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Development of a single dose tetanus toxoid formulation based on polymeric microspheres: a comparative study of poly(D,L-lactic-co-glycolic acid) versus chitosan microspheres

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Abstract

Stable polymeric microspheres capable of controlled release of tetanus toxoid (TT) for periods ranging from days to over months were developed. TT was stabilized, encapsulated in microspheres prepared from poly(D,L)-lactide-co-glycolide (PLGA) and chitosan by using protein stabilizer (trehalose) and its immune response was compared. The influence of co-encapsulated protein stabilizer on tetanus toxoid's stability and release from the microspheres was studied. The protein stabilizer (trehalose) prevented structural losses and aggregation of microencapsulated TT. To neutralize the acids liberated by the biodegradable lactic/glycolic acid-based polymer, we also co-incorporated into the polymer an antacid, ($Mg(OH)_2$), which neutralized the acidity during degradation of the polymer and also prevented TT structural losses and aggregation. The in vitro release experiments with PLGA and chitosan microspheres were performed and the release of TT was increased up to 80–90%. The antigen integrity was investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue staining. The SDS-PAGE analysis confirmed that antigen integrity was not affected by the encapsulation procedure. In addition, the immunogenicity of PLGA and chitosan microspheres based single dose vaccine was evaluated in guinea pigs and compared with multiple doses of alum adsorbed TT. Results indicated that a single injection of PLGA and chitosan microspheres containing TT could maintain the antibody response at a level comparable to the booster injections of conventional alum adsorbed vaccines. The both PLGA and chitosan based stable vaccine formulations produced an equal immune response. Hence chitosan can be used to replace the expensive polymer PLGA. This approach should have potential application in the field of vaccine delivery.

Keywords: Chitosan microspheres; PLGA microspheres; Protein stability; Tetanus toxoid; Single shot vaccine

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1. Introduction

Sustained release of macromolecular drugs from polymeric matrices has received increasing attention in recent years. In view of the fact that many of the future drugs are going to be of recombinant DNA origin. Vaccines that are required to be given in multiple divided doses are not efficacious if only one dose is given without boosting. In developing countries, the drop-out rates from individuals receiving the first dose, but not successive doses is high. In order to be effective, most vaccines require two or three booster doses after primary immunization. Therefore, the conversion of multiple dose vaccines into single dose vaccines may represent an important advance because recombinant proteins are expensive. It should also lead to improved vaccination coverage as well as reduced costs (Aguado, 1993). Among the various tentatives for improving the administration of proteins, the microencapsulation into biodegradable polymers represents a practical and promising approach. The biodegradable and biocompatible polyester, poly (lactide-co-glycolides) (PLGA) and chitosan are the primary candidates for the development of single dose vaccines, because they have been used in humans as suture material and as controlled release delivery systems for peptide drugs (Okada and Toguchi, 1995; Aguado and Lambert, 1992). Microspheres (<10 μm) promote their entry into lymph nodes and provide a high level concentration of antigen over an extended time-period. It also promotes the interaction of encapsulated antigen with antigen presenting cells (APCs) such as macrophages, dendritic cells (DCs) (Eldridge et al., 1991; O'Hagan et al., 1993). Various therapeutic proteins have been formulated using PLGA and chitosan microspheres and some are also undergoing clinical trials (Paul and Sharma, 2000; Illum et al., 2001; Shi et al., 2002; Fini and Orienti, 2003). Various PLGA microsphere formulations are in the market including: Lupron Depot®, Enantone Depot®, Decapeptil® and Parlodel LA®. Though microspheres have high potential for the controlled release of macromolecules, there are some difficulties, which explain to a certain extent, the limited number of formulations in the market. These drawbacks include; low encapsulation efficiency, protein inactivation during the encapsulation process and difficulties for controlling the release of the active protein (Alonso et al., 1993; Schwendeman et al., 1996; Zhu et al., 2000). The prospect of stabilizing antigens and administering primary and secondary doses in a "single shot" by formulating vaccines in these polymers is a promising development that might well revolutionize vaccination in both developed countries and the third world (Perez et al., 2002). The stability of TT was improved by using protein stabilizer (gelatin) (Chang and Gupta, 1996), succinic anhydride (Schwendeman et al., 1996) and albumin, trehalose, etc. (Johansen et al., 1998). In the present study, PLGA and chitosan microspheres based formulations were redesigned by co-encapsulating TT with potential stabilizer such as trehalose and compared the immune response between PLGA and chitosan based TT formulations. PLGA is very expensive polymer when compared with chitosan polymer, which will increase the cost of PLGA based vaccines considerably. In view to reduce the cost, chitosan can be used to replace the expensive polymer (PLGA) since both PLGA and chitosan microsphere based formulations produces an equal immune response. Advances in this line will be illustrated using a model protein ovalbumin (OVA) and the antigen tetanus toxoid (TT). Tetanus toxoid represents an ideal protein that would substantially benefit from the microencapsulation technologies. Moreover, because of its easy availability and characteristics, TT appears as a suitable antigen to attempt alternative approaches to the existing vaccines.

