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PLGA microspheres containing bee venom proteins for preventive immunotherapy

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ABSTRACT

Bee venom (BV) allergy is potentially dangerous for allergic individuals because a single bee sting may induce an anaphylactic reaction, eventually leading to death. Currently, venom immunotherapy (VIT) is the only treatment with long-lasting effect for this kind of allergy and its efficiency has been recognized worldwide. This therapy consists of subcutaneous injections of gradually increasing doses of the allergen. This causes patient lack of compliance due to a long time of treatment with a total of 30–80 injections administered over years. In this article we deal with the characterization of different MS-PLGA formulations containing BV proteins for VIT. The PLGA microspheres containing BV represent a strategy to replace the multiple injections, because they can control the solute release. Physical and biochemical methods were used to analyze and characterize their preparation. Microspheres with encapsulation efficiencies of 49–75% were obtained with a BV triphasic release profile. Among them, the MS-PLGA 34 kDa-COOH showed to be best for VIT because they presented a low initial burst (20%) and a slow BV release during lag phase. Furthermore, few conformational changes were observed in the released BV. Above all, the BV remained immunologically recognizable, which means that they could continuously stimulate the immune system. Those microspheres containing BV could replace sequential injections of traditional VIT with the remarkable advantage of reduced number of injections.

1. Introduction

In developed countries, about 20–30% of the population has allergy (Ring et al., 2001). One of the potential allergen sources is represented by insect venoms, such as those from honey bee stings. This kind of allergy is potentially more dangerous for the allergic individual because a single bee sting may induce an anaphylactic reaction, eventually leading to death (Jilek et al., 2004; Steen et al., 2005). Although allergy treatment with corticosteroids and antihistamines can efficiently ameliorate IgE-mediated symptoms, they are not preventive. In this way, venom immunotherapy (VIT) currently is the best treatment with long-lasting effect for the bee venom (BV) allergic patient, and its efficiency has been recognized worldwide (Muller, 2003). The therapy consists of subcutaneous injections of gradually increasing doses of the allergen. This shifts the immune response against the allergen from a predominantly IgE production towards an IgG production (Venarske and deShazo, 2003; Till et al., 2004). Some limitations had caused patient lack of compliance, and prevented its use, such as the long time of treatment with a total of 30–80 injections administered over years. This is also a high cost treatment (Durham et al., 1999). As it has been proved for other allergens (Batanero et al., 2003; Gómez et al., 2009), microspheres (MS) prepared with biodegradable polymers, such as, poly lactide-co-glycolide acids (PLGA) would provide an excellent controlled delivery system to encapsulated BV for using in VIT.

MS-PLGA has the capacity of delivering proteins in either continuous or pulsatile way over prolonged periods. They can induce protective immunity after a single subcutaneous injection, thereby simulating the sequential injections of the traditional VIT (Thomasin et al., 1996; Cleland et al., 1997). The encapsulation of allergens within MS-PLGA represents the most promising approach to protect those allergens from degradation by natural proteases in the organism, by increasing their bioavailability to stimulate immune system. Furthermore, the MS protects the organism from the deleterious effects caused by the direct contact with the BV toxins. Finally, they target these allergens to the antigen presenting cells (Waeckerle-Men and Groettrup, 2005).

A major problem hindering the progress of MS-PLGA-based allergen formulations for human usage is protein stability dur-

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ing microencapsulation, storage and release (Schwendeman et al., 1996; Uchida et al., 1996; Cleland, 1998; Jiang et al., 2005; Namur et al., 2009). Once the troubles for encapsulating BV proteins within MS-PLGA were circumvented (Trindade et al., 2011), the *in vitro* characterization of these formulations, such as, size, morphology, release profile, allergen stability and antigenicity during their microsphere residence time were studied.

2. Materials and methods

2.1. Materials

Lyophilized bee venom (BV) was purchased from Sigma Chemical Co., St. Louis, MO, USA. PLGA polymers with different molecular weights with free terminal-carboxyl (12 kDa-H, 34 kDa-H and 63 kDa-H) and methylated terminal-carboxyl (12 kDa-Me, 34 kDa-Me and 63 kDa-Me) were purchased from Boehringer Ingelhein (Germany). Anti-*Apis mellifera* venom antibody; monoclonal anti-rabbit immunoglobulins (IgG, IgA, IgM) peroxidase conjugates; lactate standards and reagents and polyvinyl alcohol (PVA Mw 49,000 – Mowiol® 40–88) were obtained from Sigma–Aldrich®. Dichloromethane was obtained from Aldrich Chemical® – Milwaukee – USA. All other chemicals used were of analytical reagent grade.

2.2. Preparation of BV-loaded microspheres

A water-in oil-in water ($W_1/O/W_2$) double-emulsion solvent evaporation (Langer, 1990) adapted method (Namur et al., 2006) was employed to prepare microspheres containing bee venom proteins. A 250 μL of BV (50 mg/mL in PBS) was mixed with 4 mL of dichloromethane containing PLGA, and emulsified (T 25 Basic IKA Labortechnik–Ultra Turrax®) at 24,000 rpm for 2 min. The resultant first emulsion was added to 20 mL of PVA (2%), under agitation, and emulsification continued at 13,500 rpm for 2 min. The emulsion was stirred continuously for 3 h at room temperature and atmospheric pressure until complete solvent evaporation. After the microspheres had formed, they were collected by centrifugation for 10 min at 2000 × *g*, rinsed with water three times and then resuspended with 2 mL of 1% PVA, freeze-dried for 24 h and stored at −20 °C.

