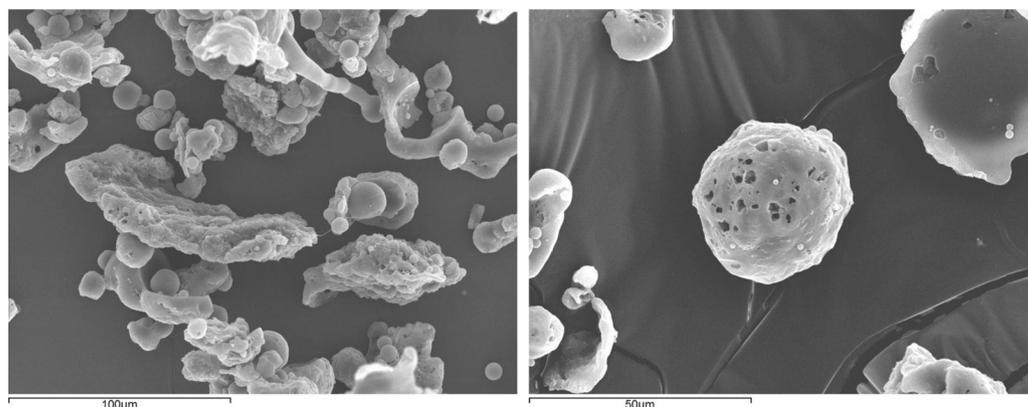


Fig. 5. SEM images of ALB polymeric microparticles obtained with ethyl acetate (left scale bar = 100 μm and right scale bar = 50 μm).

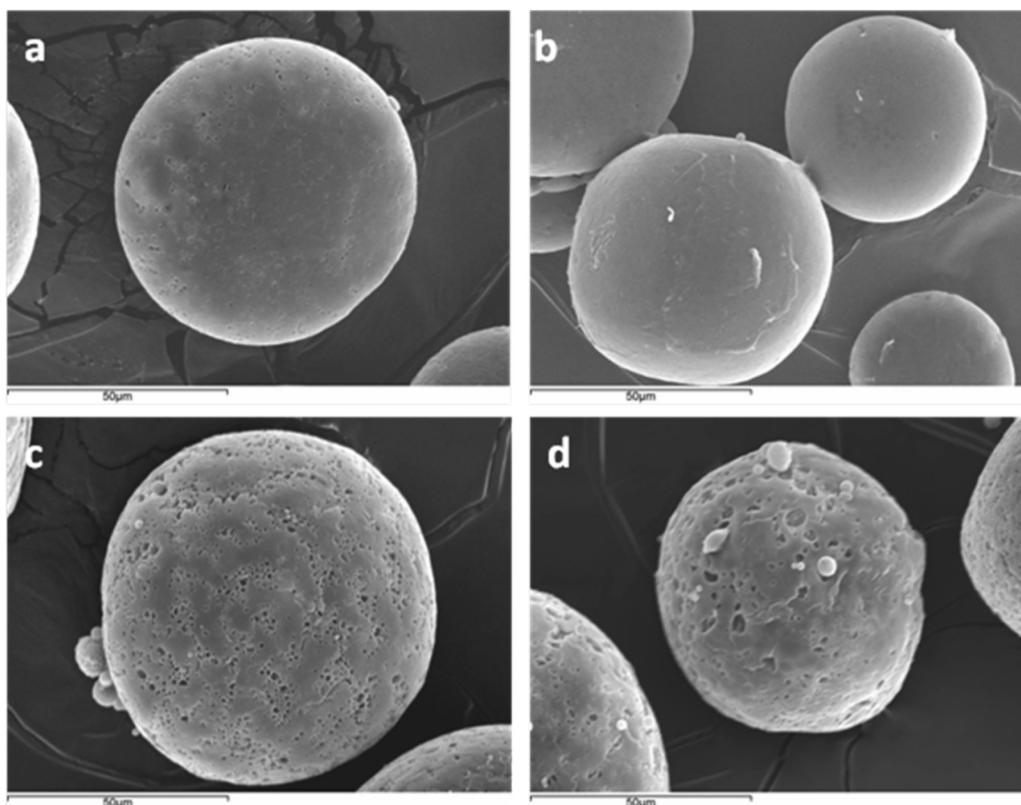


Fig. 6. SEM images of ALB polymeric microparticles obtained by adding PEG in the formulation (scale bar = 50 μm). Microparticles obtained with (a) PEG 400 in the inner aqueous phase, (b) PEG 4000 in inner the aqueous phase, (c) PEG 4000 in the organic phase and (d) PEG 400 in the inner aqueous phase plus PEG 4000 in the organic phase.

lead to the expected results, since highly porous microparticles were obtained with a non-detectable protein loading.