2. Materials and methods

2.1. Materials

Poly(D,L)-lactide-co-glycolide (PLGA) with a lactide/glycolide (L/G) ratio of 50:50 (MW 40,000–75,000), poly vinyl alcohol (PVA) (MW 30,000–70,000) and chitosan were procured from Sigma Chemical Company, USA. Tetanus toxoid (MW 150 kDa) having concentration 1250 Lf U ml^{-1} (Limes flocculation, the international unit for vaccines) and standard tetanus anti-toxin were obtained from Pasteur Institute of India, Coonoor, India. All other chemicals and reagents were of analytical grade and purchased from commercial vendors.

2.2. Preparation of TT-PLGA microspheres

PLGA microspheres were prepared by double emulsion method at room temperature aseptically followed

a method reported by Shi et al. (2002) with minor modifications. In brief, 800 μl of TT containing 1.5% (w/v) trehalose and 2% (w/v) $Mg(OH)_2$ was suspended in 10 ml of 5% (w/v) PLGA in methylene chloride and sonicated for 10 s at 50 W in an ice bath (Branson Sonicator® 250). To this water-in-oil emulsion, 40 ml of 10% (w/v) aqueous PVA was added and mixed at high speed to obtain a w/o/w emulsion with an Ultraturrax T-25 homogenizer for 10 s. The w/o/w multiple emulsion was poured into 50 ml of 0.3% (w/v) aqueous PVA with vigorous stirring for 1 h. The microspheres were collected by centrifugation, washed with distilled water and lyophilized to obtain free flowing powder. The dried microspheres were stored in a sealed glass vial and placed in a dessicator at 4 °C.

2.3. Preparation of TT-chitosan microspheres

Chitosan microspheres were prepared following the techniques reported by Jameela et al. (1994) with minor modifications. Briefly, 1.5% (w/v) aqueous solution of chitosan was prepared in 3% (w/v) acetic acid containing 2% (w/v) sodium chloride. The resulting solution was stirred (Remi Instruments, Mumbai, India) for 1 h at 3000 rpm to form a gel that was kept overnight for stabilization.

A dispersion phase was prepared by mixing 50 ml of toluene and 5 ml of span-80 at 3000 rpm for 10 min. To this dispersion phase, 4 ml of chitosan gel with 1 ml of 0.01% hydrochloric acid and 2 ml of TT solution containing 1.5% (w/v) trehalose were added. At the end of second hour, 5 ml of glutaraldehyde saturated toluene (GST) was introduced drop wise while stirring was continued up to 2 h followed by addition of another 5 ml of GST. The stirring was further continued for 2 h. At the end, the suspension of microspheres were centrifuged and separated. The pellet obtained was washed five times with 5 ml volumes of toluene followed by three times washing with 5 ml volume of acetone. The microspheres were suspended in 10 ml of acetone and dried at room temperature. The dried microspheres were stored in a sealed glass vial in a desiccator.

2.4. Estimation of the TT content in the PLGA microspheres

Ten milligrams of TT-PLGA microspheres was placed in centrifuge tubes and acetonitrile was added to dissolve the polymer (Volkin et al., 1991; Schwendeman et al., 1996). The mixture was vortexed, centrifuged and then the supernatant was withdrawn. Complete extraction was ascertained by treating the particles with acetonitrile for four times and the residual solvent was removed under vacuum. The remaining solid protein was reconstituted in phosphate buffer saline (PBS, pH 7.4, 0.1 M). The total antigen content in the extract was determined by ELISA. The estimation of the TT content in the PLGA microspheres was further validated by exposing mixtures of plain microspheres and free TT to the same extracting conditions. Placebo microspheres (without antigen) containing trehalose and $Mg(OH)_2$ were used as control.