2.3. Morphology and size of BV-loaded PLGA-MS and non-loaded PLGA-MS through scanning electron microscopy (SEM) and laser diffraction

The microspheres were metalized with gold, and observed in a Jeol JSM 840A scanning electron microscope at an intensity of 25 kV. The size of non-loaded and BV-loaded MS-PLGA was determined from granulometric distribution by laser diffraction obtained in the Mastersize 2000 equipment (Malvern Instrument, Malvern, UK). For this analysis, the lyophilized MS-PLGA was resuspended in distilled water. The size of each sample was measured at least five times and the mean value was calculated. The average cumulative undersize distributions (D_{10}, D_{50}, and D_{90}) were determined on a volume basis, which represents diameters equivalent to 10%, 50%, and 90% of cumulative amount, respectively. The polydispersivity index (PI) was calculated by using the following equation:

$$\mathrm{PI} = \frac{D_{90} - D_{10}}{D_{50}}$$

2.4. Encapsulation efficiency (EE)

EE was determined after MS alkaline digestion with some modifications. Briefly, 20 mg of the freeze-dried MS was added to 5 mL of 0.1% SDS (Sodium dodecylsulfate)–0.1 M NaOH solution (Sharif and O'Hagan, 1995). The mixture was incubated under gentle stirring for 24 h at 37 ± 1.0 °C. After centrifugation and filtration in 0.22 μm membranes, the clear supernatant liquid was assayed by the Lowry method. The amount of BV was determined using the standard curve equation ($y = 1.00208X$, $r = 0.99931$). The encapsulation efficiency (EE) can be calculated as follows:

$$\mathrm{EE}(\%) = \frac{\text{Protein found in microspheres}}{\text{Initial protein added}} \times 100$$

Table 1
Size distributions and polydispersity index (PI) of non-loaded and BV-loaded MS prepared with different PLGAs.

PLGA	MS size distribution				
	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	VMD[a] (μm)	PI[b]
12 kDa-COOH	6.19	18.23	47.47	23.15	2.26
12 kDa-$COOCH_3$	6.77	19.56	42.16	22.55	1.81
34 kDa-COOH	6.12	17.24	34.54	19.07	1.65
34 kDa-$COOCH_3$	11.27	21.89	38.53	23.50	1.25
63 kDa-COOH	6.82	19.77	40.32	21.97	1.70
63 kDa-$COOCH_3$	8.92	19.89	38.38	21.93	1.48
12 kDa-COOH/BV	4.41	21.49	56.91	26.61	2.44
34 kDa-$COOCH_3$/BV	9.25	21.42	40.82	23.38	1.47
63 kDa-COOH/BV	14.70	30.20	56.06	33.10	1.37
12 kDa-$COOCH_3$/BV	11.07	21.02	37.05	22.69	1.24
34 kDa-COOH/BV	15.50	29.48	52.26	31.89	1.25
63 kDa-$COOCH_3$/BV	10.94	21.70	39.50	23.66	1.32

The cumulative averages of different populations (D_{10}, D_{50} and D_{90}) were determined on a volume basis.
[a] Volume mean diameter.
[b] Polydispersity index.

2.4.1. Protein assay

The method (Lowry et al., 1951; Peters, 1985; Peterson, 1979) was adapted and developed at our laboratory. 50 mL of each sample of BV were added in an ELISA plate containing to 100 mL of the following 1:1:1 solution: (a) 0.1% cupper sulfate; 0.2% sodium and potassium tartarate; 10% sodium carbonate, (b) 10% Sodium dodecylsulfate and (c) 1 N NaOH, respectively. After 10 min at room temperature 100 mL of Folin solution were added. After 30 min at room temperature, the plate was automatically read at a Titerteck Multiskan MCC/340. Bovine sera albumin at 1 mg/mL was used as standard protein.

2.5. Adsorption of BV in the MS-PLGA surface

10 mg of each blank MS-PLGA (all six types of polymers) were incubated with BV proteins (1 mg/mL) dissolved in $MgCl_2$ solution (pH 6.5). The incubation vials were maintained at 37 ± 1 °C under agitation of 50 rpm for 24 h. After this time, the vials were centrifuged for 30 min at 3354 × *g*. BV proteins were measured in the supernatant by the Lowry method. Bound BV protein was calculated from the difference between the initial (total) and remaining (free) BV protein concentrations. Controls: only BV or PLGAs. Data are presented as average with the standard deviations.

2.6. Degradation study of MS-PLGA through lactic acid production

The assays for the produced lactic acid were performed by an enzymatic method (Tietz, 1995; Westgard et al., 1972). Lactate is converted to pyruvate and hydrogen per-

