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Encapsulation of Naja –Naja Oxiana Snake venom into Poly (lactide-co-glycolide) microspheres

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ABSTRACT

One small-scale double emulsion technique for incorporation of Naja- Naja oxiana venom into Poly (lactide-co-glycolide) (PLGA) microspheres were developed and optimized. The effects of high speed homogenization on the double emulsion stability, microsphere size, entrapment efficiency and In vitro release of venom were studied. A stable double emulsion was verified by homogenization method. Slow removal of the organic phase allowed measurement of the size of the emulsion droplets and subsequent predication of the size which resulting microspheres. Microspheres in the size range of 1-10μm were prepared using homogenization technique, but this technique was sensitive to changes in the operating time, speed and volume of outer aqueous phase. Snake venom was released in vitro in a triphasic manner. After immunization of guinea–pig with a single IM injection, the PLGA-venom microspheres elicited an antibody response very high as that elicited with conventional method. These results indicate that the antigenicity of venom was retained after incorporation into PLGA microspheres using homogenization technique.

***Keywords*:** Venom, microsphere, double emulsion.

INTRODUCTION

Hyperimmunization is important process in production of therapeutic sera, because gammaglobulins produced against intended antigens (snake venom and so on) are active ingredients of this antisera (Anthony 1991). In order to increase of venom or other antigens immunogenicity, use of different adjuvants became routine and more efficient. At present for immunization against snake venom various type of adjuvants, as a solution, suspention or emulsion systems, are used in different centers (Fu *et al* 1999). Above mentioned systems dose not considerable stability and can not protect antigens against environmental factors. In the other hand, their preparation process is time consuming and costly. These adjuvants metabolized with high rate after injection in animal, so can not have considerable sustained release properties. Therefore, it is necessary to design novel forms of adjuvants. The microencapsulation has been noticed since 1950, but its application in antigen delivery and pharmacy has been initiated in recent years, and their application for preparation of the sustained and controlled release drug delivery and simultaneously multi-antigen delivery systems is interested. Biodegradable polymers are used as one of the most important vehicles. (Pries & Longer 1979, Davis *et al* 1987). The use of microparticles as a antigen delivery system is one of the most reasonable procedures for

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antigens protection and their sustained release (Jones *et al* 1996, Change & Gupta 1996, Xing *et al* 1996, Yan *et al* 1995 and Esquisabel *et al* 2000). However, antigens are sensitive to organic solvent, drying process, stress pending of miroencapsulation process, acidic medium produced during polymer degradation in body or microencapsulation process. Thus a suitable process should be designed in respect to physicochemical and biological properties of each specific antigen (Fu *et al* 1999). Already, different polymers have been used for this purpose which biodegradable polymers were main polymer among them (Xing *et al* 1996, Yan *et al* 1995). One of the most important polymers for preparation of toxoid microcapsules were polylactides and polyglycols (Change & Gupta 1996, Xing *et al* 1996, Yan *et a*l 1995 and Fu *et al* 1999). Molecular weight of them is often under 40 KD which are biodegraded to lactic and glycolic acids inside the body, then this metabolites entered to Krebs cycle and converted to CO2 and H2O. These polymers are used as a monomer or copolymer with different ratio. Hydrophobic nature of PLGA decrease tendency of water soluble antigens to it. Addition of monomers or hydrophilic polymers such as polyethylenglycol to structure of PLGA increase antigen entrapment efficiency by enhancement of hydrophilicity of polymer (Deng *et al* 1999). Microparticle preparation methods generally classified into two groups, chemical (simple and complex coaservation, interface polymerization,…) and mechanical procedures (spray dryer, spray in cold environment, coating in air bed, …) (Benoit *et al* 1996). Whereas antigens activity is depend on structural conformation of them such as other proteins, therefore during microparticle preparation with current methods (for vigorous changes in temperature, pH, ion concentration and solvent effect), it is possible conformation changes which generally is reversible (Change & Gupta 1996). Modified type of this method has been presented for preparation of proteins and vaccines containing microparticles from this polymer, which used in our study (Viswanathan *et al* 1999, Chattaraj *et al* 1999, Yan *et al* 1995 & Sturessun *et al* 1999). There is also a report on DNA-loaded biodegradable microparticles by PLGA for vaccine delivery, in order to increase the potency of DNA vaccination in vivo (Samantha Jilek *et al* 2005) and recently PLGA microparticles used for Co-Delivery of Ovalbumin and CpG Motifs as a safer alternative to current specific immunotherapy (Beatriz *et al* 2009). Prestrelski and cowokers reported "Microparticle formulations for sustained-release of bioactive compounds" succesfully (Prestrelski 2005). To manufacturing of antivenin the immunogenecity of Naja Naja Oxiana snake venom is weak, due to its low molecular weights of toxic fractions, so even by use of conventional adjuvants could not considerable increased the immunogenecity of venom. The aim of this study was to improve immunogenecity of Naja Naja Oxiana snake venom by entrapment of the venom in PLGA microparticles, and to evaluate their potential as antigen delivery system.