Finally, in batch 13, PEG was added both in the internal aqueous phase (PEG 400) and in the organic phase (PEG 4000). The microencapsulation yield was low and the microparticles showed a highly porous surface (Fig. 6). Besides, as happened in batches 10–12, larger microparticles were obtained. But importantly, encapsulation efficiency and protein loading values were high (55.9 μg ALB/mg microparticles), very close to those of batch 9.

Yeh et al. (1995) and Dorati et al. (2005, 2008) analyzed the effect of increasing the viscosity of the inner water and organic phase by adding PEG: blending of PEG 8000 with PLGA results in a dramatic improvement of protein loading into poly(D,L-lactide-co-glycolide) microparticles (Yeh et al., 1995). Therefore, it is to be concluded that PEG improves protein loading in PLGA microparticles but only if added to both the internal aqueous phase and the organic phase.

3.2. In vitro release assay

In vitro release assays were carried out with the two batches with the highest protein loading: batch 9 (with PVA in the internal aqueous phase) and batch 13 (with PEG 400 in the internal aqueous phase plus PEG 4000 in the organic phase).

Results obtained are depicted in Fig. 7. With batch 9, about 100% of microencapsulated ALB was released by day 130. This release fitted a zero order kinetic from day 15 up to day 75 ($r^2 = 0.981$), with a constant release rate of 3.48 μg ALB/mg microparticles/day. From day 75, the release rate increased up to day 95.

Typically, proteins are released from PLGA microspheres in three phases: an initial burst, a diffusion-driven controlled release, and an erosion-driven controlled release. The initial burst phase is

a consequence of the rapid release (within a few days) of the protein located at or near the surface of the microspheres (Dorati et al., 2008). In the diffusion-driven controlled release phase, the protein diffuses through pores or channels of the microspheres, and in the erosion-driven controlled release phase; the protein is released as a consequence of the gradual degradation of the polymer by hydrolysis (Cho et al., 2000).

In the case of microparticles of batch 9, the initial burst phase was negligible, since the amount of ALB released was less than 10% within the first two days. Later the phase of constant release could correspond to the diffusion-driven phase; and the last stage of faster release to the erosion-driven phase.

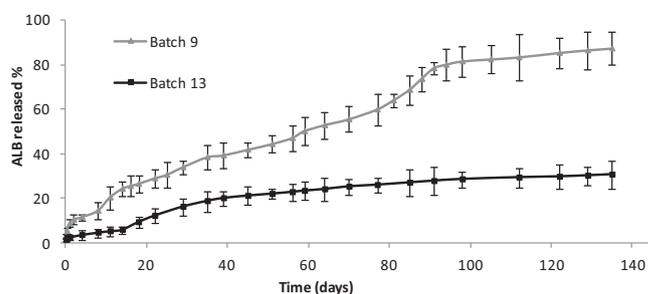


Fig. 7. Release profile of ALB from polymeric microparticles obtained from batches 9 and 13. For the *in vitro* release studies, microspheres were incubated in PBS (pH 7.4) and maintained in a shaking incubator at 37 °C. At predetermined time intervals, supernatants were withdrawn and the media was replaced. The concentration of ALB in the release medium was quantified by micro BCA. Data correspond to the cumulative amount of drug released at the indicated time points, and are expressed as mean percentage of dex released relative to the total amount of ALB in microspheres ($n = 3$).