2.5. Estimation of TT content in the chitosan microspheres

Ten milligrams of TT-chitosan microspheres was powdered in an agate mortar and the TT was extracted with 6 ml of PBS (pH 7.4) in screw-capped vials by rotating the tubes head to tail in a haematology mixer for 7 days (Jameela et al., 1994). The total antigen content in the extract was determined by ELISA. Complete extraction was ascertained by further treating the microspheres with 5% (v/v) HCl in ethanol again for another 2 days, if any, by ELISA. The estimation of the TT content in the chitosan microspheres was also validated by exposing mixtures of plain microspheres and free TT to the same extracting conditions. Placebo microspheres (without antigen) containing trehalose were used as control.

2.6. Enzyme-linked immunosorbent assay of tetanus toxoid (ELISA)

The amount of TT was measured by ELISA as described by Johansen et al. (1998) with modifications. Briefly, 96-well flat-bottomed NUNC-Immuno Plates (Inter Med, Denmark) were coated at room temperature overnight with 100 μl of 2 international units (IU) ml^{-1} of horse anti-tetanus IgG in 50 mM carbonate buffer (pH 9.6). The plates were washed three times with 300 μl of PBS containing 0.05% Tween 20. Standard TT with known concentration and samples were diluted at two-fold steps in coated plates using PBS with 0.5% BSA. The plates were held at room temperature for 2 h followed by addition of horse-

radish peroxidase conjugated anti-sheep tetanus IgG (in PBS with 0.5% BSA) at room temperature for 2 h. Finally, 100 μl of 0.2 mg ml^{-1} peroxidase substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonate) in 100 mM NaH_2PO_4 solution was added to the plates. The plates were read on an ELISA reader after 30 min at a wavelength of 410 nm. The concentration of antigenic TT in release medium samples was determined against standard TT using the four parameters logistic equation of ELISAID software (Robert Maciel & Associates, Arlington, MA).

2.7. In vitro release of antigens from the microspheres

The release of antigen from the PLGA and chitosan microspheres was studied in PBS (pH 7.4) containing 0.02% (w/v) Tween-80 (PBST). Several vials containing 10 mg of microspheres and 5 ml of PBST were incubated at 37 °C on a constant shaking mixer. The content of a vial was withdrawn for estimation of released antigen on days 1, 3, 7, 14, 28 and 35. The microsphere suspension was centrifuged at 8000 rpm for 10 min the supernatant was collected and used for protein estimation by ELISA. Placebo microspheres (without antigen) containing trehalose were used as control.

To test whether $Mg(OH)_2$ could neutralize the acidic environment inside the PLGA microspheres, PBS (pH 7.4) medium containing 5 mg PLGA microspheres were incubated at 37 °C for 2 weeks and pH was measured. The degradation half-life of a PLGA microspheres and percentage aggregation was also determined as described by Zhu et al. (2000).

2.8. Estimation of antigen integrity

The extracted TT antigen solution was run on SDS-PAGE gel (Bio-Rad, USA) using standard protocols. A gel was prepared with stacking and separating gels of 4 and 8% polyacrylamide, respectively. Equal concentrations of samples were loaded carefully into the gel wells with the help of a micropipette and it was run at a constant voltage (150 V) until the dye band reached the gel bottom. The gel was stained with a 0.1% coomassie blue fixative solution, destained using an aqueous solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

2.9. Immunogenicity studies

Guinea pigs (350–400 g) were used for in vivo studies. Animals were housed in groups of five ($n = 10$) with free access to food and water. They were deprived of food 3 h prior to subcutaneous (sc) immunization. The Institutional Animals Ethical Committee of Dr. Hari Singh Gour University approved the study. The studies were carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. TT-PLGA microspheres as well as TT-chitosan microspheres based formulations (15 mg) were diluted with 1.0% solution (sterile) of sodium carboxy methyl cellulose in normal saline individually to get a single dose of 0.5 Lf ml^{-1} (1/10 of single human dose) and injected subcutaneously into male guinea pigs. The control group received the equivalent dose of alum adsorbed TT and booster dose was given after 4 weeks of primary immunization. After 2 weeks of gap following administration of booster, animals were bled by cardiac puncture at 0, 15, 30, 45, 60, 90, 120 and 150 days and the serum was separated and used for determination of antibody levels.