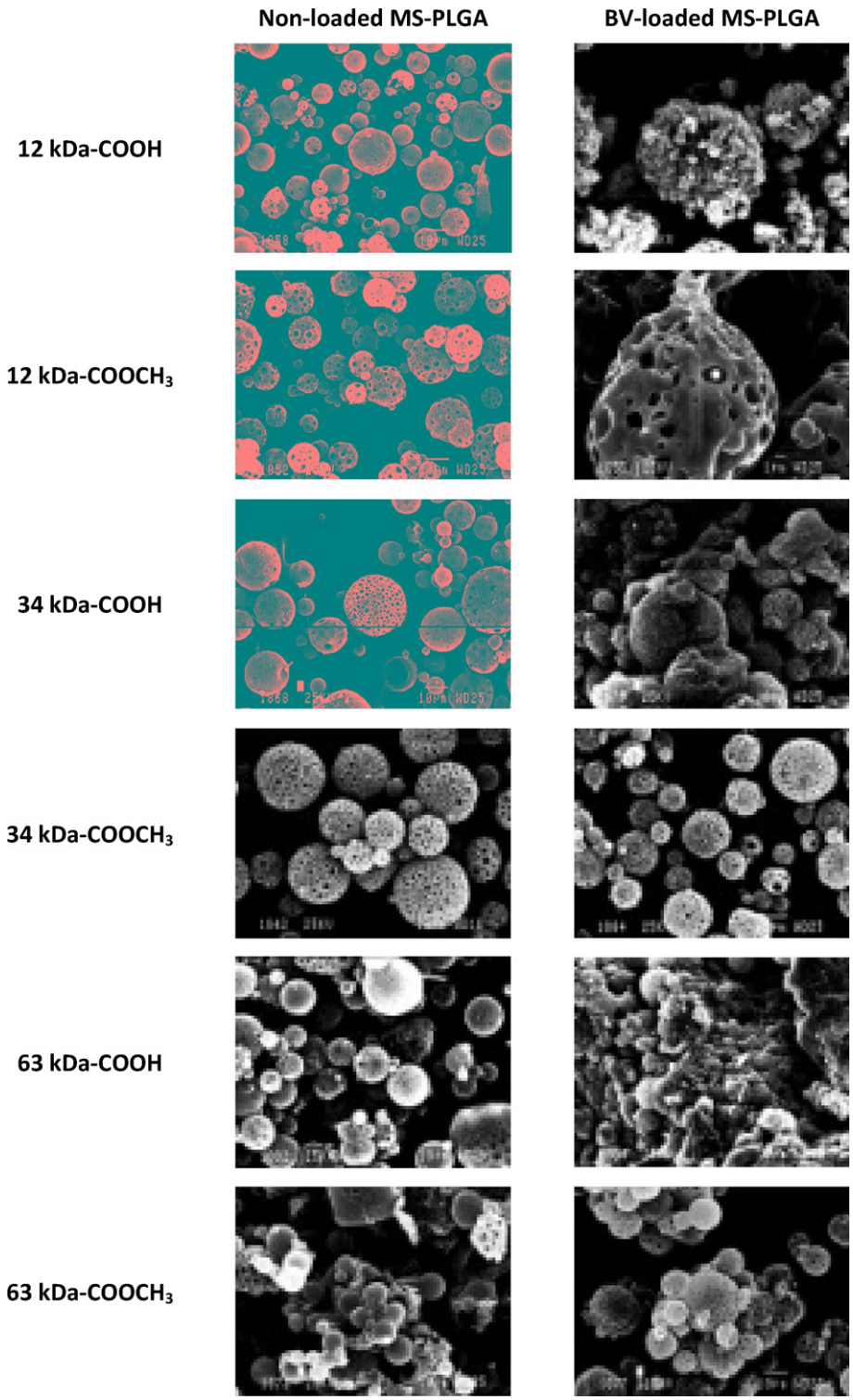

Fig. 1. Effect of molecular mass and carboxy-terminal (free or methylated) on the morphology of MS-PLGA by SEM (1000×).

oxide by lactate oxidase. In the presence of the H_2O_2, horseradish peroxidase catalyzes the oxidative condensation of chromogen precursors to produce a colored dye with absorption maximum at 540 nm. Measurement was started by adding 10 μL of samples to 1 mL of testing reagent and incubating for 5–10 min. The amount of released lactate in the medium was determined using the standard curve equation ($y = 0.03216X$, $r = 0.98512$) prepared with lactate standards.

2.7. In vitro BV release kinetics from MS-PLGA

BV-loaded MS (20 mg) was added to 2 mL PBS buffer (pH 7.4) containing 0.02% sodium azide. They were incubated at 37 °C with continuous agitation at about 50 rpm. Periodically, the supernatant was withdrawn and the amount of BV protein released was evaluated by the Lowry method. Experiments were performed in triplicate. Data are presented as the average with the standard deviations.

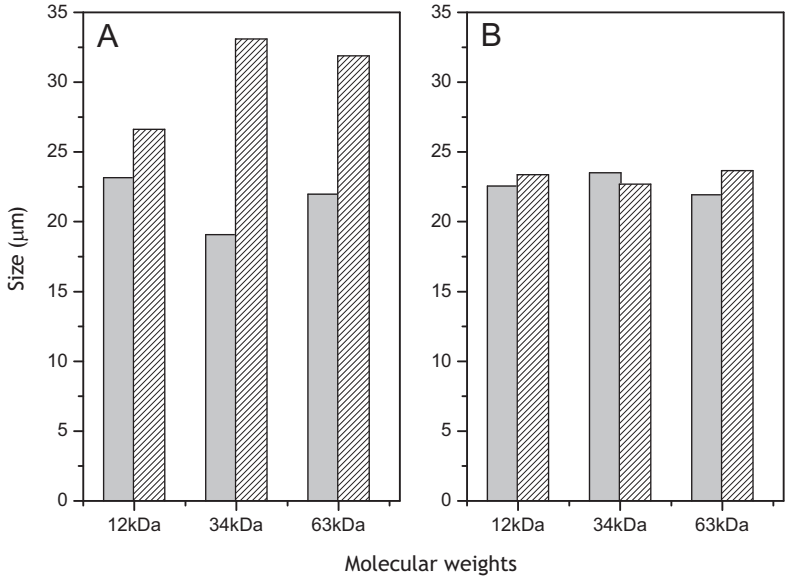

Fig. 2. Effect of molecular mass and carboxy-terminal (free or methylated) on the MS size prepared with different PLGAs. (A) MS-PLGA–COOH; (B) MS-PLGA–$COOCH_3$. Filled bars are non-loaded MS and dense bars are BV-loaded MS.

2.8. Immunological BV recognition along in vitro release

Equal amounts of medium where BV proteins were released (100 μL) were added to 96 well-plates, followed by blockade with PBS containing Tween 20 and 1.0% dry milk. After 4 h of incubation at 37 °C, the plates were washed and incubated for 4 h at 37 °C with 100 μL of a 1:500 dilution of rabbit anti-*Apis Mellifera* (honey bee) venom. Subsequently, the plates were washed and incubated with 100 μL of peroxidase-conjugated anti-rabbit immunoglobulins (IgG or IgA or IgM) for 2 h at 37 °C. Again, the plates were washed, and the reaction was developed with addition of 100 μL of tetramethylbenzidine previously dissolved in dimethyl sulfoxide and 5 mL of citrate buffer (pH 5.0) containing 5 μL of H_2O_2 30%. The reaction was developed at room temperature and protected from light. After 15 min, the reaction was stopped by addition of 50 μL of H_2SO_4 1 M. The absorbances were measured at 450 nm in a Titerteck Multiscan MCC/340. Positive (crescent amounts of recently prepared native BV) and negative (only PBS) controls were also included. The amount of immunologically recognized released BV was compared with a standard curve constructed with positive controls ($y = 0.30863X$, $r = 0.97332$).

