

## Extended Immunization of Rats using Microencapsulated Cobra Venom

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**Abstract:** Production of a vaccine with an extended release kinetic and with a high immunization response has an economic value in the third world countries. In this study, production and evaluation of bovine serum albumin microspheres that are incorporating cobra venom were carried out. The microcapsules were prepared using modified emulsification-polymerization technique. They were evaluated for their size using photon correlation spectroscopy and for their morphology using scanning electron microscopy. The release kinetics of the venom from the chosen formula was following Higushi model with  $r^2 > 0.97$ . The average microsphere size of the best formula was 5.6  $\mu\text{m}$  and the encapsulation efficiency approached 82%. The pharmacokinetic parameters of the microencapsulated cobra venom showed a significant difference from the free venom formulations in terms of prolonged stimulation of *in-vivo* immune response (production of antibodies). The method of preparation and evaluation of microspheres were characterized by being easy to perform, yielding high entrapment efficiency and maximum induction of immune response, which enables availability of a safe, effective and economic vaccine.

**Key words:** BSA, Copra venom, ELISA, microspheres, sustained release

### INTRODUCTION

Micro and nano-carriers such as microspheres, liposomes, polymeric micelles and nanoparticles have many advantages concerning drug delivery and targeting. These advantages include high drug loading, lack of chemical interaction with drug, which is necessary for encapsulation and considerable protection of the drug molecules (Crommelin *et al.*, 2001). Microspheres having a size range from 4-6 micrometers have been used extensively for encapsulation of proteins and peptide drugs. PLGA (poly lactide co-glycolide) microspheres containing recombinant hepatitis B core antigen prepared by a w/o/w emulsion/solvent evaporation method were evaluated for the possibility of acting as a potent long-acting carrier for hepatitis B core antigen in mice (Uchida *et al.*, 1998).

Considering the protection of drug molecules, one of the most important carriers is albumin microspheres. Either Bovine Serum Albumin (BSA) or Ovine Serum Albumin (OSA) has been used for microsphere preparation because they are biodegradable, biocompatible and nontoxic. One example which made a benefit from using this carrier was the production of celecoxib-loaded albumin microspheres for prolonged treatment of arthritis (Thakkar *et al.*, 2005).

The release characteristics of drugs loaded into microspheres is greatly influenced by the methodology

used in design of such delivery systems. Gupta *et al.* (1986) studied the release of adriamycin from heat-stabilized bovine serum albumin microspheres. Their results indicated that release from the microspheres can be adequately described by bi-exponential, first-order, bi-phasic, zero-order and Higuchi's square-root of time equations. The manufacture of microspheres was developed to prevent particles coalescence and stabilize the primary emulsions. Inclusion of lactide and polyethylene glycol to form microspheres resulted in more stable particles with high encapsulation efficiency (Wei *et al.*, 2008). Numerous immunoassay techniques such as Enzyme Linked Immunosorbent Assay (ELISA), radioimmunoassay, immunofluorescence, and immunoelectrophoresis are employed in the field of immunization research. ELISA was found to be the most versatile immunoassay technique that is applied to the field of venom research, its main advantages over other methods including relatively high levels of sensitivity and specificity, reproducibility, simplicity and ease of sample collection. It can also be readily modified into kit form and is easily adapted for use in large scale epidemiological studies and for accurate retrospective diagnosis of snake bite. None of the other assay systems considered fulfill these criteria to the same extent (Theakston, 1983). The efficacy of immunotherapy was evaluated after envenomation of rabbits using non-lethal doses of snake venom. A rapid and sensitive Enzyme-

Linked Immunosorbent Assay (ELISA) method for measuring venom antigens after an experimental snake bite was proposed by (Labrousse *et al.*, 1988).

Production and evaluation of anti-rabies antibodies (Redwan *et al.*, 2009) using ovine antibody had lower immunogenic effects than human and horse antibodies when injected into the mouse. When tested in envenomed patients they induced potent and sustained serological response with very high antibody avidity (Cook *et al.*, 2010). Encapsulation of *Naja naja oxiana* Cobra venom within nanoparticles fabricated from chitosan nanoparticles and tripolyphosphate (TPP) using ionic gelation process was reported (Mohammadpourounghi *et al.*, 2010).