2.9.1. Determination of protective antibody levels

The pooled guinea pig serum from each group was assayed by toxin neutralization method (Gupta et al., 1985). Briefly, the pooled sera were diluted to obtain various end points by using normal saline. These mixtures were incubated with tetanus toxin (limes paralyticum (Lp)/10 dose) at room temperature for 40–60 min and injected subcutaneously into LACA mice ($n = 6$ per group) and observed for any paralytic effect up to 4 days (Gupta et al., 1985). The anti-TT antibodies were also determined by ELISA. To determine the presence of anti-TT antibodies in guinea pig sera, 50 μl of TT (3.3 Lf ml^{-1}) were fixed onto 96-well flat-bottomed NUNC-Immuno Plates (Inter Med, Denmark) in 0.1 M borate buffer (pH 8.3), overnight at 4 °C. Wells were blocked with PBS containing 1% BSA (PBS-BSA) for 1 h and washed with PBS containing 0.05% (v/v) Tween 20, the coated wells were incubated for 1 h at 37 °C with serial dilutions of guinea pig serum. Bound IgG was detected by addition of goat anti-guinea pig IgG (diluted 1:1000) in PBS-BSA coupled to horse-radish peroxidase (Bio-Rad lab,

Richmond, CA). The final reaction was visualized by incubation with the peroxidase substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) (Sigma, St. Louis, MO, USA). The resulting absorbance was measured at 405 nm. The titer of anti-TT antibodies in serum samples was obtained by using the four parameters logistic equation of ELISAID software (Robert Maciel & Associates, Arlington, MA).

2.10. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.) for each group of guinea pig. Statistical analysis was made with SPSS 10.1 for windows® (SPSS®, Chicago, USA).

3. Results and discussion

3.1. Physical characterization of TT-PLGA and TT-chitosan microspheres

PLGA and chitosan microspheres were prepared by double emulsion method and emulsion cross-linking method, respectively. The surface of these microspheres as observed under scanning electron microscopy (SEM) (JEOL 6100, Japan) (Figs. 1 and 2) was free from any pores or cracks. The actual size of individual microspheres was in the range of 1–10 μm and few microspheres were measured of size up to 50 μm. Microspheres that are less than 10 μm in size are reportedly taken up by the APCs (macrophages and dendritic cells) and thus considered to be most suitable for optimum adjuvant effect (Eldridge et al., 1991; O'Hagan et al., 1993). Microspheres had earlier been standardized using ovalbumin (OVA) as a model protein to obtain uniform and reproducible batches of microspheres (results not shown). A 1.5% (w/v) solution of chitosan was selected, which was suitable for making small microspheres (10 μm). Similarly, the PLGA polymer concentration was selected on the basis of the size of microspheres. A 4–6% (w/v) polymer solution was found to be ideal for making small microspheres (<10 μm). The loading efficiency for both PLGA and chitosan microspheres containing trehalose varied from 80 to 90% as determined by ELISA, whereas, 30–40% of TT payload was observed with PLGA and chitosan microspheres without trehalose. This could be due to inactivation of the TT at w/o interface (first emul-

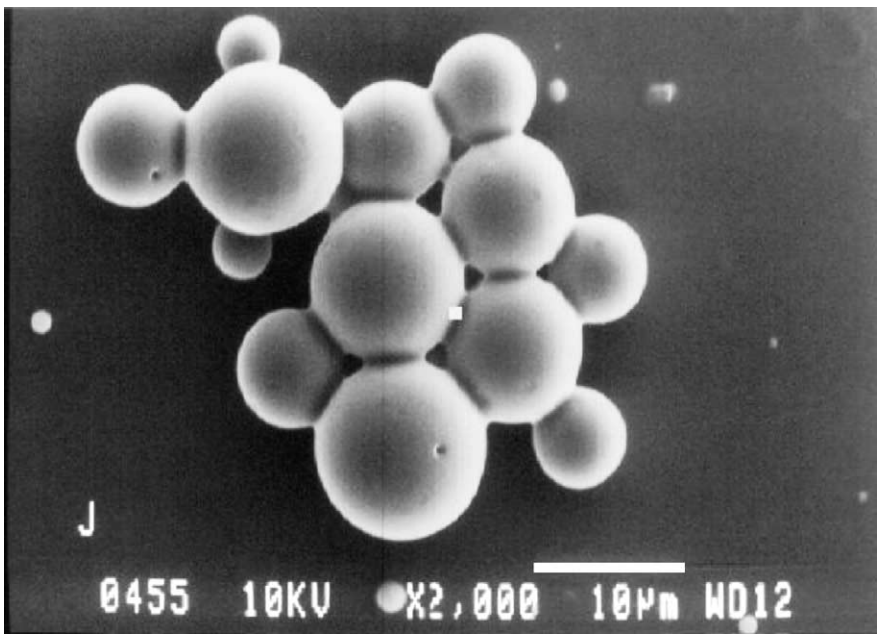

Fig. 1. SEM photograph of stabilized TT-PLGA microspheres.