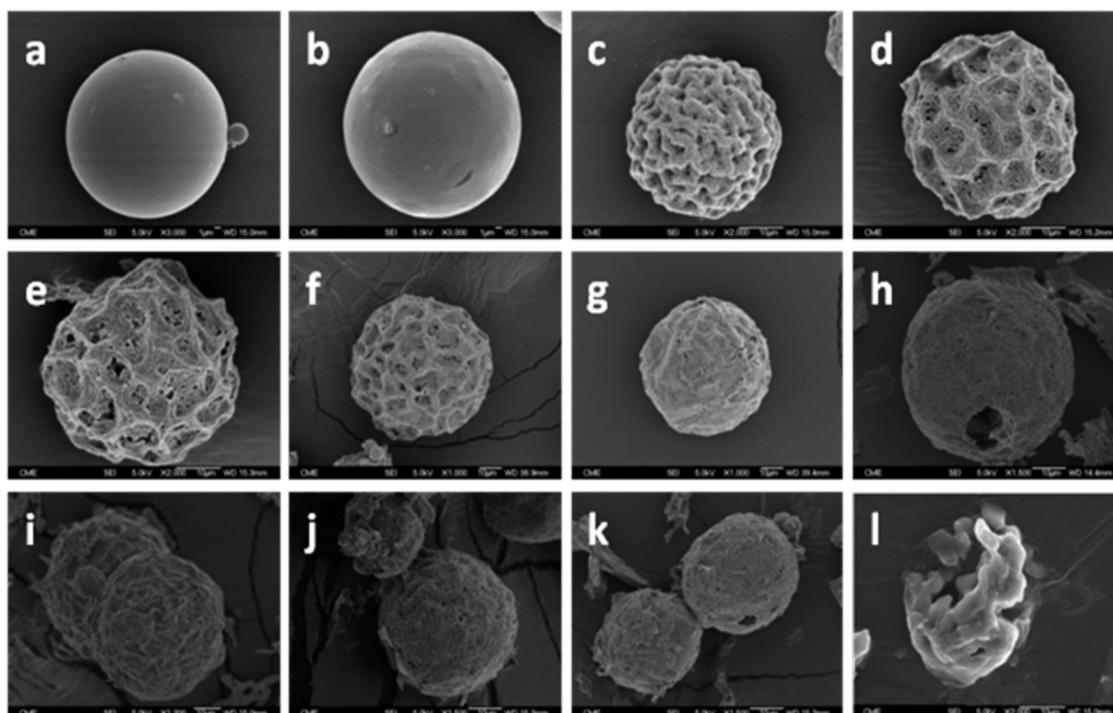


Fig. 8. SEM images of ALB polymeric microparticles throughout the *in vitro* release studies: time 0 (a), 10 days (b), 20 days (c), 30 days (d), 40 days (e), 54 days (f), 64 days (g), 76 days (h), 86 days (i), 96 days (j), 106 days (k) and 120 days (l) (scale bar = 10 μm).

Indeed, the polymer degradation throughout the protein release from batch 9 microspheres was analyzed by SEM, being the obtained images presented in Fig. 8. Initially the microparticles showed a smooth non-porous surface. After 10 days in PBS the microparticles swelled, increasing their volume. Later on some pores appeared, becoming larger and larger with time and the surface of the microparticles turned irregular, maybe because short polymer chains were shattered. After 76 days holes were clearly seen, as well as polymer remainders around the microparticles, which were smaller and irregular. These changes were noticed at the exact point when ALB release kinetic turned faster, starting off the erosion-driven phase. Finally, after 120 days the microparticles had lost totally their spherical shape due to the massive degradation of the polymer.

When the ALB released from microparticles of batch 13 was determined, completely different results were obtained, since after the same time period of 130 days, only 30% of microencapsulated ALB had been released, in spite of the high porosity exhibited by microparticles of this batch.

Indeed, as it has been previously established, the addition of PEG to microparticles gives rise to more porous structures. In addition, introducing PEG would increase the hydrophilicity of the polymeric carriers, and thus their degradation rate, and would decrease the acidity of degraded products. Hydrophilic PEG segments could also enhance the diffusivity of water inside the polymeric matrix and consequently the diffusivity of drug molecules in the surrounding medium. This should contribute to get more linear protein release from microparticles and to reduce the burst effect. However, in spite of all these hypotheses, ALB release from batch 13 was significantly slower than from batch 9. An explanation to this slower release could be found by analysing the effect of PEG on protein distribution into the microparticle and on the polymer hydration grade. On the one hand, PEG 400 increases the viscosity of the aqueous internal phase, increasing droplet size of the primary W/O emulsion. These larger droplets would avoid an uniform distribution of the protein

in the polymer matrix, staying in deeper layers of the microparticle. On the other hand, PEG 400 and PEG 4000 lead to water retention during microparticle elaboration, enhancing the hydration of the polymer matrix and increasing microparticle size. These more hydrated microparticles would show less avidity by the release medium than anhydrous ones (just as happens with anhydrous and hydrated compounds).