2.9. Fluorescence analysis of BV during their release in vitro

The spectra of weekly BV protein released from MS-PLGA were recorded in a Hitachi F 2000 fluorimeter using quartz cuvettes with 1 cm optical path. The BV protein concentration was set at 0.1 mg/mL before analysis. The samples were excited at 280 nm and the fluorescence emission measured between 280 and 480 nm. Control: native BV at 0.1 mg/mL.

2.10. Statistical analysis

The means and standard deviations (SD) from, at least, three measurements were obtained for all analytical experiments. Results from the experiments were used as variables and analyzed

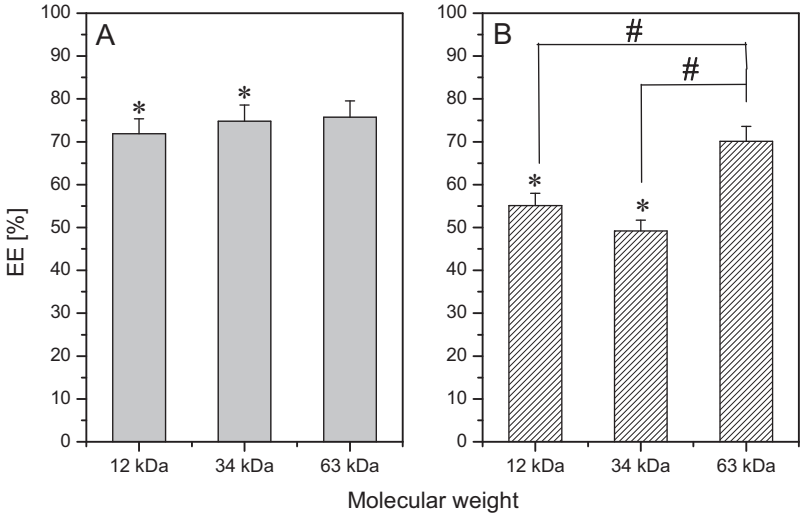

Fig. 3. Effect of molecular mass and carboxy-terminal (free or methylated) on the efficiency of encapsulation of BV within MS prepared with different PLGAs. (A) MS-PLGA–COOH; (B) MS-PLGA–$COOCH_3$. *comparisons between different carboxy-terminal within the same Mn PLGA ($p < 0.05$) and #comparisons between different Mn within the same carboxy-terminal PLGA ($p < 0.05$).

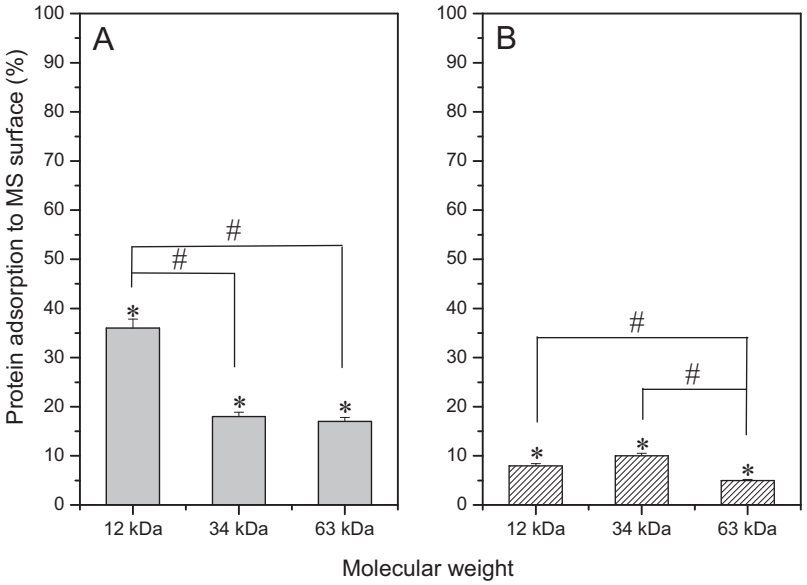

Fig. 4. Effect of molecular mass and carboxy-terminal (free or methylated) on adsorption of BV on surface of different MS-PLGA. (A) MS-PLGA–COOH; (B) MS-PLGA–$COOCH_3$. *comparisons between different carboxy-terminal within the same Mn PLGA ($p < 0.05$). #comparisons between different Mn within the same carboxy-terminal PLGA ($p < 0.05$).

by using a one-way analysis of variance (ANOVA) from GraphPad® software. When statistically significant differences were found, Tukey tests were performed. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Morphology and size

We obtained spherical microspheres with smooth, porous surface with all polymers (Fig. 1). The porosity was independent of either molecular weight or type of carboxyl-end (free or methylated). Furthermore, the MS morphology was independent of BV presence in the formulations (Fig. 1).

MS with polydisperse size distributions were obtained, showing a predominant size ranging from 19 μm to 33 μm (Table 1). Highest polydispersity index were found in MS prepared with lowest molecular weights (12 kDa) polymers with –$COOCH_3$ carboxyl-end (Table 1). Low polydispersity index means better homogeneity in size distributions. It was observed that MS size was independent of either molecular weight or carboxyl-end (–COOH or –$COOCH_3$). The presence of BV induced an increase in MS size in all studied polymers. This phenomenon was more evident in the MS prepared with polymers –COOH (Fig. 2).

