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Chitosan Microparticles as Oral Delivery System for Tetanus Toxoid

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Systemic and local immune response against Chitosan encapsulated tetanus toxoid (CS-TT) microparticles is studied, prepared by ionic cross-linking using Sodium Tripolyphosphate (STPP). Final formulation was evaluated in terms of release of TT in 0.1 N HCl and PBS (pH 7.4), sedimentation profile and stability. CS-TT microparticles, TT in PBS and plain CS microparticles were orally administered to mice and TT (adsorbed) was administered through intramuscular route. Sera were analyzed for anti-TT IgG and intestinal lavage, faeces, intestinal washings for anti-TT IgA levels using an ELISA. Entrapment efficiency of about 100% was obtained. A dose dependent immune response was observed in mice vaccinated with Chitosan-TT microparticles. A strong enhancement of the systemic and local immune response against TT were found when compared with oral feeding of TT in PBS. The study shows the efficacy of chitosan microparticle suspension system, containing a high molecular protein (TT), in inducing the IgA in intestine and IgG in systemic circulation. This demonstrates that chitosan microparticles can prove to be a promising oral vaccine delivery system for mucosal and systemic immunity.

Keywords chitosan; tetanus toxoid; mucosal and systemic immunity

INTRODUCTION

The oral route of delivery is most attractive and acceptable, but is also the most challenging and difficult for proteins, peptides and other high molecular weight molecules. Hence, due to factors like the degradation of the antigen in the gastrointestinal (GI) tract and low uptake by the gut associated lymphoid tissue (GALT) there is considerable interest in the development of novel delivery systems for oral administration of vaccines, which can be used to package and deliver a range of antigens from important pathogens. Mainly for reasons of safety, it would be desirable if these novel delivery systems were based on non-living carrier systems, rather than modified bacterial or viral vectors (O'Hagan, 1998).

Peyer's patches (PPs) are the main target for oral vaccine, which are present in the lower ileum (Lubben, 2003). The use of polymeric microparticles offers significant potential for the development of orally administered vaccines. Microparticles can be prepared from a range of different polymers, which can be designed to protect entrapped vaccine against degradation, to delay the gastric transit of the vaccine or to target vaccines for uptake into the mucosal associated lymphoid tissues (MALTs) of the Peyer's patches (PPs). The antigen incorporated into particulates will induce antigen-specific immune response to a greater extent than an antigen in the water soluble form. The particle-incorporated form protects them from degradation by low pH of the stomach and proteolytic activity of enzymes and bile salts in the gut, and additionally enhances the antigen uptake via phagocytic M cells (Lubben, 2003). After transport of the microparticles to the dome of the PPs, the microparticles are degraded and the vaccine is released into the lymphoid tissue. Following stimulation by an antigen in PPs and its presentation to B- and T-cells, the antigen induces B- and T-cells proliferation and these cells subsequently leave the PPs via efferent lymphatics and reach the systemic circulation through the thoracic duct (Damge, 2000; Lubben, 2003; O'Hagan, 1998; Tabata, 1996). Eldridge suggested that larger particles (5–10 μm) remained in the PPs while smaller particles (<5 μm) were transported systemically in the lymph (Eldridge, 1990).

Several types of microparticles have been demonstrated to significantly enhance the systemic and/or mucosal immune response after mucosal vaccination (Cho, 1998; Kim, 1999). In recent years, the principal polymers used for the preparation of

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microencapsulated vaccines have been the aliphatic polyesters, the poly(lactide-co-glycolides) (PLGs). However, they have several disadvantages (Barackman, 1998; Kim, 1999; Peyre, 2003). Antigen incorporation requires the use of organic solvents, which may denature the antigen. In addition, during microencapsulation, vaccine antigens may also be exposed to high shear stress, aqueous-organic interfaces, and elevated temperature making it unsuitable for the vaccine delivery systems (O'Hagan, 1998). Upon hydrolysis of the polymers, the pH decreases strongly, which also may deteriorate the antigen (Lubben, 2003).

It has been reported in our previous studies that Chitosan solution in 1% acetic acid, Sodium tripolyphosphate, Propylene glycol, Glycerol are compatible with TT when analyzed using Rocket Electrophoresis technique (Ahire, 2006). It has been shown that chitosan microparticles are able to entrap large quantities of antigens (Alpar, 1998; Lubben, 2001; Lubben, 2003). Chitosan has favorable biological properties such as biodegradability and biocompatibility. Chitosan has low oral toxicity with LD50 of 16 g/kg in rats (Radi, 2003; Singla, 2001).

The incidence of tetanus in the third world countries is quite high. Tetanus is still a major health problem in developing countries and continues to occur in countries with high medical standard (Coppi, 2002). In countries where primary vaccination has been carried out for years tetanus is mainly observed among elderly and non-immunized (Simonsen, 1987). Tetanus kills on an average 140 times more individuals in poor developing countries than in rich developed countries. Currently, in the world neonatal deaths reported are about 200,000 per year, in spite of the fact that an economical, effective, and safe prophylactic agent has been available for more than 50 years! There are about 57 countries where tetanus is considered as a one of the major disease. India reports maximum number of cases in the world every year, and leading the list of class "C" countries which are declared as countries wherein tetanus is still a major issue as per UNICEF (Wassilak, 2002).