Therefore the aim of this work is to formulate a sustained release vaccine in the form of microspheres intended for immunization of horses using cobra venom. We tried to combine the beneficiary properties of BSA as being biocompatible, biodegradable with its microspheres capability of high loading efficiency, venom protection, extended release characteristics as well as strong induction of immune response. Adult rats were used as model animals for reducing the cost of the study.

## MATERIALS AND METHODS

**Materials:** Bovine serum albumin (BSA) was purchased from Sigma Aldrich Ltd. (Cairo, Egypt). Cobra venom was obtained fresh from the institute of vaccination and immunization (Vacsera, Giza, Egypt). Glutaraldehyde was obtained from Fisher chemicals (Fischer Chemical (HK) Co., Ltd., Wan Chai, Hongkong). Diethyl ether was purchased from Merck (Germany). All other solutions were of analytical grade. Enzyme linked immunosorbent assay (ELISA) kit (Model EL-311, California, USA) was used for measuring the optical density of the venom and antivenom immunity and the antimouse conjugate peroxidase labelled was used as substrate (Sigma Aldrich, USA).

**Preparation of microspheres:** The BSA microspheres containing cobra venom were prepared in the Institute of immunization and vaccination (Vacsera) Dokki, Giza, Egypt in 2008. They were prepared aseptically under vertical laminar flow cabinet (Nuair biological safety cabinet: NU-425-400E, series 21, serial No.: 74424, Canada) using a modified emulsification polymerization methods (Sahin *et al.*, 2002). BSA (125 mg) was dissolved in de-ionized water (1 mL) and left to hydrate for 15 min. In another vial, cobra venom (0.44 µL) was mixed with water (0.5 mL). The two vials were then added into 100 mL corn oil (ADWEK, Egypt) at room temperature and stirred for 15 min at a rate of 1000 rpm. The mixture was then added into another 100 mL of corn

Table 1: BSA microspheres incorporating Copra venom prepared at different cross linking time and concentrations

Sample code	Amount Glutaraldehyde (mL)	Cross-linking time (min)
B1	0.1	15
B2	0.3	15
B3	0.5	15
B4	0.7	15
B5	0.1	60
B6	0.3	60
B7	0.5	60
B8	0.7	60

oil. The resulting microspheres were cross linked using 0.1, 0.3, 0.5 and 0.7 mL of glutaraldehyde solution (25% w/v) for periods of 15 and 60 min (Table 1).

### Morphological characterization of microspheres:

Scanning electron microscopy was used to evaluate the morphology of the produced microspheres. Cobra venom microspheres in Phosphate Buffer Saline (PBS) were mounted on copper stubs with double side adhesive tape and coated with gold using coated sputter (S150A Edward, England). The samples were examined under JXA-840A electron probe micro-analyser (Jeol, Tokyo Japan). Different micrographs were obtained showing variety of microcapsule sizes depending on the amount of glutaraldehyde added and the time of cross linking.

### Calculation of percentage yield of microspheres:

The yield of each formula was calculated by dividing the final weight of the microspheres produced by the weight of the BSA added. The weight of the vaccine is neglected because it was in the microlitre range. The yield was calculated according to Eq. (1):

$$\text{Yield} = \text{WM} / \text{W}_{\text{BSA}} \times 100 \quad (1)$$

Where, the WM is the final weight of the microsphere after drying whilst  $\text{W}_{\text{BSA}}$  is the initial weight of BSA added.

### Encapsulation efficiency of microspheres:

Weighed amounts of microspheres (20 mg) were pulverized using mortar and pestle. The grounded powders were then suspended in 10 mL deionized water and shaken for 4 h to extract the venom. The suspension was then centrifuged at 8000 rpm and the solid residue was removed. The optical density of the resulting reconstituted aqueous solution was measured for each sample using ELISA to determine the amount of venom encapsulated in the microspheres.

The encapsulation efficiency was measured for each sample using Eq. (2):

$$\text{EE} (\%) = \text{D microcap} / \text{D added} \times 100 \quad (2)$$

where, D microcap is representing the drug in the microsphere while the D added is corresponding to the drug added

**Evaluation of microsphere particle size:** The particle size distribution of BSA microspheres was determined using photon correlation spectroscopy (Malvern master sizer, Malvern instruments, UK). Before analysis, samples of microcapsules (10 mg) were suspended in 5 mL of deionized water and the particle size analyses were performed in triplicate.