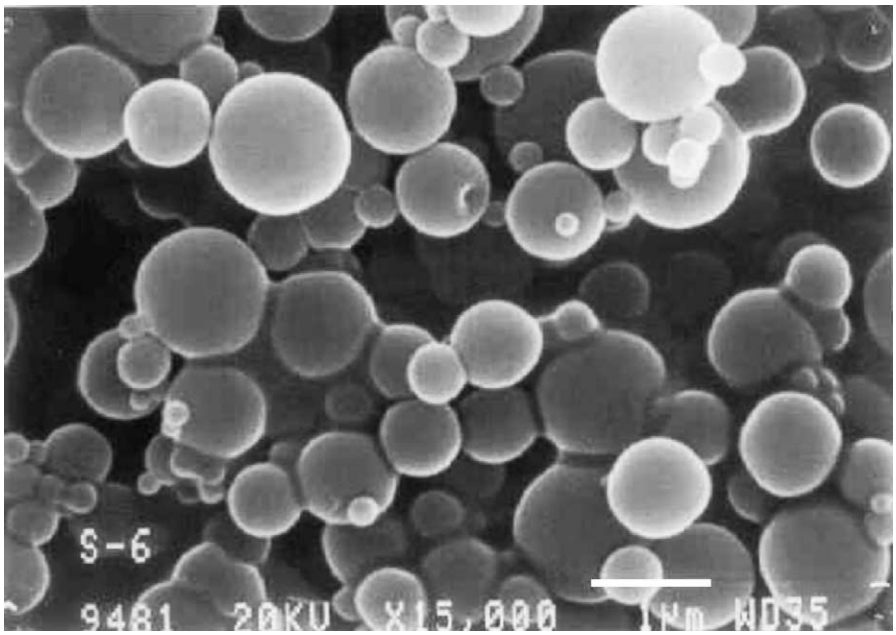

Fig. 2. SEM photograph of stabilized TT-chitosan microspheres.

sification step). Since, protein stabilizer (trehalose) has restrained the denaturation at w/o interface, the payload of TT was also increased (Cleland and Jones, 1996).

The hydrolysis of PLGA releases lactic and glycolic acids, which may produce acidic pH < 3 inside the microspheres (Mehta et al., 1994; Park et al., 1995; Brunner et al., 1999). In order to neutralize the acidic pH, an antacid ($Mg(OH)_2$) at different concentrations (0.5, 1.0, 1.5 and 2.0%, w/v) was incorporated into the PLGA microspheres, which prevented TT structural losses and aggregation for long time (Heller, 1990). To confirm the neutralization of acidic environment by $Mg(OH)_2$, the percentage aggregation, PLGA degradation and pH of release medium were examined (Table 3). As predicted, as more $Mg(OH)_2$ (2.0%) was incorporated into PLGA microspheres, percentage aggregation was shifted from 65 to 1.5%, degradation half-time was extended from 14 to 32 days and the pH value of the release medium dropped from 7.4 to 7.0, whereas pH 3.5 was noticed without $Mg(OH)_2$ (Table 3). These results suggested that the acidic environment inside the PLGA microspheres was successfully neutralized, when $Mg(OH)_2$ was co-encapsulated in the PLGA microspheres.

Table 1
In vivo TT immune responses to various species of mice model

Serial No.	Species of mice	STD tetanus anti-toxin dose ($IU\,ml^{-1}$)	
		1.0	2.0
1	LACA	1.05 ± 0.03	2.0 ± 0.02
2	Swiss albino	0.96 ± 0.03	2.01 ± 0.03
3	BALB/c	Death	Tetanic paralysis

The preparation of PLGA and chitosan microspheres needs the use of discordant solvents that may degrade the proteins. The microspheres when exposed to physiological environments (temperature, pH and osmotic strength) may also destabilize the protein molecules (Aguado and Lambert, 1992; Esparza and Kissel, 1992; Alonso et al., 1993, 1994; Crotts and Park, 1998; Perez et al., 2002). It is thus necessary to stabilize the protein during both encapsulation process and release from the microspheres. Therefore, the microspheres were co-encapsulated with potential protein stabilizer, trehalose. It was also hypothesized that protein stabilizer can shield the antigen from the organic solvent via preferential hydration of their surface, thus preventing protein-interface exposure to deleterious solvent effects.