Prevention of tetanus can be achieved by the use of a formaldehyde-inactivated tetanus toxin, i.e., tetanus toxoid, which induces production of neutralizing antibodies (Esparaza, 1992). The immunological response to TT requires more than one dose to confer protection and persistent immunity. Many difficulties exist for delivering repeated injections to induce a strong and lasting immune response. In particular, the population at highest risk lives mainly in isolated rural areas with poor access to health care services. At present, Tetanus toxoid is administered through i.m. route and should be stored at 4–8°C. Therefore, formulation, which does not require cold storage condition and which can be self-administered, would prove to be beneficial (Esparaza, 1992).

The current studies were performed to investigate the potential of chitosan microparticles for oral mucosal vaccine delivery. The induction of systemic and local immune responses after oral administration of Tetanus Toxoid (TT) associated to chitosan microparticles were investigated. Mice were orally vaccinated with TT loaded microparticles; both IgG titres in serum and IgA titres in faeces were determined. The extent of immune response from the formulation was compared with plain TT to assess improvement in oral mucosal and systemic immunity.

MATERIALS AND METHODS

Chitosan (CS) was a gift sample from Central Institute of Fisheries Technology, Cochin and used without any modification and purification (Molecular weight ~600,000 Daltons, Degree of deacetylation > 85%, pH of 1% solution in 1% acetic acid-4.5 and viscosity-132cP).

Sodium tripolyphosphate (STPP) was purchased from National Chemicals, Baroda. Tetanus toxoid (TT, 3840 Lf/mL) and Primary antibody [IgG, horse polyclonal against TT, 500 IU/mL] was a generous gift from Serum Institute of India Ltd., Pune.

Ethylenediaminetetra-acetic acid (EDTA), propylene glycol, glacial acetic acid, sodium chloride, sodium sulphate, potassium chloride, sodium bicarbonate, polyethylene glycol (PEG) were purchased from SD fine chemicals, Mumbai.

Alkaline phosphatase conjugated anti-mouse IgG, anti-horse IgG and anti-mouse IgA; Tween 80, bovine serum albumin (BSA), *p*-nitrophenyl phosphate (*p*NPP) were purchased from Sigma, St. Louis, MD, USA.

Soybean trypsin protease inhibitor was purchased from Sisco Research Lab, Mumbai and Phenylmethane suphonyl fluoride (PMSF) was purchased from HiMedia Labs, Mumbai. Flat bottomed 96 well ELISA plates (Nunc-Immuno™ with Maxisorb™ surface) were purchased from Nunc, Denmark. Bio-Rad Microplate washer, BioRad Microplate Reader 550 was used to read ELISA plates and data was analyzed using Microplate Manager™ software. All other reagents were of analytical grade.

Preparation of Plain Chitosan Microparticles

Chitosan solution, 2 mL of 2%, was diluted upto 40 mL with MilliQ water and to this 1 mg/mL solution of STPP was added drop-wise till the solution turned turbid indicating crosslinking and microparticle formation. The formation of microparticles was verified microscopically. The exact quantity of STPP required to crosslink CS was optimized.

Optimization of Amount of STPP to Cross-link CS

CS amount was fixed at 40 mg and optimum amount of STPP required to cross-link CS was determined by measuring change in percent transmittance (%T) at 600 nm using Shimadzu-1601 UV-Visible Spetrophotometer. pH was monitored during the reaction using Global digital pH meter, DPH-500. Chitosan (40 mg) was taken from stock solution of chitosan (2% in 1% acetic acid MilliQ water) and volume was made up

to 40 mL to get 1 mg/mL solution using MilliQ water. To this, 1 mL of STPP 2 mg/mL solution was added and allowed to react for 10 min under magnetic stirrer. After 10 min, %T was measured. The step was repeated till %T became constant. %T was plotted against the amount of STPP added.

Confirmation of CS-STPP Complexation Using FTIR

FTIR was taken to confirm the formation of complex between CS and STPP. After completion of complexation of CS with STPP, the dispersion was kept for freezing at –70°C in Remi Deep Freezer for 24 h and then lyophilized using Heto Drywinner Lyophilizer, Denmark. FTIR of the lyophilized powder and Chitosan powder was taken using Shimadzu FTIR spectrophotometer. FTIR of Chitosan powder was overlapped with Chitosan-STPP (CS-STPP) complex and shift or absence of peaks was examined.