**In vitro drug release:** Evaluation of the release characteristics of the venom from BSA microspheres was carried out according to the method reported by Jaganathan *et al.* (2005) with modification. Microspheres (10 mg) were suspended in 5 mL phosphate buffer saline (PBS, pH 7.4) containing 0.02% w/v Tween-80 and incubated at 37°C in a shaking water bath. At predetermined intervals, samples (500 µL) were withdrawn. Determination of venom content was undertaken using Enzyme-Linked Immunosorbent Assay (ELISA) as reported at El-Karamany (1987) and Laakel *et al.* (1996). The specimens withdrawn were replaced with equivalent fresh samples of dissolution medium. The withdrawn samples were two fold serially diluted with carbonate buffer pH (9.6) which is composed of Na<sub>2</sub>CO<sub>3</sub> (1.5 gm/L) and NaHCO<sub>3</sub> (2.93 gm/L). Plates were incubated for 18 h. Coating buffer was decanted and plates were washed using ELISA washer (Dynatech MRW, USA) at intervals (3 min) with washing buffer (200 µL/well) which is composed of Tween 80 (0.5%) in PBS. Plates were blocked using BSA (4% w/v) in PBS (pH 7.2). Standard venom (5 µg) was included into the plates as a standard sample for evaluation of released venom from test sample. Anti cobra venom (10 IU/mL) (VACSERA-Egypt) was dispensed as 100 µL/well and incubated for 1 h at 37°C, plates were washed as previous and anti horse conjugate used as 1/1000 was dispensed as 0.1 mL/well. One hour after the incubation. Plates were washed and substrate buffer was dispensed (0.1 mL/well). Developed color was measured using ELISA reader at  $\bar{e}_{\max}$  of 450 nm and the optical density were plotted against serial dilution.

The concentration of the venom in the release medium at any time interval was calculated according to Eq. (3).

$$\begin{aligned} &\text{Cumulative release of test} \\ &= \text{ODT/ODR} \times \text{R conc.} \end{aligned} \quad (3)$$

where ODT is representing the optical density of the test and ODR is the optical density of the reference. Both are multiplied by the concentration of the reference.

**Water uptake (bursting effect):** Microspheres (40 mg) were suspended in 5 mL of phosphate buffer saline (PBS, pH 7.4) containing 0.02% (w/v) Tween 80 at 37°C for one day. The initial weights of microspheres before immersion into PBS were taken as (W1). After immersion and collection by filtration, the microspheres were reweighed immediately (W2). Water uptake of microspheres was calculated using Eq. (4):

$$\text{Water uptake (\%)} = [(W2-W1)/W1] \times 100 \quad (4)$$

**Evaluation of immune response following envenomation:** Twelve adult Albino rats, Sprague-Dawley strain, with an average weight of 150-200 gm was provided by the animal house facilities (Vacsera, Giza, Egypt). All animals were handled humanly according to the Helsinki protocols (1964) and the experiment was carried out in the Institute of immunization and vaccination (Vacsera) Dokki, Giza, Egypt. The dose of formulated venom was calculated according to Paget and Barnes (1964) to be equivalent to doses used for immunization of the horses. At the time of the experiment, rats were divided into two groups; six rats in each. The first group was injected cobra venom (0.44 µg) included in BSA microspheres (10 mg/mL). The second set of rats was injected with free cobra venom in PBS (0.44 µg/mL). Formulated venoms were administered into the thighs muscles of the rats. Blood samples were withdrawn at 0, 15, 30, 45, 60, 75, 90, 105 and 120 days post immunization. Plasma was separated by cold centrifugation of heparinized blood samples. ELISA plates were coated with snake venom (1 µg/100 µL) for 24 h at 4°C. Plates were blocked and washed as previous. Anticobra venom rat was dispensed as 0.1 mL per well. Standard anticobra venom was prepared and locally evaluated according to the manufacturer (VACSERA-Sear Plant). They were processed parallel to the test samples. Student t-test was used for evaluation of the differences between average values of measured antibody titer from both free and microencapsulated venom. p-values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

**Physicochemical characterization of microspheres:** Table 2 shows particle sizes of different Cobra venom microspheres as measured by PCS. The presence of a wide variety of particle sizes in different batches may be attributed to the effect of both cross linking time and the cross linker concentration on the particle size of microspheres. It is well known that by increasing the cross linker concentration, smaller particles were produced with low polydispersity indices as shown in B1,