MATERIAL AND METHODS

Materials. Copolymer (D, L-Lactide- co – glycolide) with monomer ratio of 50:50 and viscosity $\eta = 0.25$, tween 80, 20, span 80, 20 were purchased from sigma Co. Polyvinyl alcohol with molecular weight of 7200 and methylen chloride were obtained from Merck Company. Naja-Naja Oxiana venom was taken from Razi Institute.

Preparation of PLGA microspheres. Microspheres were prepared by using of w/o/w emulsion technique. For preparation of this system, 2 ml of internal aqueous phase were added to 10 ml of external organic phase which were produced from dissolving of PLGA in methylen chloride, then were emulsified at 11000 rpm, 1 minute by homogenizer (IKA T18). This w/o emulsion was added to 15 ml polyvinyl alcohol 12 % w/v and homogenized under above conditions. In result of this process a w/o/w emulsion was produced. This formulation mixed by magnetic

stirrer (Heidolph) at 500 rpm for 24 h in room temperature, so that gradually evaporated methylen chloride and formed microspheres. Microparticles were isolated by using of centrifuge (Sorvall RT 6000D) at 5000 rpm for 20 min and washed 3 times by distilled water and then lyophilized (Christ Alpha 1-2). Lyophilized microparticles were preserved in 4 – 8 °C. Venom with concentration of 100 mg/ml was dissolved in aqueous phase before adding to organic phase.

Evaluation of emulsion properties. Double emulsion properties were evaluated by using of optic microscope (Olympus BX51TRF) and stability of emulsified systems by centrifugation at 3750 rpm for 5 h were investigated (Rieger 1986).

Determination of particle size. Lyophilized powder of microparticles was suspended and their diameter measured by particle size analyzer (Analysett 22). Particles size was evaluated on the basis of Vmd (Volume mean diameter).

In vitro release evaluation. Microparticles powder containing venom was spilled in test tubes of 20 ml and then were added 10 ml of phosphate buffer saline (PBS), pH : 7, to it and was incubated at 37 °C in shaker incubator for one month . Test tubes were centrifuged (2000 rpm, 20 min) with 1 day intervals and was sampling 1 ml of them and venom release amount of microspheres was estimated by evaluation of venom content of samples. Equal volume of fresh PBS was added to tubes for maintains of primary volume.

Evaluation of venom entrapment efficiency. For evaluation of entrapped venom content in microspheres, was dissolved definite amount of lyophilized powder under continues mixing in 0.1 M sodium hydroxide containing 5 %w/v SDS and total nitrogen content of solution estimated by microkjeldal method (United State Pharmacopoeia 2005). Then, the amount of protein entrapped in microspheres was measured.