Estimation of Tetanus Toxoid (TT) Using ELISA

Modified ELISA method was used for the estimation of TT (Chang, 1996). TT standard curve was prepared by adsorbing flat bottomed 96 well ELISA plates (Nunc-Immuno™ Plate) with two-fold dilutions of TT in 50 mM bicarbonate coating buffer of pH 9.6 and incubated at 37.5°C for 1 h. Standard curve for TT was in the concentration range of 0.0012 to 0.0386 Lf/mL. Plates were washed four times with PBS-T-0.05% washing buffer using Bio-Rad Microplate washer and blocked using 0.5% BSA in 10 mM PB, incubated for 1 h at 37.5°C followed by washing. To this 1:5000 dilution of primary antibody [IgG, horse polyclonal against TT] was added and incubated for 1 h at 37.5°C followed by washing cycle. Secondary antibodies, [antihorse IgG conjugated with alkaline phosphatase] 100 µL per well were added and incubated followed by washing cycle. To this 100 µL of p-Nitrophenyl phosphate was added and incubated at room temperature for 30 min in dark. Plate was read at 450 nm. Standard calibration plot was prepared during every analysis to compensate for the variation due the binding of the TT with the plate. The unknown samples, suitably diluted, were estimated with respect to the standard curve. The plot of optical density at 450 nm versus concentration of TT of 0.0012 to 0.0386 Lf/mL showed linear relationship with $r^2 > 0.99$.

Preparation of TT Loaded Microparticles

TT loaded microparticles were prepared by adding 1000 Lf TT to chitosan solution. To this 1 mL STPP was added at every 10 min. Different variables like viscosity of reaction medium, pH of reaction medium, and rate of addition of STPP were optimized in terms of % entrapment.

Optimization of Experimental Conditions for Entrapment of TT

The ratio of Chitosan to STPP was optimized to 1.16:1 (w:w). Other variable formulation conditions were optimized so as to entrap 1000 Lf of TT per batch. Phase A was prepared by taking 2 mL of CS from stock solution. To this, TT (1000 Lf) solution was added and volume was made up to 40 mL. To Phase A, 1 mL of 2.8 mg/mL solution of STPP was added after every 10 min. Entrapment efficiency was calculated by measuring the unentrapped TT from supernatant obtained after centrifugation using ELISA method.

Effect of Viscosity

Effect of viscosity of the reaction medium on entrapment was studied using following solvent compositions into which CS was added: Water, Propylene Glycol (PG), Glycerol, Water:Glycerol (50:50), Water:PG (50:50). Final volume of medium was 50 mL (40 mL CS + 10 mL STPP).

Volume of Water

Volume of water was optimized by determining encapsulation efficiency at different volumes i.e., 25 mL (15 mL CS + 10 mL STPP), 50 mL (40 mL CS + 10 mL STPP), and 60 mL (50 mL CS + 10 mL STPP) of the final reaction medium volume.

Effect of pH

Effect of pH on the entrapment of TT was checked at pH 4.5, 6, and 7. The pH of the reaction medium was adjusted to pH 6 and 7, using 1% sodium carbonate solution.

Effect of Rate of STPP Addition

Effect of rate STPP addition was checked at four levels: 1 mL per min, 5 mL per min, 1 mL per 5 min and 1 mL per 10 min after mixing chitosan and TT.

Method of Preparation of Chitosan-TT Microparticles

Ratio of Chitosan (CS) to Sodium tripoly phosphate (STPP) was taken as 1.16:1 (w/w). Following method was used to achieve the required particle size distribution

Phase A: CS-80 mg
TT-1500 Lf,
MilliQ water qs 100 mL
Phase B: STPP-56 mg,
MilliQ water qs-20 mL

Phase A was allowed to mix at 1000 rpm for 20 min on magnetic stirrer. To phase A, phase B was added drop wise, under continuous stirring, at the rate of 1 mL per 5 min. After completion of addition, the system was allowed to cure for 20 min under continuous stirring. After centrifugation using Remi K-70, industrial centrifuge, at 1200 rpm for 15 min, the supernatant was removed carefully. The sediment was redispersed in 20 mL propylene glycol and proper mixing was ensured. This suspension was passed through Emulsiflex C-5 microfluidizer (EC-5), two cycles without pressure, to ensure proper mixing. After cooling the system for 10 min at 4°C, it was again passed through EC-5, 2 cycles at 5000 psi pressure. The product

obtained was stored at 4°C for 15 min and to this 5 mL of 2% Sodium carboxymethylcellulose was added and processed using CAT-560 silverson type homogenizer for 12 min at 4500 rpm on an ice bath. Particle size distribution was studied using Malvern particle size analyzer, UK. Entrapment efficiency was calculated by measuring the unentrapped TT from supernatant obtained after centrifugation using ELISA.

Release Study of TT in 0.1 N HCl and Phosphate Buffered Saline (pH 7.4)

From final batch containing 1500 Lf TT, 1 mL was taken and added in 5 mL of 0.1 N HCl or PBS and placed in incubator shaker at 100 rpm and 37.5°C. Samples were withdrawn after 30, 60, 120 min and replaced by fresh 0.1 N HCl or PBS. Withdrawn samples were suitably diluted with 50 mM bicarbonate coating buffer (pH 9.6) and analyzed for TT using ELISA.