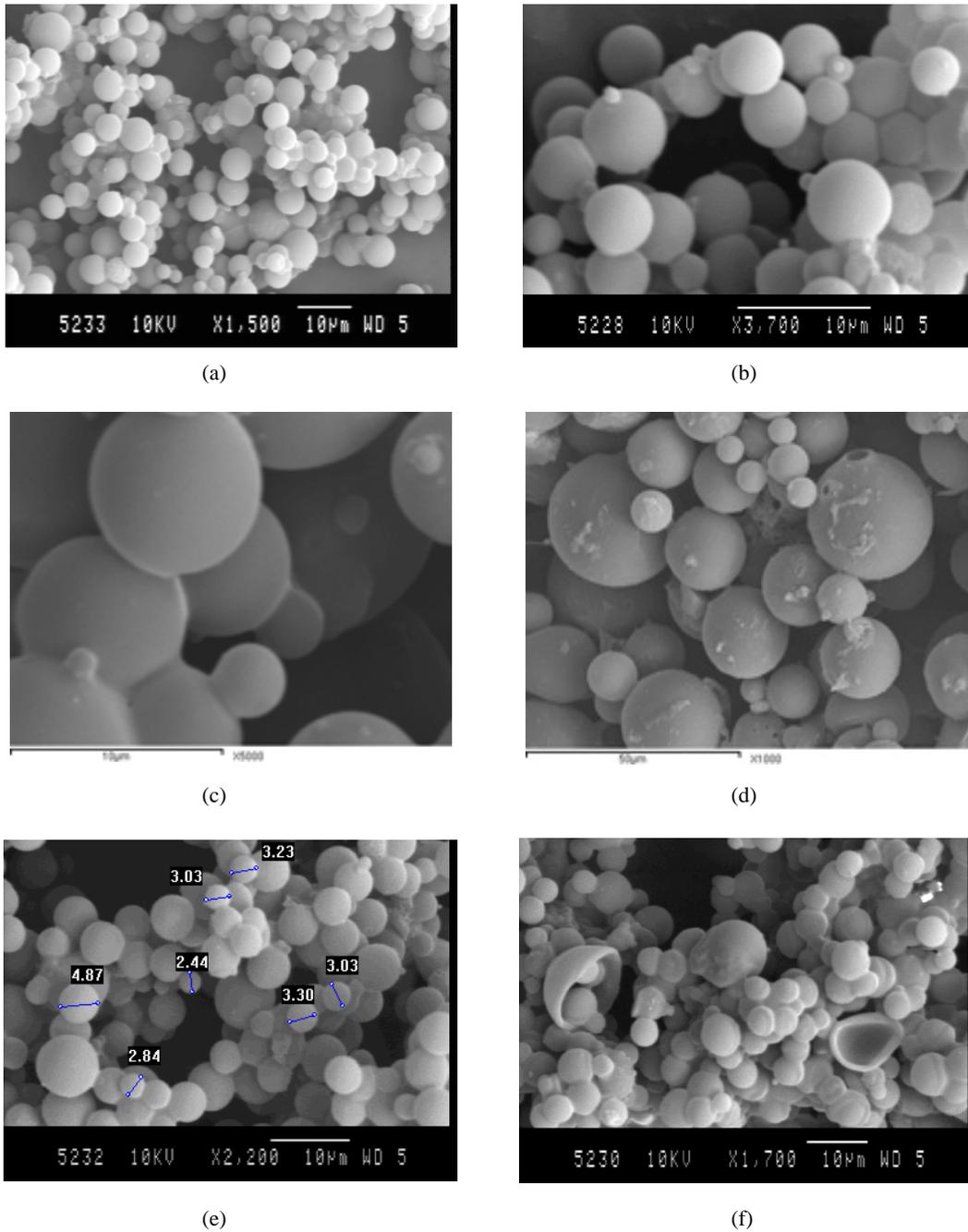


Fig. 1: Microspheres loaded with anti-cobra venom for formulations, (a) B1; (b) B2; (c) B3; (d) B4; (e) B6 and (f) B8

B2, B3 and B4. By increasing both the cross linking time and the concentration the same phenomenon was predicted as shown with B5, B6, B7 and B8. However the lowest particle size was reached when 0.3 µL of glutaraldehyde was used for 60 min to reach particle size of 5.6 µm.