Immunization protocol. In this study guinea pigs with 200 – 250 gram weight are used for evaluation of microparticles immunogenicity. The guinea pigs injected twice in a two weeks intervals received microspheres by intra muscular route and animals bleed after 0, 30, 50 days. Microspheres were suspended in normal saline before injection. In this study five group of animals were used. Each group included 3 guinea pigs and injection volume in each animal was 0.5 ml. First group of animals injected 200 µg/ml venom solution, second group venom containing microspheres with 200 µg/ml venom, third group venom free microspheres suspensions with 200 µg/ml venom, fourth group venom free microspheres suspension, fifth group fraund adjuvant emulsion (conventional) with 200 µg/ml venom and sixth group only normal saline injected .

Evaluation of immunogenicity. The European Pharmacopoeia (2005) method was used for evaluation of antibody titer in animal's blood. The different concentrations of venom were incubated with certain volumes of animal's sera at 37 °C for 1 hour. Then from each tube 1 ml injected to the mousses by Intravenous route and the mousses observe for 48 hour. The rate of mortality recorded and potency of antivenin in animal's sera estimated according to dose response curve.

RESULTS

Appearance, emulsion system stability and microparticles particle size. The microscopic evaluation showed a desirable double emulsion system after second homogenization process. The increase of homogenization time more than one min leads to destruction of double emulsion structure. The results obtained from evaluation of different emulsion systems stability shown in the table 1. The effects of emulsification duration, homogenization rate and time, aqueous phases volume to microparticles size were evaluated. As it is shown in the figure 1, when the time of homogenization increase from 1 to 6 minutes the size of particles reduced up to 60% (3.20 – 1.28 µm).

Table 1. Evaluation of double emulsions system stability.

Formulation No.	Second homogenization time (min)	Stability
1	1	+
2	1.5	±
3	2	-
4	2.5	-
5	3	-

The time of homogenization has not effect on particle size distribution. Our results showed that with increase of homogenization rate from 4000 to 11000 rpm which cause the reduction of particles size, but rates above 11000 rpm has no significant effect on mean of particles size (Figure 2).

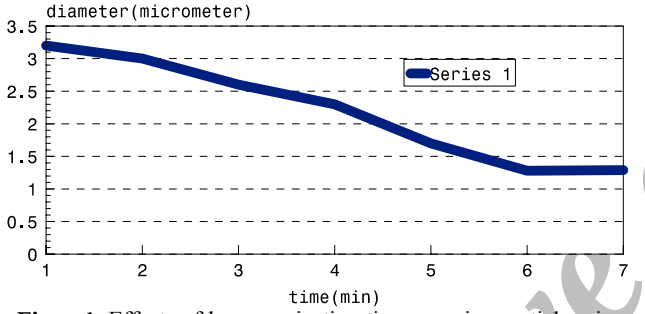

Figure1. Effects of homogenization time on microparticles size (11000 rpm), n =3.

Our investigation on effects of internal aqueous phase volume on particles size distribution has shown that increase if internal aqueous phase from 1 to 2 ml has not considerable effect on particles size.

Table 2. Effects of aqueous phases volumes on particles size.

Formulation No.	V_1(ml)	V_2(ml)	Particles Size(Vmd) µm
6	1	15	2.2
7	1.5	15	2.1
8	2	15	2.2
9	2	20	2.7
10	2	25	3.8
11	2	30	5.4

Increase of external aqueous phase from 15 to 30 ml cause increase of particles size (Table 2). The microscopic assessment of microparticles shown that the particles have globular shape with smooth surface and have relatively normal particle size distribution.

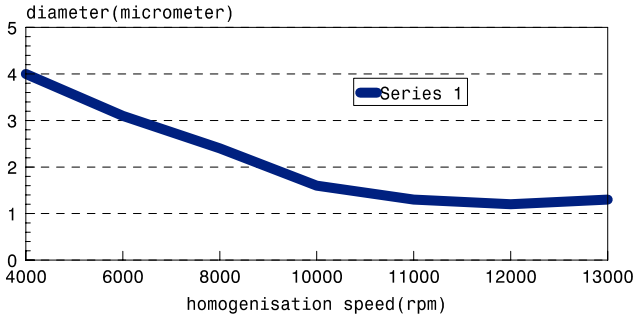

Figure 2. Effects of homogenization rate on microparticles size (time =1 min), n =3.