Oral Vaccination Studies

Local and systemic immune responses were measured in 5–6 weeks old BALB/c mice weighing 15–20 g. The Ethical Committee for Laboratory Animals of The M.S. University of Baroda, Gujarat, India, approved all animal experiments.

Systemic immune response was determined by measuring the IgG level in serum and local immune response was determined by measuring IgA level in fecal matter extracts. Following procedures were used.

Assessment of Dose Response Relationship for Systemic Immunity

Seven animal groups, each group containing five BALB/c mice were taken. First 3 groups were treated with 0.5mL of chitosan microparticle formulation containing TT 20, 40, 60 Lf, respectively; the next three groups with plain TT 20, 40, 60 Lf, respectively through oral route. Dosing was done on 1st, 2nd, 3rd, and 15th, 16 th, 17th day using oral feeding needle. In Seventh group 10 Lf TT adsorbed on aluminium phosphate was administered through *i.m.* route. Food was withdrawn 6 h before administration of the dose but water was available ad libitum. Blood was withdrawn from all animals on retro-orbital plexus on 22nd day, allowed to clot, centrifuged at 14000 rpm to remove the sera and stored at –20°C (Lubben, 2003).

Assessment of Systemic Kinetic Immune Response

Three animal groups, each containing 10 BALB/c mice were treated with chitosan microparticles containing 40 Lf TT; plain chitosan microparticles; and plain 40 Lf TT respectively, 0.5 mL of the above were administered orally on 1st, 2nd, 3rd, and 15th, 16th, 17th day using modified oral feeding needle (Lubben, 2003). Blood was withdrawn from retro-orbital plexus on 14th, 22nd, and 29th day and sera was collected and stored at –20°C.

Assessment of Local Immune Response

Two animal groups, each group containing 10 BALB/c mice were taken. In first group chitosan microparticles containing 40 Lf TT; in second group plain 40 Lf TT were administered orally on 1st, 2nd, 3rd and on 15th, 16th, 17th day. Food was withdrawn 6 h before administration of the dose but water was available ad libitum. Faeces (5 freshly voided pieces, in the morning) was collected on 4th, 8th, 14th, and 22nd day and incubated for 15 min in 4 mL of homogenization buffer (50mM EDTA, 1% BSA, 0.1 mg/mL soybean trypsin inhibitor, and 2 mM PMSF in PBS, pH 7.3) on ice, followed by mashing it with a blunt needle. The supernatants, obtained by centrifuging the suspension for 25 min at 13000 rpm at 4°C, were stored at –20°C until tested (Forrest, 1992; Grewal, 2000).

Hyperimmunization of Mice for Higher Titre of TT Specific IgG

Ten BALB/c mice were hyperimmunized by administering through intraperitoneal route 10 Lf/mL TTadsorbed on aluminium phosphate on 1st, 2nd, 3rd, and on 15th, 16th, 17th day. Food was withdrawn 6 h before administration of the dose but water available ad libitum. Blood was withdrawn from retro-orbital plexus on 22nd day and allowed to clot to collect sera, which was pooled and distributed in aliquots and stored at –20°C.

Estimation of TT Specific IgG in Hyperimmunized Mice

Modified ELISA method was used for detection of TT specific IgG (Lubben, 2003; Xing, 1996). ELISA was performed by coating flat bottomed 96 well ELISA plates with 100 µL of 1 Lf/mL TT in 50 mM bicarbonate coating buffer of pH 9.6, allowed to incubate at 37.5°C for 1 h and washed with 300 µL of PBS-T-0.05% of washing buffer and blocked using 0.5% BSA in 10 mM phosphate buffer and again incubated for 1 h at 37.5°C followed by washing. To this two fold dilutions of appropriate concentration of the primary antibody [IgG horse polyclonal against TT], was added and incubated for 1 h at 37.5°C followed by washing. Secondary antibodies, [antihorse IgG, alkaline phosphatase conjugated] 100 µL per well was added and incubated followed by washing. To this 100 µL of p-nitrophenyl phosphate was added and incubated at RT for 30 min in dark. Plate was read at 450 nm. The plot of optical density at 450 nm versus concentration of IgG (IU/mL) showed linear relationship with $r^2 > 0.99$. This linear relationship was utilized to determine IgG levels in hyperimmunized mice by using anti-mouse IgG [conjugated with alkaline phosphatase enzyme] as secondary antibodies instead of anti-horse IgG.

TT Specific IgA Estimation in Experimental Animals Using ELISA

Estimation of TT specific IgA in samples of fecal extracts was done by using ELISA by following the same method as that of IgG in hyperimmunized mice except anti-mouse IgA

conjugated with alkaline phosphatase enzyme was used instead of anti-mouse IgG conjugated with alkaline phosphatase enzyme (Lubben, 2003). Absorbance at 450 nm was used to measure the relative IgA level.