3.2. Efficiency of encapsulation (EE)

MS encapsulated between 49% and 75% of added BV (Fig. 3). The most important observation was that MS-PLGA–COOH encapsulated more BV proteins than MS-PLGA–$COOCH_3$ ones (Fig. 3). The EE of MS-PLGA–COOH was independent of their molecular weights (Fig. 3A). Furthermore, some differences in EE of the MS-PLGA–$COOCH_3$ were observed, but they did not show direct correlation with molecular weight (Fig. 3B). Also, it was observed that MS prepared with 63 kDa-$COOCH_3$ polymer encapsulated 15–21% more BV than lower molecular weight ones (12 kDa-$COOCH_3$ and 34 kDa-$COOCH_3$).

3.3. Adsorption of BV in the MS-PLGA surface

Adsorptions were observed on surface of MS prepared with all studied polymers (Fig. 4). More strong interactions were observed between BV proteins and the surface of MS-PLGA–COOH (Fig. 4A). This means that BV proteins have preference by more hydrophilic surfaces. Also, it was observed, among these polymers, that adsorptions were more accentuated in the lower molecular weight MS-PLGAs (12 kDa), which did not occur in –$COOCH_3$ ones (Fig. 4 B).

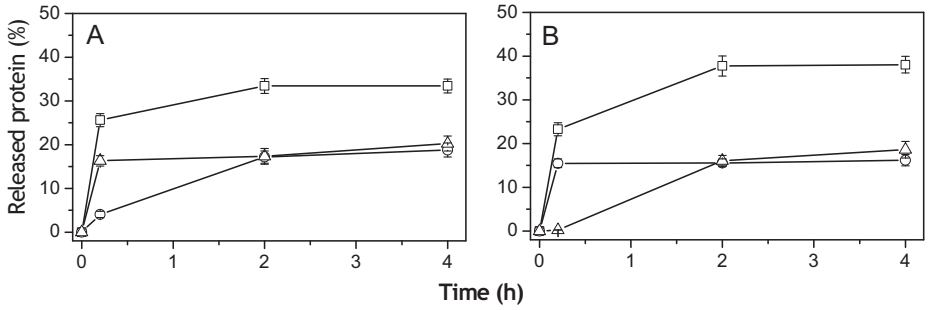

Fig. 5. Effect of molecular mass and carboxy-terminal (free or methylated) on the burst BV release (first 4 h) from MS-PLGA. (A) MS-PLGA–COOH; (B) MS-PLGA–$COOCH_3$. (□) 12 kDa; (○) 34 kDa; (△) 63 kDa.