Venom entrapment efficiency and in-vitro release profile. The amount of venom in microparticles estimated by destruction of microspheres and measurement of total protein by microkjeldal method.

Table 3. Effects of different concentrations of PLGA on venom entrapping mean.

Formulation No.	PLGA Concentration (%w/v)	Entraption amount (%)
12	6	33±3
3	12	32±3
14	18	34±3
16	24	35±4
17	30	34±8

The amount of venom entrapped in the microspheres which prepared by different concentrations of PLGA 6 , 12 , 18 , 24 and 30 %w/v in organic solvent estimated.

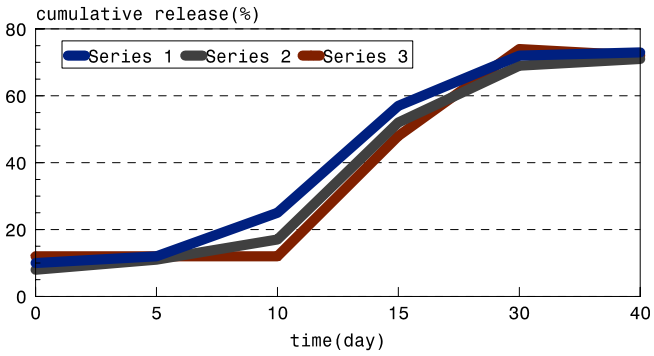

Figure3. Venom release profile from microparticles in-vitro (phosphate buffer pH: 7, T 37 °C, n =3). series 1 (PLGA 6%w/v), series 2 (PLGA 18%w/v), series 3 (PLGA 30%w/v).

Increase of PLGA, concentration causes no considerable increase of venom entrapment in microparticles (Table 3).

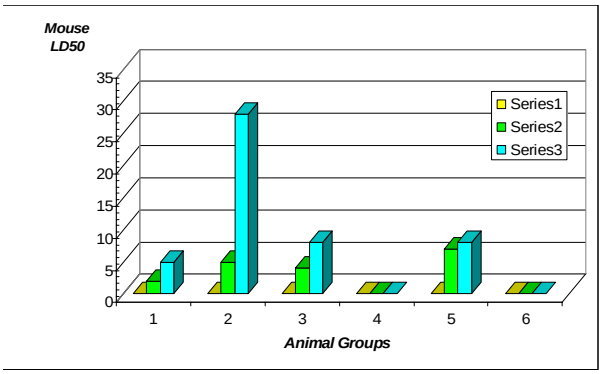

Figure 4. Antivenin titer in animal's sera. group 1: venom solution, group 2: venom containing microspheres, group 3: venom free microspheres suspensions + venom, group 4: venom free microspheres suspension, group 5: fraund adjuvant emulsion + venom, group 6: normal saline. series 1: before injection, series 2: 30 days, series 3: 50 days after injection.

The snake venom release profile from microparticles prepared with different concentrations of PLGA 6, 12, 18, 24 and 30 %w/v shown in the figure number 3. Our results indicated that, increase of polymer concentration has no significant effect on release profile of venom.

Immunization. In the present study we also studied the immunogenecity of venom loaded microparticles. The immunogenicity of microparticles containing venom has compared with fraunds adjuvant and positive and negative controls. Our results showed that 30 and 50 days after the last ingection, the level of humeral immunity significantly was high in the animal group number 2 (Figure 4).