RESULTS AND DISCUSSION

Preparation of Microparticles

Optimization of Amount of STPP to Crosslink CS

Ionic crosslinker STPP was used for crosslinking of CS. Reactions of chitosan with negatively charged components can be demonstrated by IR spectra (Mi, 1999), turbidimetric titration (Shu, 2001) or viscometry (Brack, 1998). Chitosan microparticles can be formed by the addition of either covalent or ionic crosslinker. Ionic crosslinking is a simple and mild procedure as compared to covalent crosslinking (Berger, 2004; Xu, 2003).

The crosslinking reaction can be monitored by pH measurement as addition of tripolyphosphate leads to the release of OH^- ions. To study the required amount of STPP for complete crosslinking of chitosan, change in %T was measured as a function of increased amount of STPP. %T was plotted against the amount of STPP added as shown in Figure 1.

The plot clearly shows that the %T decreased with addition of STPP. The rate of decrease of %T was slow and steady till 22 mg of STPP was added. The rate of decrease in %T was very fast after 22 mg till 28 mg of STPP was added, after which %T remained constant. The %T decreases as the free protonated amino groups are crosslinked by phosphate groups of STPP, decreasing solubility of chitosan. The reduction in %T with increasing concentration of STPP can be attributed to the crosslinking of the protonated amino groups of chitosan by phosphate ions of STPP. The increase in %T after complete crosslinking is may be due to the dilution of the system. The amount of STPP required to crosslink 40 mg of chitosan was calculated from the point at which %T remained constant and was found to be 28 mg.

The pH of the system during this phase was found to be 5.2 to 5.4. The pH of the system increased with the addition of STPP, remained constant in between and again increased in last phase. The pH was below the isoelectric point of chitosan (pH 6.5) indicating that the decrease in %T was only due to ionic interaction of the protonated amino groups of chitosan and phosphate groups of the STPP and not due to the coacervation-phase separation reaction.

Fourier Transform Infra Red Spectroscopy of CS and CS-STPP Complex

FTIR spectroscopy was used to prove the formation of ionic complex between CS and STPP (Brack, 1998; Mi, 1999). The FTIR spectra of lyophilized CS microparticles crosslinked using STPP was overlapped with that of plain CS powder as

