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In vitro Release Study of Diphtheria Toxoid Loaded Chitosan Microspheres

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The majority of available vaccines require several booster doses to induce effective immunity and these results in significance compliance problems, particularly in developing world. On basis of this, Tetanus toxoid (TT) has the model antigen encapsulated with in chitosan micropheres by emulsion cross-linking method, with an aim of targeting the drug to the site of inflammation and for controlled release. The *in vitro* release studies of Tetanus toxoid encapsulated chitosan microspheres were evaluated by using limes flocculation test. In this case Tetanus toxoid started to release only on the 8th day and there after showed a pulsed release upto 40th day. The drug was released continuously over 60 d with no burst effect with a maximum release of 83.33 %. A single administration of chitosan microspheres showed better release kinetics when comparated to markedly available vaccine. These results indicated the possibility of drug being released at a controlled rate from chitosan micro spheres and targeted at the size.

Key Words: Limes flocculation, Chitosan microspheres, Tetanus toxoid.

INTRODUCTION

For drug targeting, particulate carrier systems, including liposomes, microspheres, nano particles and aquasomes have been used[1]. Chitosan microspheres are used in parenteral drug delivery to stimulate the memory cells of lymphoid organs. Particles of size ranging from 1-7 μm can be injected through intramuscular, subcutaneous (or) intraperitoneal routes for passive targeting the memory cells[2]. Tetanus is a infective disease to the exotoxin of *Clostridium tetani*, gram positive spore bearing bacteria. The markedly available Tetanus toxoid (TT) vaccine requires several booster doses to induce effective immunity[3]. On the basis of this, the development of single dose controlled release vaccine would eliminate the need for booster immunizations through biodegradable polymers would be a

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significant advance in the efforts to protect individuals against a number of vaccine preventable diseases. In control release technology, natural biodegradable polymeric carriers after potential advantages for prolonged release of macromolecule drugs[4], like polypeptides, hormones, polysaccharides, antigens, antibodies *etc*.

EXPERIMENTAL

Tetanus toxoid (750 Lf/mL), tetanus antitoxin (100 Lf/mL), adsorbed TT was a gift sample from Pasteur institute of India, Coonoor and Chitosan from Marine Institute of Fisheries and Technology, Cochin. All other chemicals and solvents used were of analytical reagent grade and Millipore water was used throughout the study.

Preparation of chitosan microspheres: Tetanus toxoid encapsulated chitosan microspheres were prepared by emulsion cross-linking method. Based on the method followed by Jeffery *et al.*[5] various formulations were prepared by differing chitosan concentration (0.5, 1, 2, 3 %) The 3 mL of above chitosan gels were mixed separately with 2 mL of TT (750 Lf) in a cyclomixer for 10 min. This solution was separately dispersed in the dispersion medium containing 12.5 mL of linseed oil, 12.5 mL of toluene and 1 % of Tween 80. The stirring was continued for 0.5 h. To this 2 mL of 5 % tripolyphosphate solution was added and the stirring was continued at 1700 rpm for 5 h. The formed emulsion was centrifuged by suspending in toluene and acetone at 5000 rpm for 10 min. The same procedure was continued 5 times with toluene and 3 times with acetone. Many batches of microspheres thus obtained were pooled and stored in refrigerator[6].

Drug content in microspheres: The amount of TT encapsulated in micro spheres was estimated by digesting 10 mg of chitosan microspheres in 10 mL of normal saline solution and then this solution was immediately washed. To the pellet, 10 mL of 1 M NaCl was added and kept in refrigerator for 2 d[7]. The micro spheres were separated by centrifugation, supernatant containing the vaccine protein was taken and the potency was estimated by limes flocculation test. To the series of flocculation tubes containing 1 mL of sample solution[8], various volumes of (0.01, 0.02, 0.03, 0.04, 0.05 mL) of saturated antitoxin were added. The volume was made up to 2 mL using normal saline solution. The content in the tubes were mixed properly and kept in the flocculation chamber at 45-50ºC and observed for the most rapidly flocculating mixture.

Determination of the size of the microspheres: Size analysis was carried out using optical microscopic method with the help of calibrated eyepiece micrometer. The size of around 400 particles was measured. Results were tabulated. Average mean diameter was calculated.

***In vitro* dissolution study:** Tetanus toxoid encapsulated chitosan microspheres (50 mg, accurately weighed) were suspended in 50 mL of release medium (phosphate buffer pH-7.4), the flask was agitated at 90-95 rpm in an incubator maintained at 37ºC. From this 5 mL samples were withdrawn at various time intervals, fresh 5 mL of phosphate buffer pH-7.4 being replaced immediately[9]. This was continued for 60 d. The collected samples were centrifuged and supernatant solution was analyzed in terms of limes flocculation.

RESULTS AND DISCUSSION

Among the 4 preparations, maximum yield (86.67 %) was obtained with chitosan microspheres (Table-1). The shape of the tetanus toxoid incorporated chitosan microspheres was found to be spherical (Fig. 1). The size of the chitosan microspheres were found to be ranging between 1.1-2.0 µm.