DISCUSSION

The suitable adjuvant should have considerable stability and significant increasing ability of immunogenicity. In the present study PLGA microparticles prepared and used as adjuvant for immunization of animals with snake venom. The antigenicity of proteins entrapped in microparticles reduce as prepared emulsion system does not suitable stability (Cohen *et al* 1994). The report shows the increase viscosity of internal phase by using proteins such as gelatin is improved protein entrapment (Badmeier & McGinity 1988) . In the other study external oil phase which is not mixable with oil phase of primary core (w/o/o emulsion) was used in order to increase protein loading (Viswanathan *et al* 1999). The Beta-cyclodextrin , PVP and PVA used to prepared of primary emulsion core can be protect the antigen against organic solvents and increase stability during to microparticulation (shiga *et al* 1996). In order to prepare a stable double emulsion system the effect of second homogenization time was investigated in the formulations number 1 to 5. As it is shown in our results (Table 1) the suitable time for second homogenization was 1 minute. Therefore, it was expected that with this condition the antigenicity of venom could be protected. As shown in the figures 1 and 2 the mean of particles size related to homogenization time and velocity. According to our observation in the emulsification process, the best duration of homogenization in second step is 1 min and optimum rate of homogenization is 11000 rpm. As the duration of homogenization increases from 1 to 6 min the microparticles size reduced 60% and no considerable effect observed on particles size distribution (Figures 1 and 2). Increase of homogenization duration leads to turbolance in system and can be cause increase of entrance of venom from internal aqueous phase to external aqueous phase by adjacent of internal and external phases in homogenization process. As it is mentioned the 1 min is a suitable homogenization time. Increase of homogenization rate leads to significant reduction of particle size in a short time. In this study a linear correlation between reduction of particle size and homogenization rate up to 11000 rpm were observed and therefore it is considered as optimum rate. According of our results it seems that increase rate of homogenization with increase of energy input on system has significant effect on microparticles size mean. The increase of internal aqueous phase volume has no effect on particle size

but increase of external aqueous phase, due to less effective homogenization and accumulation of internal phase globules which cause emulsion with larger globules and increase the particles size (table 2). Therefore, volume of 2 ml for internal and 15 ml for external phase was suitable for preparing microparticles. If the particles size distribution come in close to unimodal state, it shows the suitable emulsion system, uniform particle size, and could leads to desirable release profile. In our study under above mentioned conditions the particles size distribution and particles characteristics were desirable. Results about the effect of polymer concentration on venom entrapment showed in table3. As it is shown the 33% antigen entrapment efficiency in the polymer solution with 6%w/v concentration and increase of polymer concentration up to 30% w/v has no significant effect on antigen entrapping, but due to higher polymer precipitation which leads to increase of particles size. Present results also confirmed by Badri and cowokers (Badri *et al* 1999). To study the profile of venom release from microparticles, the accumulated release of venom from particles in different times were investigated (figure 3). As it is shown increase of polymer concentration can cause increase lag phase of antigen release phase. Our results shows the release profile is take place in three steps. The first step is a short time as a lag phase with no release. In the second step antigen released with desirable rate and then in the third step rate of release slowly reduced. The lag phase approximately last 5 days and second step last 25 days (from 5 to 30 days) and the rate of release reduced after 30 days. As the nature of antigen molecules is protein and have high molecular weight, they are dissolvable in the polymer membrane. In order to release antigen the polymer membrane must be gradually destroy, which the primary delay of release is due to this phenomena. In the second step during the degradation of polymer the release is take place properly. After second step the considerable portion of microparticles destroyed and therefore release rate reduced. This three step type release profile is suitable for antigen delivery systems (Cohen *et al* 1994). Immunization of guinea pigs indicated that antigenic characteristics protected during microparticle preparation (figure 4). The antibody titer in the fifth group (conventional system) can be compared with third group (venom free microspheres suspensions with venom), this shows the venom is entrapped among the microspheres and gradually is released, unlike to conventional emulsion system which can be remain for short time in the body. The second group which received venom containing microparticles showed high immunity which can be concluded that , for long time remain in the body, which cause approximately three time more immunity as compare to conventional system. In our previous study the Naja–Naja Oxiana venom was loaded in chitosan nanoparticles and their properties were investigated (Mohammadpour Dounighi *et al* 2010). According to our studies this venom can be loaded in microparticles and nanoparticles, which remains intact and the both particles system have the potential for use as an alternative for traditional adjuvants. Generally, it can be concluded that PLGA microparticles with desirable particle size for use in injectional and mucosal route which can be prepared by double emulsion system. Microparticles which prepared in present study showed suitable release profile in vitro, significant immunizing potential in vivo, good stability, low venom consumption and less administration frequency as compared with conventional system.

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