Biological activity of bee venom after encapsulation within PLGA MS for Immunotherapy

Trindade, R.A. and Bueno da Costa, M.H.
Laboratory of Microspheres and Liposomes - Center of Biotechnology
Institute Butantan
Av. Vital Brasil 1500, (05503-900) Butantan, São Paulo, SP, Brazil
e-mail: rtrindade@usp.br and bdacosta@usp.br

INTRODUCTION

Biodegradable and biocompatible poly(lactide-coglycolide) (PLGA) microspheres of defined composition, size, and surface property permit delivery of protein/polypeptide antigen to an organism in a continuous or pulsatile manner, thus opening the way for single-injection vaccines (Jilek, 2004). In encapsulation process within PLGA microspheres, bee venom proteins are exposed to a number of stresses, like high pressures, temperature gradients, and shear forces. Primary emulsion has been suggested as a major cause for protein denaturation, aggregation and loss of biological activity. The formation of hydrophobic interfaces results in interfacial adsorption followed by protein unfolding and aggregation, and by consequence loss of biological activity. A strategy to reduce these effects is modifying the buffer where bee venom are dissolved using salt solutions in different concentrations (NaCl; NaH_2PO_4; $MgCl_2$; KSCN). In this study, we also verified the physical/chemistry parameters after their microencapsulation in PLGA microspheres. It was used six different polymers to evaluate the effects of terminal-carboxyl (CH_3 or H) and their molecular weight (12, 34 e 64 Mw). The venom immunotherapy is expensive and time-consuming (Breheler, 2000), with a total of 30–80 injections administered over years. New strategies to improve the safety and efficacy of this treatment, with a reduced number of injections would, therefore, be of general interest (Müller, 2003), as it would improve patient compliance and provide socioeconomic benefits. Our strategy is to use PLGA microspheres as a controlled delivery system to reach this goal.

OBJECTIVE

To evaluate the effects of first emulsification on biological activity of bee venom proteins which may indicates loss of conformational structure and to verify some parameters used as a marker of efficiency of process.

MATERIAL AND METHODS

Relative hemolysis: Hemolytic activity was tested against human erythrocytes by using a standardized concentration of 1.5×10^8 HRBCs (human red blood cells)/mL of PBS. Native BV (bee venom) or post-emulsified BV recovered from salt solutions were dissolved in PBS to a concentration of 0.1 mg/mL. To 100 µL of each solution were added 50 µL of the stock erythrocytes in PBS and then it was filled up with PBS to reach a final volume of 1.0 mL. The resulting suspensions were incubated at 37°C for 60 minutes with gentle mixing. The samples were then centrifuged and the absorbance of the supernatant measured at 414 nm. For controls, zero hemolysis (blank) and 100 % hemolysis consisted of erythrocytes suspended in PBS and in 1% Triton X100, respectively (Blondelle and Houghten, 1991).

Proteolytic activity: It was added 125 µL of BV solution (0,1 mg/mL) from different salt solution, pH 7.2 to 250 µL of casein solution (1 % m/v) in PBS 0.1 M, pH 7.0. These mixtures were incubated at 37ºC for 30 minutes. The reaction was stopped with 500 µL of trichloroacetic acid (5% m/v), then they were kept under environment temperature for 30 minutes. Once finished the reaction, the samples were centrifuged and absorbance of the supernatant measured at 280 nm (De Lima, 2000).

Microspheres preparation: The PLGA particles were prepared using the $(W_1/O)/W_2$ double emulsification solvent evaporation method. The microspheres were collected by centrifugation at 2000 g for 10 min, rinsed with water three times and then ressuspended with 2 ml of 0.1% PVA, lyophilized for 24 hours and stored at −20 ºC (O'Donnel, 1997).

Size and Surface morphology: Microsphere mean size was measured by laser X-ray diffraction in a equipment Mastersize 2000 (Malvern Instrument, Malvern, UK). The surface morphology and microspheres size were observed by scanning electron microscopy (SEM). The microspheres were covered with gold particles (100–150 Å) and observed in a Jeol JSM scanning electron microscope, Model 840A, at 25 kV of intensity.

Efficiency of encapsulation: Loading efficiencies were determined by dissolving 10 mg of microspheres in 2 ml of 0.1 % SDS - 0.1 M NaOH solution. The mixture was incubated under gentle stirring for 24 hours at 37 ºC. After centrifugation and filtration in 0.22 µm membranes, the BV was measured by the Lowry method.