The results of SEM micrographs were comparable to those of the Photon Correlation Spectroscopy (PCS) as

shown in Fig. 1. However, the results of the average diameter of all preparations seem to be much lower than those given by the PCS. The SEM condition was conducted in a dry environment however the PCS is conducted in the aqueous medium.

In Fig. 1, B4 and B6 showed smallest microsphere size among other formulations. Their polydispersity indices (PIs) were in the acceptable level i.e., far below

Table 2: Mean sphere diameters and polydispersity indices as measured by photon correlation spectroscopy

Formula code	<sup>a</sup> Z-average ± <sup>b</sup> S.D (μm)	Polydispersity index ± S.D.	Yield (%)	<sup>c</sup> E.E (%)
B1	25±6.7	0.67±0.02	88	67
B2	10.8±4.2	0.73±0.06	76	34
B3	7.1±3.5	0.88±0.01	70	88
B4	4.5±7.8	0.28±0.07	62	37
B5	21.4±7.7	0.65±0.08	81	78
B6	5.6±1.7	0.31±0.01	80	82
B7	14.2±2.1	0.36±0.32	60	92
B8	15.7±2.1	0.48±0.33	62	96

a: Z-average represents the mean diameter of the microspheres; b: S.D represent the standard deviation; c: E.E represents the encapsulation efficiency; f: B represent the formula code

unity. B4 and B6 have PIs of 0.28±0.07 and 0.31±0.01, respectively.

The micrograph of formula B8 (Fig. 1f) showed that the prepared microspheres had hollow cores, which may provide a higher space for encapsulating higher amounts of venom into the microspheres. On the other hand, the electromicrograph of B4 (Fig. 1d) shows that the excess amount of the crosslinking agent (glutaraldehyde) was deposited on the surface of microspheres (left for 15 min only during preparation), whilst formula B8 showed no deposits on microspheres' surfaces. B8 was cross-linked for longer time (60 min) than B4, which may be the reason for inclusion of all cross linker into the microspheres after hydration and fusion into the walls of microspheres during the time of the experiment.

**The yield of microspheres:** The yield of many batches of microspheres was quit low. The lowest value was for the B7 (60% of the added BSA). However, some of prepared batches namely B2, B6, B5 and B1 showed quit high yield. The highest yield was for B1 with 88% of the added BSA. The reason for low yield in most of the series may be attributed to adsorption of small sized BSA microspheres on the walls of the glass vessel. However, the experiment was conducted at pH 7.4, which is well above the pKa of BSA. Therefore, BSA was mostly carrying a full negative charge at this particular pH which could lead to repulsion between the microspheres and the glass vessels rather than adsorption. Since using high concentration of the cross linker may have led to masking of a large number of amino groups on the surface of the microspheres thus reducing the negative charges on the surface of the spheres. Therefore, this behavior resulted in a higher probability of adsorption of the microspheres to the glass vessels thus lowering the yield.

**Microencapsulation efficiency:** The results indicated that formulae 3, 6, 7 and 8 had the highest encapsulation efficiency (Table 2). Increasing the cross-linking time from 15 to 60 min in some instances such as with B2 led to doubling of the encapsulation efficiency of the venom.

Table 3: Percentage of water uptake by microspheres after 24 h

Formula code	% Water uptake/24 h
B1	79.8±2.7
B2	77.4±4.5
B3	34.1±2.9
B4	24.2±5.4
B5	66.9±6.1
B6	34.9±5.1
B7	25.9±7.3
B8	40.1±3.1

Table 4: Average microsphere size before and after suspension in PBS for two weeks

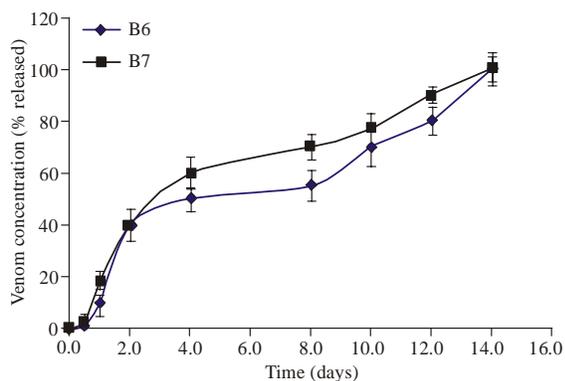
Formula Code	<sup>a</sup> Z-average (μm) Fresh microspheres	<sup>a</sup> Z-average (μm) after 14 days
B1	25±6.7	52.1±3.2
B2	10.8±4.2	20.5±4.4
B3	7.1±3.5	15.4±3.9
B4	4.5±7.8	10.4±3.1
B5	21.4±7.7	31.7±2.5
B6	5.6±1.7	7.4±2.4
B7	14.2±2.1	17.6±8.5
B8	15.7±2.1	18.7±13.9