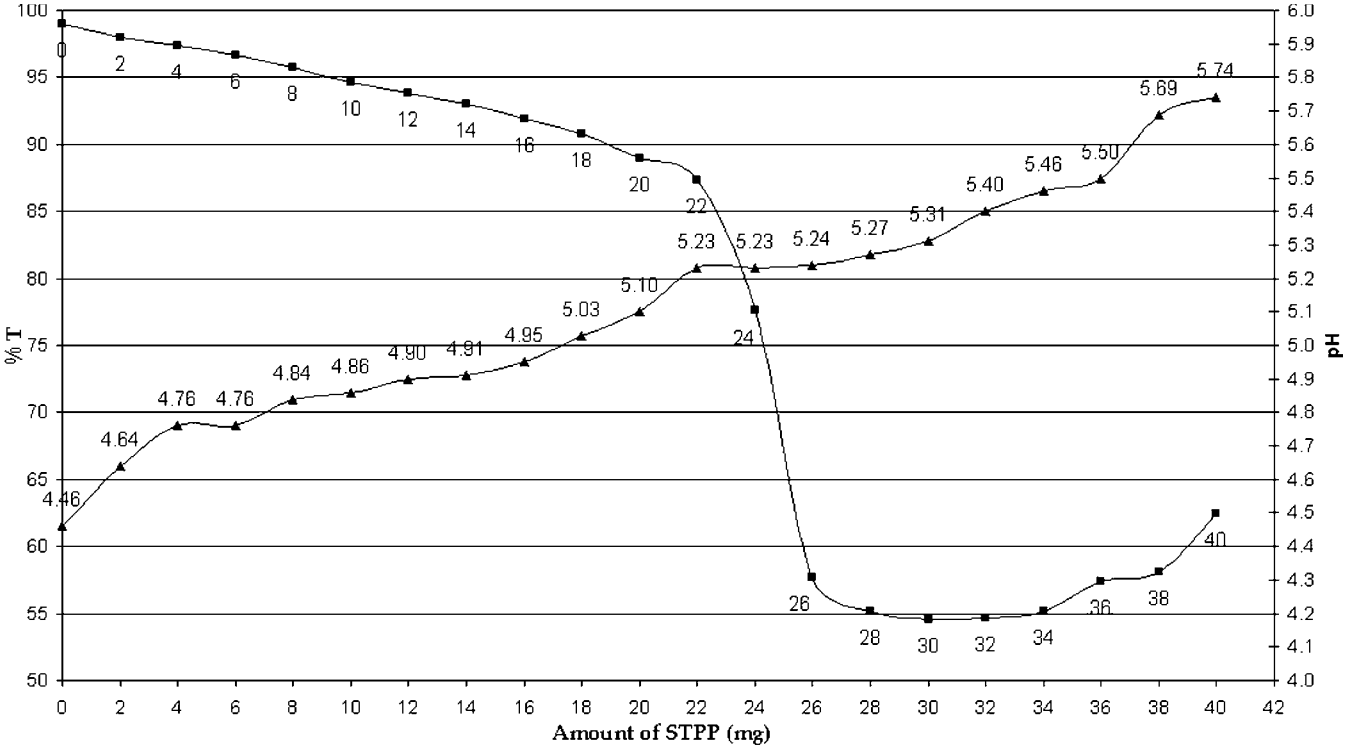

FIGURE 1. Effect of addition of STPP on pH and %T of the CS.

shown in Figure 2. A band at 3408 cm^{-1} has been attributed to –OH group stretching vibration in Chitosan matrix. In chitosan microparticles, a shift from 3408 to 3298 cm^{-1} was observed, and the peak of 3298 cm^{-1} had widened, which may be due to increased hydrogen bonding. Yu reported same observations for formation of chitosan films (Yu, 1999). In CS-STPP complex, instead of the shoulder peak of 1650 cm^{-1}, a new sharp peak at 1631 cm^{-1} was observed. The 1600 cm^{-1} peak of –NH2 bending vibration observed in chitosan powder shifted to 1535 cm^{-1} in CS–STPP complex, which may be attributed to the tripolyphosphate group of STPP linking with amino group of chitosan. Knaul observed similar results in the study of Chitosan film treated with phosphate (NaH_2PO_4), and attributed it to the linkage between phosphate and amino group (Knaul, 1999). Hence, the FTIR studies indicate that the ionic interaction between phosphate groups of STPP and amino groups of CS were responsible for formation of complex between STPP and CS. These observations are in line with results of FTIR study reported by Xu for interaction between CS-STPP for formation of CS nanoparticles (Xu, 2003).

Optimization of Experimental Conditions

Various experimental conditions were optimized to achieve maximum TT loading and desirable product characteristics.

Viscosity of the Reaction Medium

Viscosity of the reaction medium was changed by different solvent compositions and its effect on entrapment of TT was studied. The results showed that entrapment efficiency decreased with increase in viscosity of the reaction medium (Table 1). This may be due to reduction in interaction between the CS and STPP due to increased viscosity, leading to low entrapment and also the extended uncoiled conformation (Liu, 2002) of chitosan may be hindered due to high viscosity reducing the interaction with TT and STPP due to steric hindrances. The entrapment was found to be maximum in water as compared to the other solvent compositions. Therefore, water was selected as the reaction medium.