RESULTS AND DISCUSSION

It was verified that the biological activity did not show a large decrease when compared to bee venom protein, in the same conditions, but not submitted to emulsification. Stressed BV (BV after emulsification) retained about 80% of hemolytic activity when previously dissolved in the most used salt. The worst activities were founded with BV in the presence of 50 – 150 mM NaH_2PO_4, The best proteolytic activities were found when it was used $MgCl_2$ as salt (proteolysis above 50 %) (Figure 1).

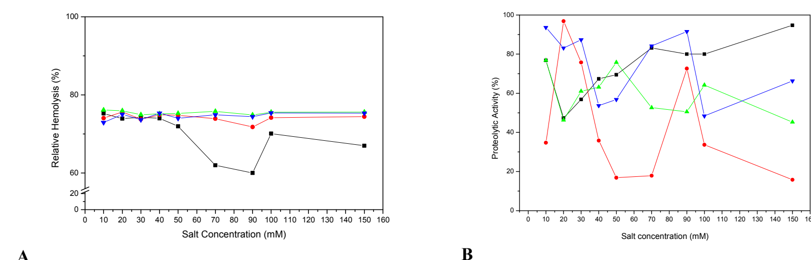


Figure 1. (A) Relative hemolysis (%) and (B) Proteolytic activity (%) of BV proteins in different salt concentrations (mM). The supernatants were analyzed in the presence of (-■-) NaH_2PO_4, (-●-) NaCl, (-▲-) KSCN and (-▼-) $MgCl_2$ after emulsification in the presence of CH_2Cl_2.

Table 1. PLGA microspheres sizes.

Polymer	Average cumulative undersize distributions (µm)			
	D_{10}	D_{50}	D_{90}	VMD*
H-12kDa	6.19	18.23	47.47	23.15
Me-12kDa	6.77	19.56	42.16	22.55
H-34kDa	6.12	17.24	34.54	19.07
Me-34Kda	11.27	21.89	38.53	23.50
H-64kDa	6.82	19.77	40.32	21.97
Me-64kDa	8.92	19.89	38.38	21.93
H-12kDa/BV	4.41	21.49	56.91	26.61
Me-12kDa/BV	9.25	21.42	40.82	23.38
H-34kDa/BV	14.70	30.20	56.06	33.10
Me-34Kda/BV	11.07	21.02	37.05	22.69
H-64kDa/BV	15.50	29.48	52.26	31.89
Me-64kDa/BV	10.94	21.70	39.50	23.66

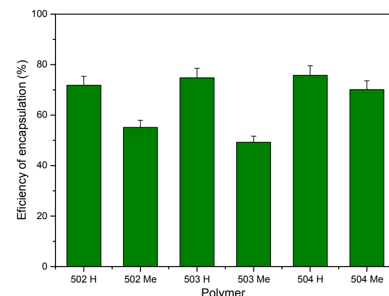


Figure 2. Efficiency of encapsulation of BV within PLGA microspheres prepared with different molecular weights and with terminal-carboxyl free (H) or methylated (CH_3) Polymers. 502 (12 kD); 503 (34 kD); 504 (64 kD).

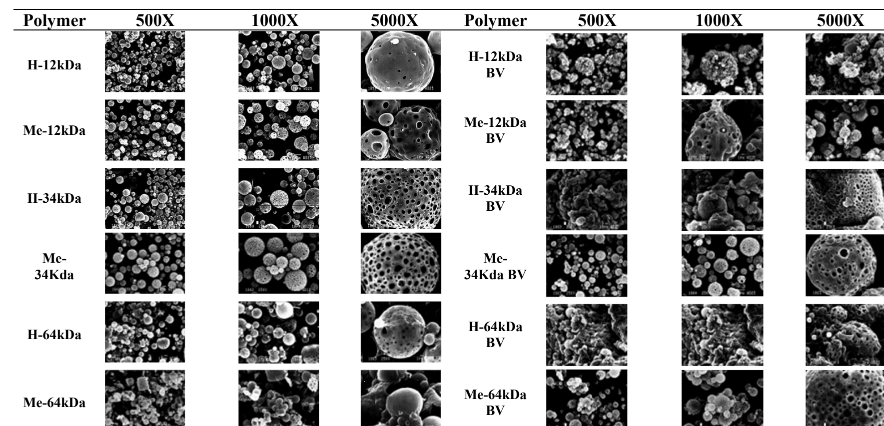
It is important to stress that a VIT formulation must contain all these components to produce antibodies against the venom proteins. Since bee venom encapsulation could cause changes in protein conformations, we used *in vitro* biological methods to follow the extent of these possible structural variations. The hemolytic activity is a good indicator of presence of a whole mellitin and phospholipase (PLA2), that is the major components of bee venom proteins, and also responsible for deleterious effects on surface of membrane cells. To remain these characteristics is important to guarantee that there was not protein denaturation during encapsulation process (Figure 1).