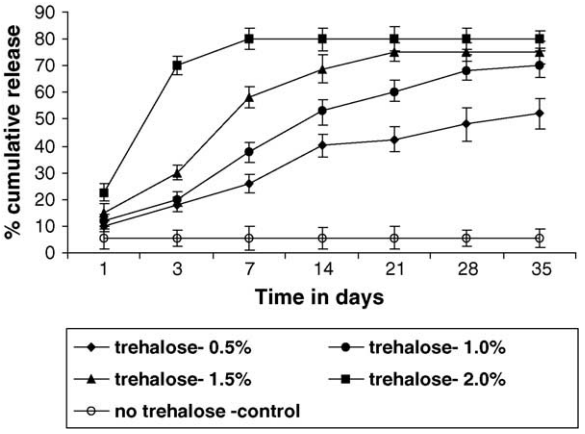

Fig. 3. In vitro cumulative release of TT antigen from PLGA microspheres stabilized with 0.5, 1.0, 1.5 and 2.0% trehalose.

3.2. In vitro release of antigens from the microspheres

The TT release pattern from the PLGA and chitosan microspheres stabilized with trehalose at different concentrations (0.5, 1.0, 1.5 and 2.0%, w/v) was compared with microspheres without trehalose. TT-PLGA and TT-chitosan microspheres without trehalose released only 5.5 and 4.5% of the loaded TT, respectively, and no further release was recorded up to 35 days (Figs. 3 and 4). This could be due to the antigen inactivation or aggregation at w/o interface (first emulsion step) (Perez et al., 2002). So, the best approach is to prevent interface-induced TT denaturation and aggregation is addition of protein stabilizer (trehalose), which will shield the TT from the organic solvent via a preferential hydration of their surface, thus preventing protein-interface contacts (Cleland and Jones, 1996). Since, protein stabilizer (trehalose) has restrained the denaturation at w/o interface, the payload of TT was increased, which is reflected in augmented cumulative percent release. Moreover, trehalose is having appreciable solubility in aqueous media. They dissolve rapidly from matrix leaving porous matrix, which in turn releases antigen/bioactive relatively faster. As trehalose (protein stabilizer) concentration increased from 0.5 to 2.0% (w/v) during encapsulation, release of TT was also increased up to 80–90% in TT-PLGA and TT-chitosan microspheres. However, a higher concentration of trehalose (2.0%, w/v) increased the initial burst of antigen i.e., >70% of TT was released within 3 days. But microspheres stabilized with 1.5% trehalose increased the release of TT slowly up to 35 days as compared to higher concentration (2.0%) (Figs. 3 and 4). Therefore, we chose a 1.5% trehalose for further studies and provided better stable environment for TT during encapsulation and subsequently during its release from the system. Other amphipathic excipients (protein stabilizers), such as bovine serum albumin (BSA), mannitol and sucrose were also co-encapsulated in our previous experiments and only trehalose was useful in maintaining protein integrity and also better release of the antigen from microsphere formulations (results not shown).

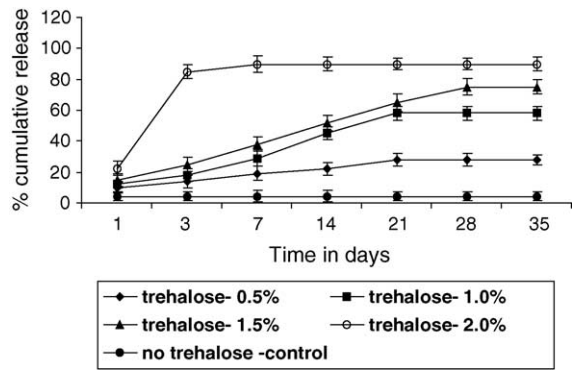

Fig. 4. In vitro cumulative release of TT antigen from chitosan microspheres stabilized with 0.5, 1.0, 1.5 and 2.0% trehalose.

3.3. Antigen integrity

The antigen integrity of TT antigen before and after encapsulation was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis followed by coomassie brilliant staining. The antigen integrity of the TT within microspheres containing trehalose was intact as shown by SDS-PAGE of the antigen before and after microencapsulation (Fig. 5). But the antigen integrity of the TT without trehalose was altered after microencapsulation, which may be because of antigen unfolding or aggregation at the o/w interface. These results provided a concrete evidence that protein stabilizer (trehalose) has the role in preventing the inactivation of TT antigen during the process of encapsulation (Cleland and Jones, 1996).