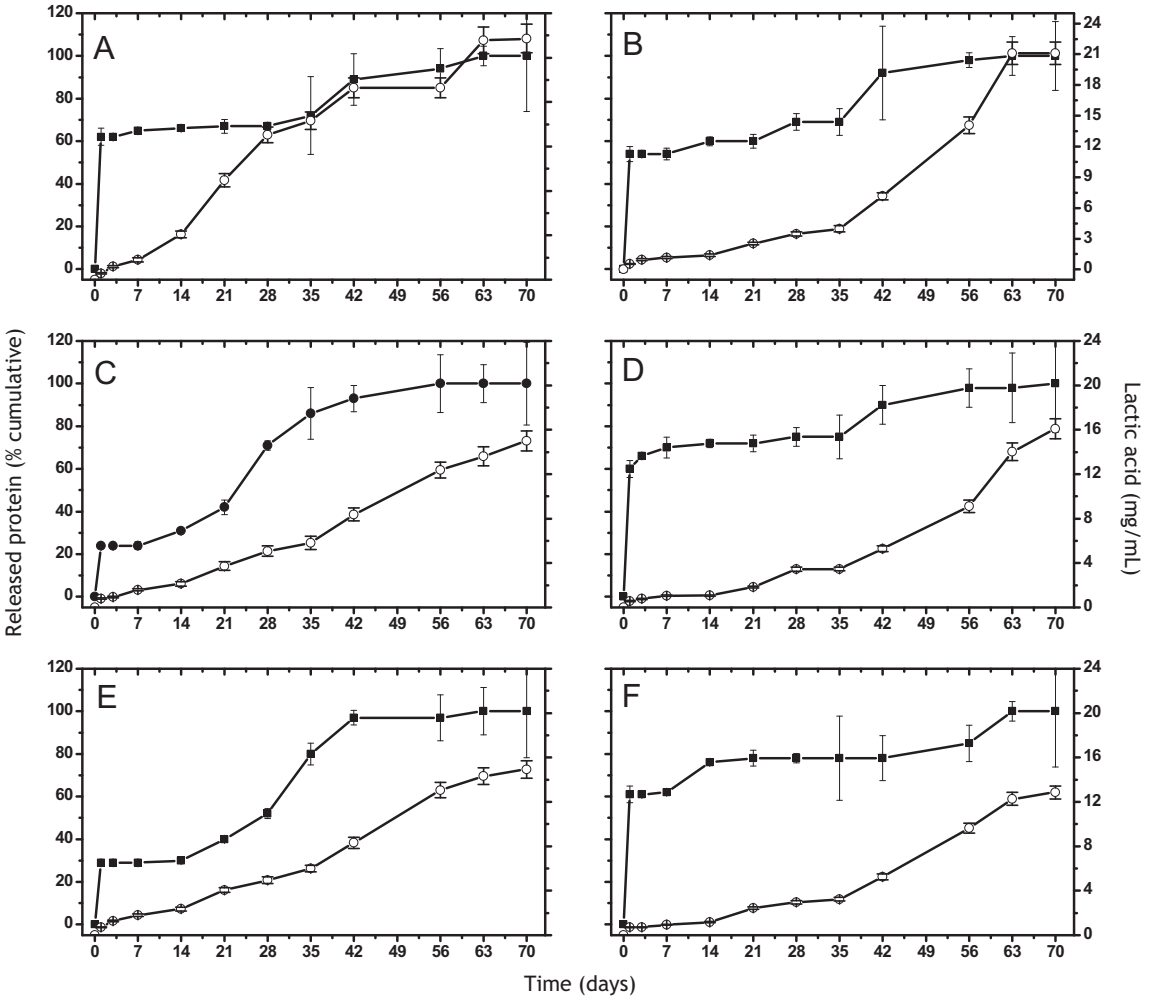

Fig. 6. Degradation profiles of MS-PLGA and BV release in function of time. (A) 12 kDa-COOH; (B) 12 kDa-$COOCH_3$; (C) 34 kDa-COOH; (D) 34 kDa-$COOCH_3$; (E) 63 kDa-COOH; (F) 63 kDa-$COOCH_3$. (■) BV protein release; (○) lactic acid production.

3.4. Burst effect, MS degradation study and release kinetics of BV from microspheres

A 35% of burst effect was observed in the first 4 h of incubation (Fig. 5) from MS-PLGA prepared with 12 kDa polymers independent of carboxyl-terminal (–COOH or –$COOCH_3$), and about 17% in the other PLGAs (Fig. 5).

The degradation of the polymer matrix was accompanied in all formulations by measuring of lactate released to the medium (Fig. 6 right axis). The lactate release showed a sigmoidal like profile in all formulations, representing three different phases (Fig. 6). The most evident was observed with the MS produced with 12 kDa-COOH and 12 kDa-COOCH3 PLGAs (Fig. 6) where it was observed that methylation retarded the velocity of degradation. This meant that, in mass, the particles formulated with 12 kDa-COOH; 34 kDa-COOH and 63 kDa-COOH PLGAs produced 36%; 13% and 20% (respectively) more lactate than their methylated pair.

The BV proteins were released by diffusion in the first studied times, as described in the literature for other proteins. A burst released proteins mainly in the particles prepared with 12 kDa PLGAs (Fig. 6). The lag phases of BV release from each polymer varied greatly. They were 28 days (12-kDa-COOH); 38 days (12 kDa-$COOCH_3$); 7 days (34-kDa-COOH); 35 days (34 kDa-$COOCH_3$); 14 days (63 kDa-COOH); and 42 days (63 kDa-$COOCH_3$). From those days on, there was a sharp increase in BV release, which was controlled probably by the particle degradation added by diffusion phenomenon. The analyses of these types of kinetics are neither simple nor trivial (Fig. 6).

3.5. Immunological recognition of BV

The BV proteins released from these formulations were maintained immunologically recognizable (Fig. 7). Furthermore, as the proteins accumulated in the releasing medium, the ELISA units also increased, meaning that even those proteins released on the last moments, where effects of the acidic environment and polymer degradation on them was more pronounceable, continued showing antigenicity.

3.6. Conformational analysis of BV by fluorescence

Exposition of BV protein hydrophobic residues to the medium was observed. It is known, from literature, that at this wavelength this exposition corresponds to tryptophan (W) and can be directly related to protein conformation changes. The expositions were directly proportional to the molecular weights of the respective MS-PLGA, mainly within –COOH series (Fig. 8A, C and E), due to acid liberation to the media. Once more, during degradation of microspheres, the cleavage of polymeric chains occurs. This cleavage leads to formation of monomers and oligomers. Thus, the pH inside the MS starts to be controlled by degradation products (glycolic and lactic acid), and this phenomenon has a base-acid function-