a: Z-average represents the mean diameter of the microspheres

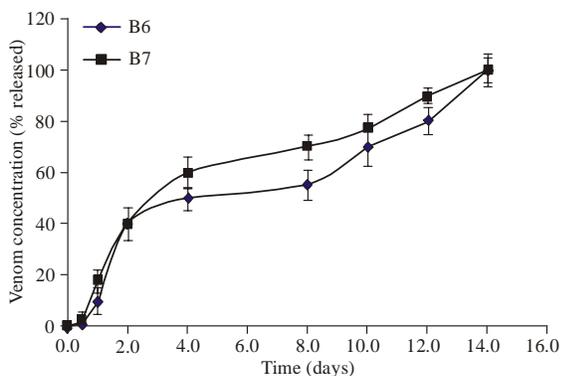
This may be attributed to the formation of narrower micro-tubular matrix within the microspheres, reducing the tendency of the venom to escape during the process of the cross-linking. Employing highest concentration of cross-linking agent with highest time in formulation B8, demonstrated greatest encapsulation efficiency approaching to 96% of the originally added venom.

**Microsphere water uptake:** Results of water uptake by microspheres after 24 h showed that formulations containing small amount of the cross-linking agent glutaraldehyde demonstrated maximum water uptake (formulations B1 and B5) as shown in Table 3. There was a trend of decreased % water taken up by microspheres with increased content of glutaraldehyde in formulations. The B4 showed the lowest % water uptake level among all investigated batches. The only odd result was observed with formula B8 which may be attributed originally to the higher microsphere average size and polydispersity index. When the formulations were kept for two weeks in PBS solution and particle size was re-evaluated, the results indicated that, formulations B1 and B4 microspheres demonstrated two fold increases in the average size (Table 4). Formulations B6 and B8 showed less increase in average microsphere size (<33%). B6 retained its smaller size than other formulations. This formula showed only 24% increase in size, which is still within the injectable average size of microspheres (less than 10 μm). This behavior revealed that B6 is the most resisting preparation that is less prone to rupture or sudden loss of contents upon suspension in a vehicle.

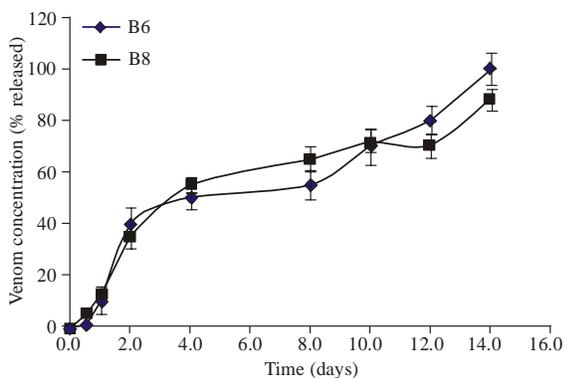
**In vitro release characteristics of venom-loaded microspheres:** *In-vitro* release characteristics were



(a)



(b)



(c)

Fig. 2: *In vitro* release profiles of microencapsulated Cobra venom, (a) B4 and B6; (b) B6 and B7, and (c) B6 and B8

calculated for the best chosen formulations based on polydispersity index and encapsulation efficiency namely formulations B4, B6, B7 and B8. The results showed that all formulations prolonged the release rate for 14 days. Formula B6 demonstrated the slowest release rate and the maximum amount of released venom reached at the 14<sup>th</sup>