3.3. Cell proliferation assay

ALB polymeric microparticles were studied in terms of cell viability to determine the effect of the microencapsulation procedure on the stability of the protein, comparing these effects with those obtained with free ALB. Plasma proteins such as albumin are the main energy and nutrition source for tumor growth. Indeed, some references describe the stimulating effect on cell proliferation of high doses of BSA (bovine serum albumin) and ALB, and even a protective effect against cell apoptosis (Gallego-Sandín et al., 2005). This effect was used in this work to determine the biological stability of ALB after microencapsulation. The microencapsulation procedure comprises the use of organic solvents as well as high shear forces during agitation steps that could lead to the loss of protein activity that cannot be detected by quantitative analysis.

The assay was performed throughout 24 h. A single administration of ALB polymeric microparticles (batch 9) was compared with the administration of free ALB in solution. In a previous assay ALB solutions from 0.25 to 10 μM were used to determine the effect of ALB on PC12 cell proliferation. From these results an ALB concentration 0.5075 μM was selected to evaluate the effect of ALB microparticles. The amount of ALB microparticles to release the same amount of ALB was calculated from the results of the release study.

As it is shown in Fig. 9, both free and microencapsulated ALB produced a rise in cellular viability of about 50% after 1 day of

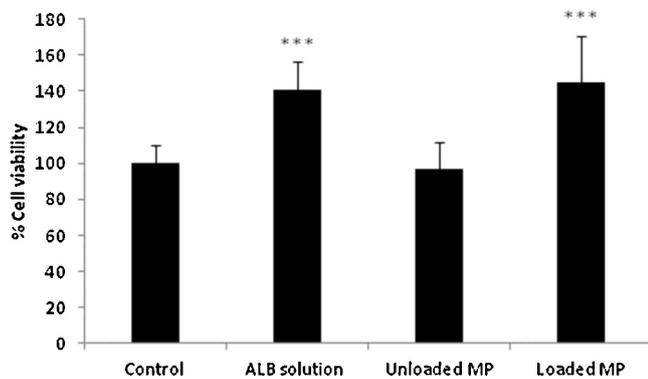


Fig. 9. Proliferation assay of ALB solution, unloaded and ALB microparticles. ALB solution and loaded microparticles increased significantly cell proliferation in the period tested. Unloaded microparticles were not affect the cell viability. Data correspond to the means \pm SEM for 3–6 wells for three different batches of cells. *** $P < 0.05$ with respect to control and unloaded microparticles cell viability (ANOVA).

exposure. Unloaded microparticles did not affect cellular viability. These results allowed us to conclude that the integrity and stability of ALB was not affected by the microencapsulation process developed and optimized.

4. Conclusions

Different formulation and elaboration process factors involved in optimizing protein loading, release kinetics and protein biological stability of PLGA-protein microparticles were evaluated.

Polymer properties highly influence protein loading into microparticles: the higher the polymer hydrophilicity and the lower its molecular weight, the lower the encapsulation efficiency; thus obtaining the most drug-loaded microparticles when using RG504.

The addition of sodium chloride (NaCl) to the external aqueous phase significantly increased process yield and encapsulation efficiency, without noticeable changes in microparticle morphology. The increase of the concentration of PVA in the external aqueous phase led to microparticles with smaller size, greater tendency to aggregate and slightly lower protein loading and encapsulation efficiency. When ethyl acetate was used as solvent of the organic phase instead of dichloromethane, highly porous and broken microparticles were obtained, with unquantifiable protein loading.

But the most determining attribute to increase protein loading was the use of viscosity enhancers (PVA, PEG 400 and PEG 4000) in the inner aqueous phase and in organic phase. When PEG was added to the aqueous internal phase, more porous microparticles with higher size were obtained. When PVA 0.5% was used no change in the size and morphology of the microparticles was detected but protein loading and encapsulation efficiency significantly increased. These microparticles released the protein in a sustained way over a period of 130 days. Finally, when PEG was added both in the internal aqueous phase (PEG 400) as in the organic phase (PEG 4000), microparticles with a high protein loading were obtained. However, only 30% of the protein was released from these microparticles after 130 days in an *in vitro* assay.

Last, the activity of the microencapsulated protein was demonstrated by an assay of cell proliferation, where ALB released from microparticles showed a similar effect on cell viability to ALB solution.

In conclusion, according to these investigations a procedure to elaborate ALB microparticles with adequate size for parenteral

administration and high protein loading was optimized. This microencapsulation procedure did not affect the biological stability of the microencapsulated protein. Protein-loaded microparticles exhibited a sustained release over 130 days.

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