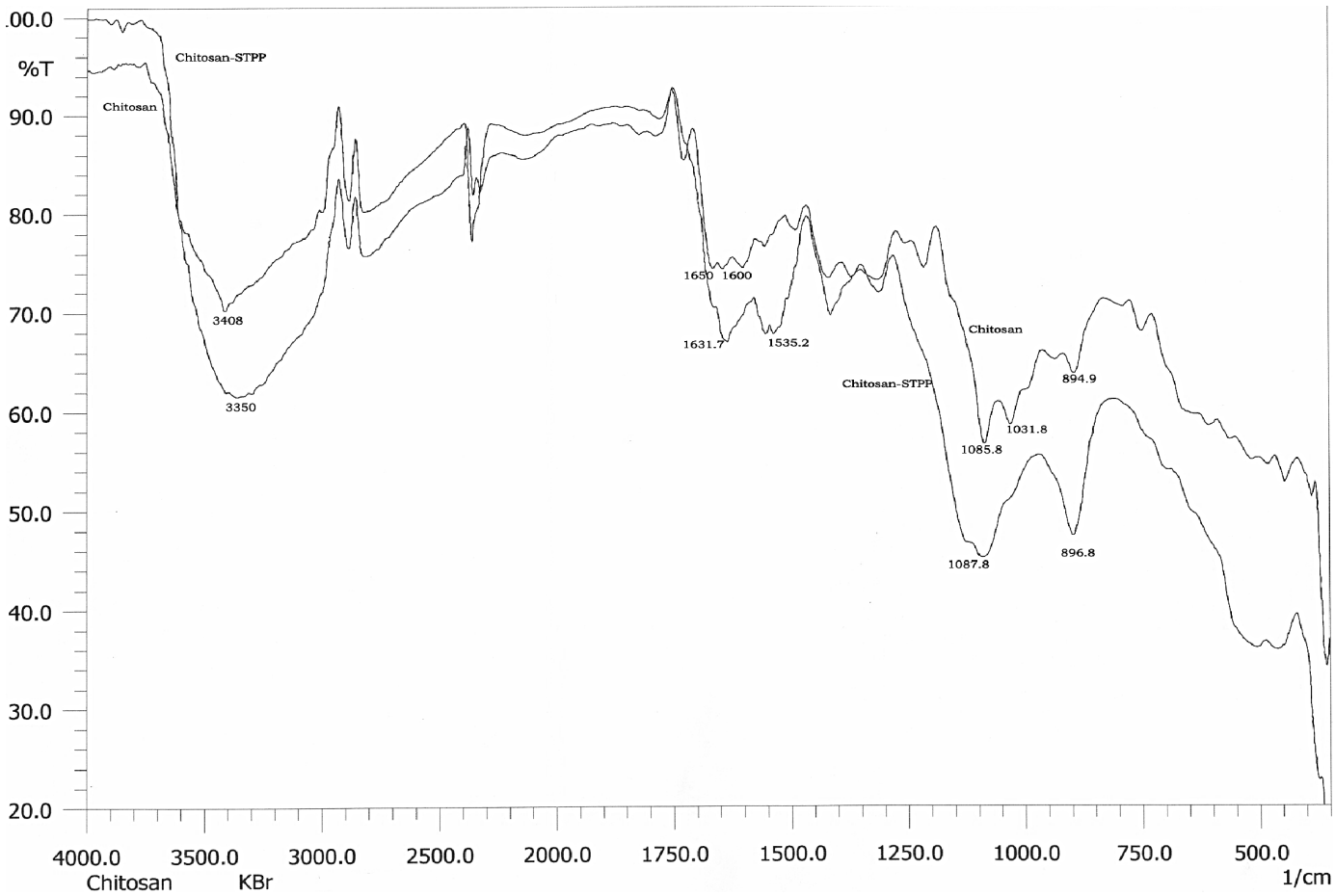

FIGURE 2. FTIR of chitosan and chitosan-STPP complex.

TABLE 1
Effect of Viscosity of Reaction Medium on the % Entrapment Efficiency of TT

Reaction Medium Composition	Viscosity (cps)	%EE ± *SD*
Glycerol	377.9	21.54 ± 0.72
Water: Glycerol (50:50)	185.0	46.34 ± 0.61
Propylene glycol	48.00	47.81 ± 0.90
Water: PG (50:50)	22.50	61.20 ± 0.87
Water	9.600	83.50 ± 0.12

Volume of the Water

Effect of volume of water as a reaction medium was studied from 25 to 60 mL. The entrapment was found to be nearly 100% in 60 mL volume. This can be attributed to the extended conformation of chitosan due to dilution effect at higher volumes enabling maximum interaction between CS-TT and CS-TT-STPP. (Table 2)

pH of Reaction Medium

Among the formulation variables that could affect the chitosan microparticles, pH appears to be the one of important parameters because pH is known to influence the amount of protein incorporated into polymer nano- and microparticles (Barichello, 1999; Calvo, 1997; Elgersma, 1999). It also affects chitosan conformation (Roberts, 1992) and its reaction with tripolyphosphate anions (Mi, 1999) required for preparation of chitosan microparticles. At the same time, pH also has significant effect on the chemical and conformational changes and stability of proteins (Brange, 1992) and would govern its interaction with STPP and chitosan during microparticle formation (Ma, 2002). The entrapment decreased as the pH shifted towards neutral pH. (Table 2) This observation strengthens the importance of maintaining the reaction medium pH below pKa value of CS, which is 6.5. As the pH approaches the pKa value, CS will precipitate due to the reduced solubility rather than reduced availability of the ionic amino groups due to ionic interaction with STPP. Due to this, the ionic interaction between the CS and STPP will remain incomplete, thus giving low entrapment. The pH of the reaction medium is also important from TT-CS interaction point of view. The isoelectric point (pI) of TT is in between 4.8–5.0. Below pI value, the toxoid will be negatively charged, giving rise to maximum ionic interaction with positively charged CS. Proteins can adsorb very efficiently onto polymers at pH around their isoelectric point (pI) because of the minimization of electrostatic repulsion, increased conformational stability, and smaller specific surface area (Coppi, 2002) during the encapsulation process. At the same time, the presence of zwitterionic toxoid molecules in the globular state can favor their hydrophobic association with chitosan along with H-bonding. pH 4.5 was chosen as the initial pH of the reaction. As the initial pH of the CS solution is 4.5 and pH, after addition of required STPP was 5.3, which is below the pKa of CS and just above the pI of toxoid, the reaction medium was used as such without adjusting the initial pH.

TABLE 2
Effect of Volume of Water, pH of Reaction Medium, and Rate of Sodium Tripolyphosphate (STPP) Addition on %Entrapment Efficiency (%EE) of TT

Variable	%EE ± *SD*
Volume of water (mL)	
25	61.70 ± 0.92
50	85.20 ± 0.63
60	99.10 ± 0.05
pH of reaction medium	
4.5	99.20 ± 0.98
6	83.10 ± 0.12
7	76.80 ± 1.02
Rate of addition of STPP	
5 mL/min	85.07 ± 0.26
1 mL/min	71.20 ± 0.13
1 mL/5 min	99.10 ± 0.05
1 mL/10 min	99.24 ± 0.12

Rate of STPP Addition

Rate of STPP addition was checked as the change in pH is directly proportional to rate of addition of STPP. It was observed that %EE increased as rate of addition decreased (Table 2). Faster the rate of addition of STPP and lesser will be the interaction period between CS-TT and CS-TT-STPP and lower will be the entrapment. Hence, a slow addition rate of 1 mL per 5 min was optimized as it gave 99% EE.