Other important feature during encapsulation process is the amount of proteins or antigens that is entrapped within microspheres. The encapsulation efficiency of the protein should be high. The ratio of the protein to the polymer should be such that the largest amount of protein is encapsulated in the minimal amount of polymer. This reduces the mass of the material to be administered. It is observed that encapsulation efficiency was above 50% in all six polymers used in these formulations, and polymers with free terminal – carboxyl showed the best capacity of entrapping bee venom proteins (> 70%) (Figure 2).

Microparticle size is critical not only in determining its release and degradation behavior but also in determining the efficacy of the therapeutic agent by affecting tissue penetration or even intracellular uptake. It is known that the rate of proteins or antigens release tend to decrease with increasing sphere size, so that, larger microparticles (10-50 µm) releases their content in a lowest speedy compared to smaller spheres (<10 µm), as the particle size is reduced, more surface area is available for entry of water into microparticles, resulting in faster degradation and release of therapeutic agent. All of microspheres prepared in our experiment showed size of about ± 25 µm (Table 1).

The better definitions in terms of surface morphology of microspheres were found in the polymers with carboxyl-terminal CH_3, nevertheless these polymers showed a reduced EE (Table 2).

Table 2. Morphology of PLGA microspheres prepared with different mass weights: 502 (12 kD); 503 (34 kD); 504 (64 kD), and with terminal-carboxyl free (H) or methylated (CH_3). They were analyzed by SEM.



Polymer	500X	1000X	5000X	Polymer	500X	1000X	5000X
H-12kDa				H-12kDa BV			
Me-12kDa				Me-12kDa BV			
H-34kDa				H-34kDa BV			
Me-34Kda				Me-34Kda BV			
H-64kDa				H-64kDa BV			
Me-64kDa				Me-64kDa BV			

CONCLUSIONS

PLGA methylation, used to increase protein stability, decreased the encapsulation efficiency. In terms of biological activities, the worst hemolytic and the best proteolytic activities were obtained when the BV was emulsified in the presence of NaH_2PO_4 and $MgCl_2$, respectively. A balance between these results should be taken in consideration in terms of formulation to be used in venom immunotherapy.

Acknowledgements: FAPESP (2005/04514-2, 2002/07293-9, 2000/14228-3), CNPq (300648/2005-7) and Fundação Butantan. R.A. Trindade has a doctoral fellowship from CNPq.

REFERENCES

Blondelle, SE, Houghten, RA (1991). *Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin*. Biochemistry 30:4671–4678.

Breheler, R, Wolff, H, Kutting, B, Schnitker, J, Luger, T (2000). *Safety of a two-day ultrarush insect venom immunotherapy protocol comparison with protocols of longer duration and involving a larger number of injections.* Journal of Allergy Clinical and Immunology 105:1231–1235.

De Lima, PRM, Brochetto-Braga, MR, Chaud-Netto, J (2000). *Proteolytic activity of africanized honeybee (Apis mellifera: hymenoptera, apidae) venom*. Journal of Venomous Animals and Toxins, 6(1).

Jilek S, Walter E, Merkle HP, Corthésy B (2004). *Modulation of allergic responses in mice by using biodegradable poly(lactide-co-glycolide) microspheres.* Journal of Allergy Clinical And Immunology, 114: 943-950.

Müller, UR (2003). *Recent developments and future strategies for immunotherapy of insect venom allergy*. Current Opinion Allergy Clinical and Immunology, 3:299–303.

O'Donnel, PB, McGinity, JW (1997) *Preparation of microspheres by solvent evaporation technique.* Advances Drug Delivery Reviews, 28:25-42.