TABLE-1
CHARACTERISTICS OF TETANUS TOXOID INCORPORATED CHITOSAN MICROSPHERES

Drug incorporated as	Polymer used Chitosan (%)	Sample code	Actual loading Lf/mg	Theoretical loading Lf/mg	% loaded	Particle size in micron	Uniformity	Maximum drug release (%)
Liquid	0.5	TT1	7	13.043	53.66	1.87	More crystal and irregular	70.25
Liquid	1.0	TT2	10	11.538	86.67	1.89	Regular and spherical shape	83.34
Liquid	2.0	TT3	5	9.375	53.33	1.92	Small, smooth surface	71.87
Liquid	3.0	TT4	3	7.894	38.00	1.924	Clumping without spherical shape	68.29

From the *in vitro* release studies, it was observed that the drug was released continuously over a period of 60 d without any burst effect, releasing a maximum of *ca.* 83.3 % from it encapsulated chitosan microspheres. The maximum percentage of drug loading for all the 4 preparations is shown in Table-1. Further, percentage of drug loading was higher in case of 1 % drug loaded microspheres than 2 and 3 %. Thus among all, because of higher yield and drug content, the maximum drug release, chitosan microspheres with 1 % drug loading, containing drug as a solution (TT2) was considered as the best product, the release profile (Fig. 1 and Table-2).

Dissolution profiles of chitosan microspheres with 0.5, 1, 2 and 3 % of drug loading (Fig. 2) containing drug in the form of solution.

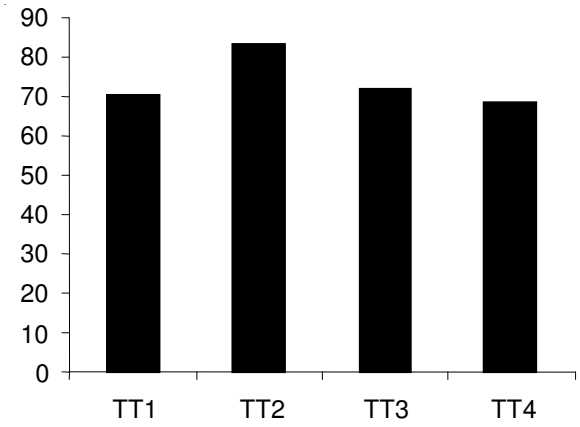

Fig. 1. Chart based on maximum drug release obtained from four batches

TABLE-2
In vitro RELEASE STUDY OF FOUR BATCHES OF TETANUS TOXOID ENCAPSULATED CHITOSAN MICROSPHERE

Days	Cumulative Percentage release			
	TT1	TT2	TT3	TT4
0	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000
8	10.000	25.000	15.230	18.523
12	12.500	27.500	17.520	20.157
16	27.500	52.750	28.230	22.765
20	29.500	55.275	30.180	25.170
24	30.500	55.528	32.750	31.761
30	33.520	55.553	35.170	50.171
34	35.730	80.555	39.560	52.717
40	38.953	83.056	39.560	56.012
45	40.215	83.306	40.261	56.012
50	40.512	83.331	43.516	57.331
55	40.553	83.333	43.518	57.333
60	40.557	83.333	43.520	57.333

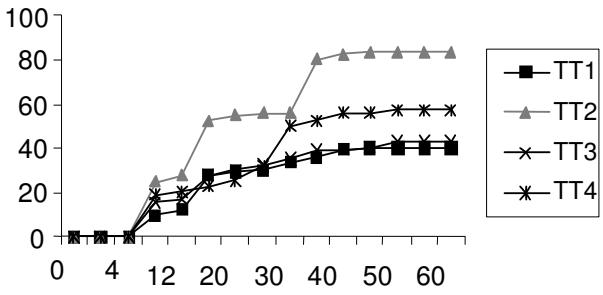

Fig. 2. Dissolution profiles of four batches of chitosan microspheres

From the plots of cumulative percentage release *vs.* number of days, the rate of release, the data fitting was done to zero and first order models for the best product *i.e.* TT2 based on the comparative dissolution profiles. Scanning electron micrograph of chitosan microsphere (TT2) (Fig. 3) taken at a magnification of 1000 times.

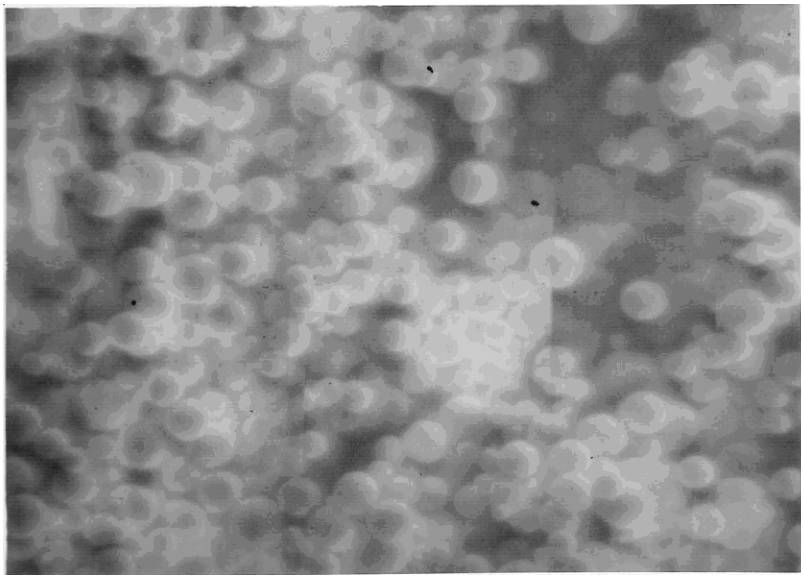

Fig. 3. Scanning electron micrograph of TT encapsulated chitosan microsphere (magnification × 1000 times)

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