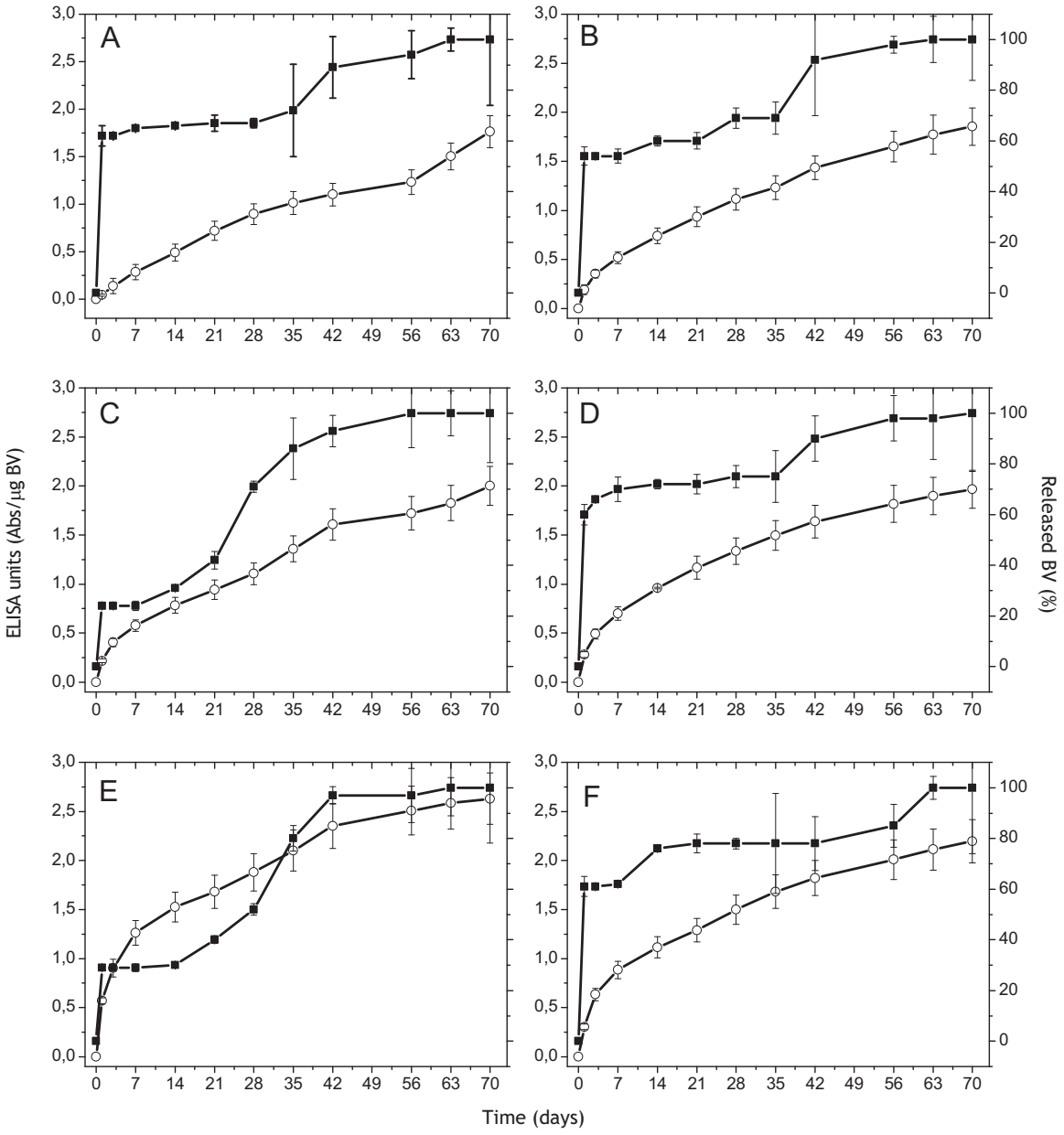

Fig. 7. ELISA of BV released from different PLGA-MS preparations in function of time. (A) 12 kDa-COOH; (B) 12 kDa-$COOCH_3$; (C) 34 kDa-COOH; (D) 34 kDa-$COOCH_3$; (E) 63 kDa-COOH; (F) 63 kDa-$COOCH_3$. (■) BV protein release; (○) ELISA units.

ality. It is known that protein conformations depend on the pH of the medium. For this reason, a more accentuated conformational changes in BV released from MS-PLGA–COOH was observed (Fig. 8A, C and E). Oligomers are produced by all formulations, which interact with BV proteins, and make them more susceptible to conformational changes. But, with no doubt, the degradation products originated from MS-PLGA–COOH exerted more strong influences on BV proteins than MS-PLAG–$COOCH_3$ ones (Fig. 8B, D and E).

4. Discussion

For the formulation of microspheres from biodegradable polymers it is essential to select an ideal controlled release system such as microspheres with desired size, high yield and entrapment efficiency, low burst release with preferred release profiles, and stability of the encapsulated protein during their release from microsphere formulations (Sinha and Trehan, 2003; Ravi et al., 2008). In this work, the MS morphologies were dependent on neither molecular weight nor carboxyl terminal group, which contrast with other authors who have reported some influences of these characteristics on the structure of microspheres (Batanero et al., 2002; Cai et al., 2009). A possible explanation for these discordant results is that the preparation process may exert stronger influence on those morphological characteristics than the type of polymer (Namur et al., 2006; Ravi et al., 2008; Mahdavi et al., 2010). Also, the MS morphologies were not modified by the presence of BV. The MS size changes after protein encapsulation is an open subject in the literature. While some authors report an increase in MS size after protein encapsulation (Dos Santos et al., 2009) in agreement with our results, others do not observe this phenomenon for protein encapsulation (Yang et al., 2001; Cai et al., 2009) or even other drugs (Torres et al., 1996; Barichello et al., 1999; Pandit et al., 2009).

The efficiency of encapsulation is enormously affected by the interaction between polymer and the medium where the solute is diluted, such as pH, ionic strength, and solute charge (Sandor et al., 2001; Cui et al., 2005). It is evident that, for PLGA con-