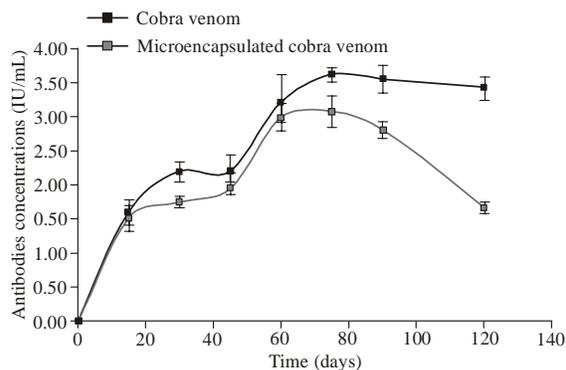


Fig. 3: Evaluation of immune response post free-cobra and micro-encapsulated cobra venom using ELISA at 15 days Intervals

day (Fig. 2). This formula also showed small polydispersity index ( $0.31 \pm 0.01$ ), small average microsphere size ( $5.6 \pm 1.7$ ), encapsulation efficiency of 82% and very low water absorption. Formulations B7 and B8 showed comparable release characteristics to formula B6 and better encapsulation efficiency, however their polydispersity indices and microsphere average size were higher than those for formula B6. All formulations showed Higuchi diffusion release kinetics with  $r^2 > 0.96$ . However the overall release kinetics were governed by the diffusion mechanism, interference with other types of release cannot be excluded. This might be due to adsorption of the venom molecules on the surface of microspheres leading to interference with the release kinetics in many instances.

***In vivo* evaluation of the immune response:** The immune response was developed post immunization of rats using cobra venom either capsulated or not. Antibody level determined in IU/mL was calculated based on the value of locally prepared standard antivenom (5 IU/mL). In Fig. 3, the immune response of cobra venom ( $0.44 \mu\text{g/mL}$ ) in BSA microspheres (B6, 10 mg/mL) was evaluated in comparison to the control free cobra venom solution injection ( $0.44 \mu\text{g/mL}$ ). The vehicle used was PBS (pH 7.4) in both cases. Results represented as means  $\pm$ SD were subjected to non-compartmental analysis using WinNonlin software (version 1.5, Pharsight Corporation, USA). After injection of the microspheres into the lower limb of the male rats, the optical density of the anti-Cobra venom antibodies was measured using ELISA. The maximum serum concentration of the antibodies  $C_{\text{max}}$  was equal to  $3.617 \pm$  IU/mL, whilst for the control, the  $C_{\text{max}}$  concentration was found to be  $3.073 \pm 1$  IU/mL. The terminal half-life of the test formula was  $578.8 \pm 0.8$  days and  $49.07 \pm 3.5$  days for the control. The  $\text{AUC}_{0-\infty}$  of the test formula was  $3159 \pm \text{IU} \cdot \text{Day/mL}$  and the total Mean

Table 5: Pharmacokinetic parameters measured for free and microencapsulated venom following injection into mice

Parameter	Free venom	Micro-encapsulated venom
C <sub>max</sub> (IU/mL)	3.073	3.61700
K <sub>elim</sub> (days <sup>-1</sup> )	0.0141	0.00120
t <sub>1/2</sub> (days)	49.0716	573.862
AUC <sub>0-∞</sub> (IU. day/mL)	375.435	3159.00
MRT (days)	106.066	858.1639

Residence Time (MRT) was 858±1.44 day. On the other hand, the AUC<sub>0-∞</sub> of the control was 375±IU.day/mL and the total MRT was 106±1.7 day. The previous results show that microencapsulation resulted in prolongation of anti-Cobra venom antibodies' concentrations, which was detected from the ratio between the MRT of the test formula to the MRT of the control, which was found to be equal to 7.9. The elimination rate of the tested sample (0.0012 per day) was much lower than that of the control (0.014 per day). It is clear from the results that the microcapsules were successful in modulating the release of cobra venom in plasma. During the first two months following envenomation both formulations showed comparable immune response (Fig. 3, Table 5). Starting from day 60 to day 120, statistically significant differences between amounts of elicited immunity due to microencapsulated formulations and their free venom counterparts were demonstrated (p = 0.04). This behavior may be attributed to stronger potentiation of the immune response obtained from microencapsulated venom due to inclusion of BSA both microsphere former and immune stimulator.

### CONCLUSION

The modified less expensive technique used for production of microspheres has generated a good yield of microspheres. These have got sufficient loading capacity and stability which enabled proper stimulation of immune response with high titre of antibodies over a prolonged period of time reaching 4 months. Therefore the feasibility, economic property, good stability and high yield of microspheres makes it possible for better control of snake bites episodes in low economy endemic areas.

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