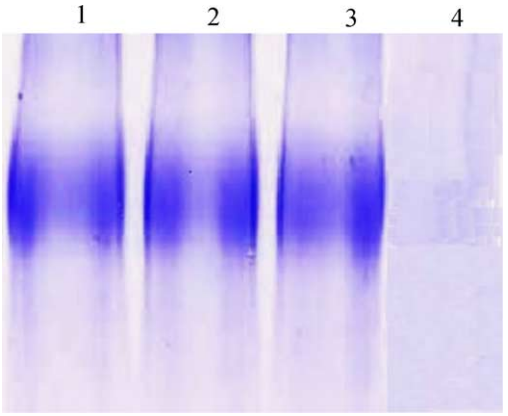

Fig. 5. SDS-PAGE analysis of TT; lane 1: standard TT antigen; lane 2: TT antigen extracted from PLGA microspheres;lane 3: TT antigen extracted from chitosan microspheres; lane 4: TT antigen extracted from microspheres containing no trehalose.

3.4. Immunogenicity studies

Dose-dependent response was studied by immunizing guinea pigs with various doses of alum adsorbed TT (0.3, 0.5, 0.7, 0.9 and 1.1 Lf/dose) and the antibody levels were measured after 2 weeks of booster dose. The results indicated that 0.5 Lf/dose triggered highest levels of antibodies while immunization with >0.5 Lf/dose showed decreasing antibody production (Fig. 6). Thus 0.5 Lf/dose was used for the immunization schedule. Lp/10 dose was determined (Gupta et al., 1985), which is the smallest quantity of the test toxin, which when mixed with 0.1 unit of the standard anti-toxin and injected subcutaneously into mice caused tetanic paralysis within 4 days. The same dose was used to challenge the animals. The serum samples from immunized guinea pigs were titrated for tetanus anti-toxin against Lp/10 dose by toxin neutralization method as per the method discussed under immunogenicity studies in various species of mice models such as LACA, Swiss albino and BALB/c. The Lp/10 dose of toxin was mixed with known units of standard anti-toxin (1 and 2 IU ml^{-1}) and incubated for 40–60 min and injected into LACA, Swiss albino and BALB/c mice models. Then the mice were observed for tetanic paralysis. Results suggested that LACA and Swiss albino mice model responded to real anti-toxin levels (1 and 2 IU ml^{-1}), whereas BALB/c suffered tetanic paralysis even on injecting 2 IU ml^{-1} of tetanus anti-toxin (Table 1). Therefore, LACA and Swiss albino mice models can be utilized for the tetanus toxoid vaccine studies.

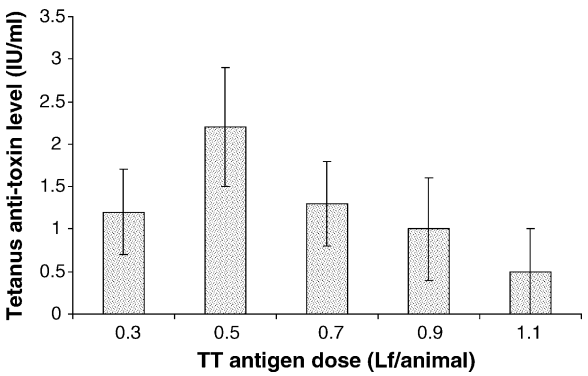

Fig. 6. Dose–response curve to alum adsorbed TT.

The potency of stabilized tetanus toxoid microspheres formulation was determined by assessing the efficacy of the vaccine to stimulate the production of tetanus anti-toxin in guinea pigs. The serum of the guinea pigs was examined for anti-toxin activity using toxin neutralization method by comparing their ability to protect the mice from the paralytic effects when a fixed dose of tetanus toxin (limes paralyticum/10 dose) was administered and compared with the dose of standard preparation of tetanus anti-toxin required to provide the same degree of protection (Gupta et al., 1985). The presence of specific anti-TT antibodies in the sera was further confirmed by ELISA. The data shown in Table 2 and Fig. 7 indicate that single

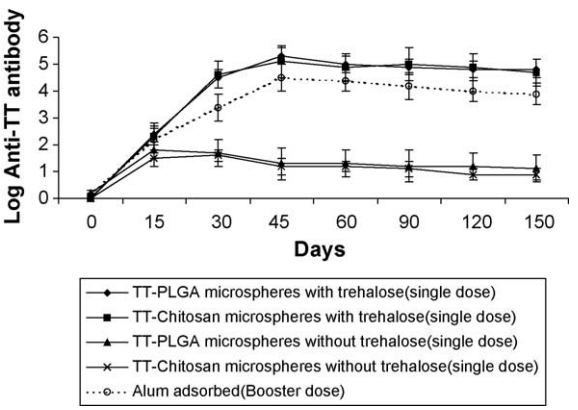

Fig. 7. Anti-TT antibodies response of TT-PLGA and TT-chitosan microspheres based formulations.