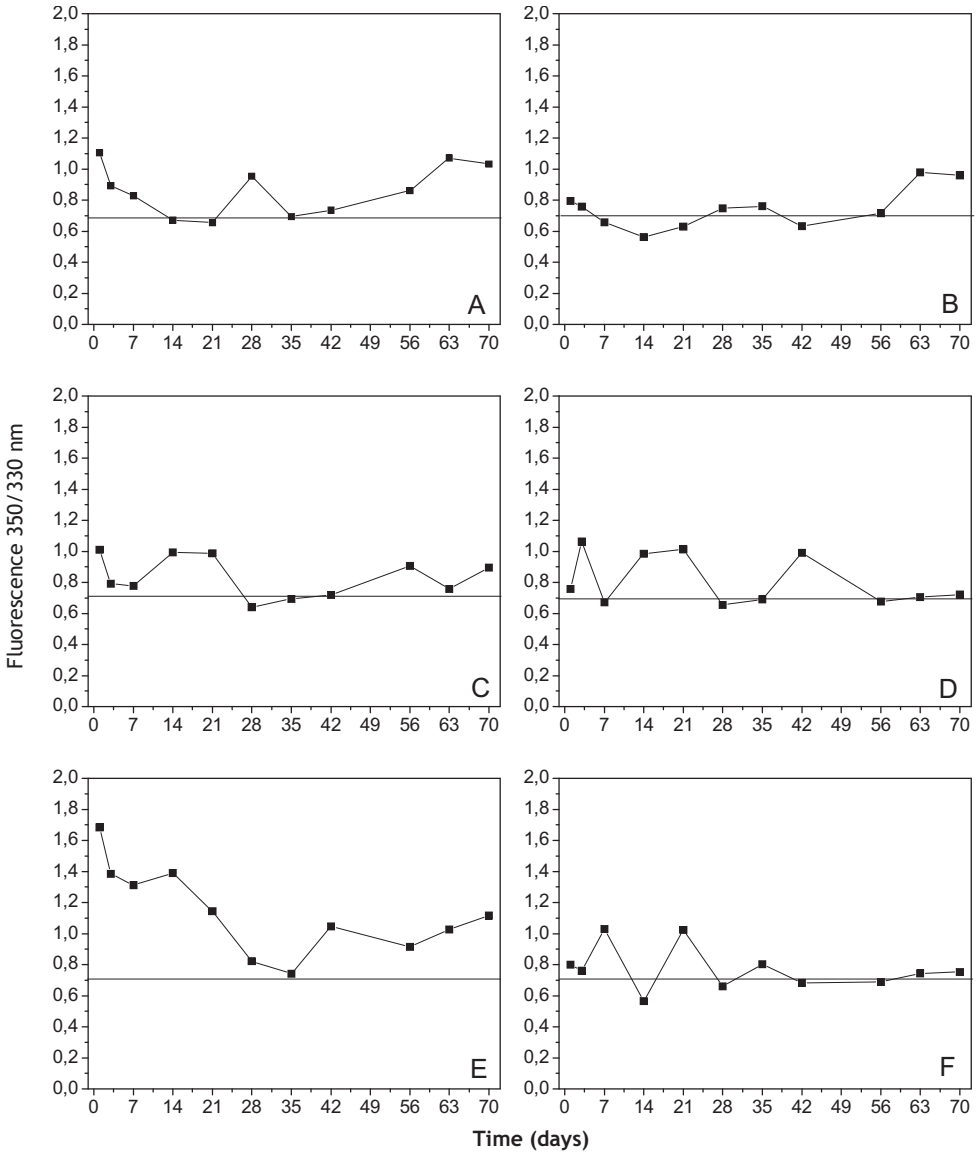

Fig. 8. $I_{350\,nm}/I_{330\,nm}$ of BV released from different PLGA-MS preparations in function of time. (A) 12 kDa-COOH; (B) 12 kDa-$COOCH_3$; (C) 34 kDa-COOH; (D) 34 kDa-$COOCH_3$; (E) 63 kDa-COOH; (F) 63 kDa-$COOCH_3$.

taining carboxy-terminal free (–COOH), the interaction between hydrophilic proteins and the polymer is influenced by the solute net charge (Blanco and Alonso, 1997; Park et al., 1998; Kwon et al., 2001; Bilati et al., 2005) as observed in our results. The efficiency of encapsulation was highest when the BV was encapsulated within MS-PLGA with carboxy-terminal free (–COOH) when compared with the polymer with the same Mn but containing –$COOCH_3$ (carboxy-terminal methylated), which is in agreement with other studies (Ravi et al., 2008). There was a statistically significant difference ($p < 0.05$) in the entrapment efficiency of BV protein among free and methylated carboxy-terminal of PLGA polymers. The methylated polymers interact hydrophobically with proteins close to their pI (Ruzgas et al., 1992; Tsai et al., 1996a,b). This kind of interaction could have been obtained for BV, whose pI is ~8.7 (Cavagnol, 1977), only if the preparation was done at pH 8.7. However, this was not interesting because at its pI the solubility of BV protein is the lowest, thereby facilitating the adsorption to water/organic solvent interface during the first emulsion of the microencapsulation process (Namur et al., 2009; Trindade et al., 2011). The main interest in this drug delivery system is to maintain the protein with maximum solubility to make possible its release from the particles. The predominantly ionic interaction between BV and –COOH free PLGAs was corroborated by the adsorption assay where it was observed that the venom adsorbed more efficiently on this more hydrophilic polymer. Among the three used –COOH polymers (12 kDa; 34 kDa and 63 kDa) the one that adsorbed more BV was the 12 kDa PLGA. Adsorption assays will always vary depending on both specific pI of each used protein and hydrophobicity properties of polymers (Tsai et al., 1996a,b; Lacasse et al., 1998; Jeong et al., 2000).

The higher MS degradation rate was observed in those particles prepared with –COOH PLGAs as also observed by other authors (Baker, 1987; Vert et al., 1991; Grizzi et al., 1995; Fu et al., 2000; Siepmann and Göpferich, 2001; Blanco-Prıeto et al., 2004; Zolnik and Burgess, 2007). The presence of BV did not affect the MS degradation profile.

The relevance of BV/PLGA interactions also were studied in the protein release profiles. The higher observed burst (12 kDa-COOH), in the first 2 h of incubation, was related to an external adsorption phenomenon which occurred with this polymer and BV proteins. This was also was observed in previous studies (Ravi et al., 2008). During the entire release assay, 10 weeks, the BV release showed a

triphasic profile in all formulations. The more evident differences were seen in the first 48 h, this was followed by a lag phase similar for all polymers. Among different polymers, the most adequate release profile for VIT was shown by the 34 kDa and the 63 kDa MS-PLGA–COOH, because they showed lower initial BV release, followed by a continuous liberation in the time until reaching a plateau after the 45th day. The reasons for these results may be explained by the strong interaction between positively charged BV and negatively charged polymers which probably helped to delay the BV release. The most relevant result in this study was that the released proteins maintained their immunological epitopes recognizable by specific antibody during the entire release assay in spite that these proteins had exposed hydrophobic residues as observed by fluorescence. These results are also in agreement with other authors that had reported alterations in either structural integrity or immunogenicity of encapsulated allergens during their release from MS-PLGA (Sharif et al., 1995; Batanero et al., 2002). The results obtained with BV proteins encapsulated into MS-PLGA showed that the allergens may still be effective in the induction of an immune response and consequently, may be a good new formulation for VIT for BV allergic patients.

5. Conclusion

Encapsulation of BV proteins within MS-PLGA represents an intelligent alternative to circumvent the troubles related to traditional VIT. BV was encapsulated within six different MS-PLGAs with varied, but adequate controlled releases, with the preservation of their biological recognition, which is a crucial factor for VIT.

Conflict of interest statement

The authors have declared no conflict of interest.

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