Table 2
In vivo antibody levels of different formulations of TT encapsulated into PLGA, chitosan microspheres and alum- adsorbed vaccine

Serial No.	Formulations	Tetanus anti-toxin (IU/ml^{-1}) (value ± S.D.)							
		Days							
		0	15	30	45	60	90	120	150
1	TT-PLGA microspheres with trehalose (single dose)	–	–	1.08 ± 0.04	3.06 ± 0.03	4.06 ± 0.03	4.15 ± 0.08	4.11 ± 0.4	3.06 ± 0.03
2	TT-chitosan microspheres with trehalose (single dose)	–	–	1.3 ± 0.09	2.15 ± 0.05	4.15 ± 0.07	4.05 ± 0.03	3.5 ± 0.07	3.05 ± 0.07
3	TT-PLGA microspheres without trehalose (single dose)	–	–	0.45 ± 0.05	0.43 ± 0.04	–	–	–	–
4	TT-chitosan microspheres without trehalose (single dose)	–	–	0.483 ± 0.01	–	–	–	–	–
5	Alum adsorbed (booster dose)	–	–	1.18 ± 0.09	2.11 ± 0.07	4.06 ± 0.033	3.11 ± 0.03	3.08 ± 0.07	2.05 ± 0.02

injection of TT-PLGA and TT-chitosan microspheres with trehalose resulted into an immune response comparable or better than the multiple injections of alum adsorbed TT. Microspheres formulated without trehalose showed very poor production of antibody titer. This effect may be attributed to the denaturation of tetanus toxoid during the process of microencapsulation (Aguado and Lambert, 1992; Alonso et al., 1993; Esparza and Kissel, 1992; Cleland and Jones, 1996; Crotts and Park, 1998; Perez et al., 2002). The results suggested that a single dose of stabilized TT-PLGA and TT-chitosan microspheres could generate an antigen depot from where the toxoid may gradually release at the site of injection. Moreover, the antibody titre generated with a single injection of TT-PLGA and TT-chitosan microspheres stabilized with trehalose was significantly higher than the booster doses of alum adsorbed vaccine. Hence, these stabilized TT-PLGA and TT-chitosan microspheres can open the door for the practical development of single-shot vaccines. The adjuvant activity of PLGA microspheres and chitosan microspheres was found to be comparable. But PLGA is very expensive polymer when compare with chitosan, which will increase the cost of PLGA based vaccines considerably. In view to reduce the cost, chitosan could be used to replace the expensive polymer PLGA since both PLGA and chitosan microsphere based formulations produced an equal immune response.

Table 3
Neutralization effect of $Mg(OH)_2$ on the behavior of TT-PLGA microspheres

Property	No $Mg(OH)_2$	$Mg(OH)_2$
Aggregation (%)[a]	65 ± 6	1.5 ± 0.5
PLGA degradation $t_{1/2}$ (days)[b]	14	32
pH of the medium[c]	3.5	7.0

[a] TT was extracted from microspheres after incubation in PBS (pH 7.4) at 37 °C for 2 weeks (mean ± S.E.M., $n = 4$).

[b] $t_{1/2}$ is the time when the PLGA Mr was reduced to half of the original Mr (determined by GPC) during incubation in PBS (pH 7.4) at 37 °C.

[c] PBS (pH 7.4) medium containing 5 mg polymer microspheres after incubation at 37 °C for 4 weeks.

4. Conclusions

The aim of this study was to stabilize, encapsulate and compare the potency of TT in PLGA and chitosan microsphere based formulations. TT was successfully encapsulated in microspheres with the help of protein stabilizer and stable TT was recovered upon release from microspheres. Protein stabilizer has the role in preventing the inactivation of antigen during the process of encapsulation and its release. A single-shot injection of stabilized TT-PLGA and TT-chitosan microspheres resulted in good tetanus anti-toxin levels, which mimiced booster injections of alum-formulated vaccine. The results indicate that TT encapsulated microspheres have potential application as a vaccine delivery module. Moreover, chitosan can be used to replace the expensive polymer PLGA. This technology, if successful, may also be applied to other vaccines such as DPT and hepatitis-B vaccine.

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