Method of Preparation Chitosan-TT Microparticles

The initial formulation, prepared in water, was found to have large particles with mean diameter of 125.76 μm and very broad particle size distribution. The particle size was reduced when the batch was prepared in glycerol or propylene glycol instead of water but the entrapment was very poor. So the batch prepared in water was processed for particle size control using Emulsiflex C-5 microfluidizer, Canada and CAT-560 silverson type homogenizer, Switzerland, either alone or in combination, in the presence of sodium carboxymethyl cellulose.

For non-loaded particles (CS Plain) 90% particles are below 25.451 μm and mean volume diameter is 15.234 μm. For TT loaded particles (CS-TT) 90% particles are below 23.652 μm and mean volume diameter is 15.154 μm as shown in Figure 3, indicating that there is no change in particles size of plain and microparticles containing TT.

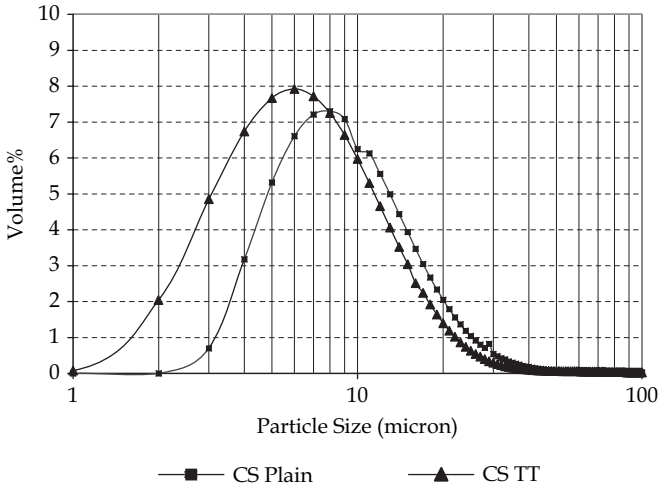

FIGURE 3. Particle size distribution of CS-plain and CS-TT microparticles.

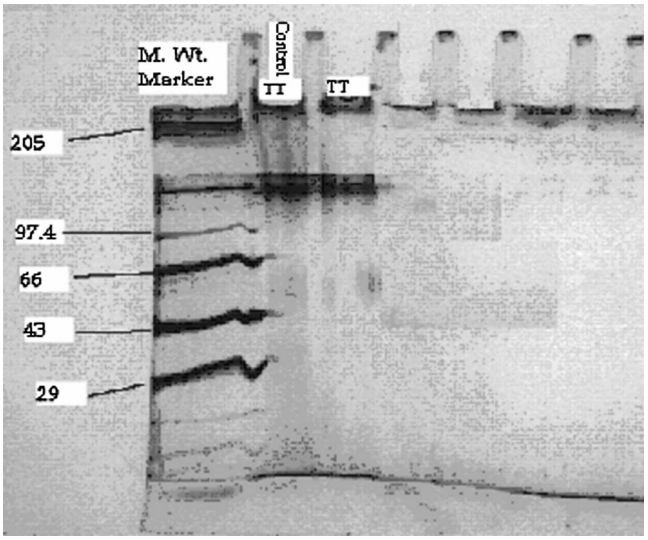

FIGURE 4. SDS-PAGE of control TT and TT, processed using Emulsiflex C-5 and Silverson type homogenizer.

Effect of High Shear Rate on the Conformational Changes

There was a possibility that the high shear rate used in the particle size reduction using Emulsiflex C-5 and CAT-560 Silverson type Homogenizer, may cause conformational changes or fractionation in the TT structure adversely affecting its antigenicity. Hence, the toxoids were subjected to similar homogenization procedure as followed for particle size optimization and estimated using SDS-PAGE. TT (1500 Lf) was taken and volume was made up to 8mL using PG, to this 2mL 2% SCMC was added and processed using the particle size optimization procedure. The sample processed were analyzed using HPLC and SDS-PAGE, as it has been used as a main tool for studying the stability of entrapped antigen and confirming protein integrity. (Lavelle, 1999; Spiers, 2000). The results (Figure 4) of SDS-PAGE of processed TT, showed no change as compared to control indicating that there is no formation of high molecular weight aggregates as well as low molecular weight fragments during the process.

HPLC chromatogram of control sample of TT (Figure 5) and processed sample of TT (Figure 6) are shown. Chromatogram of control sample of TT showed main peak of TT at Rt of 7.24 min. The main peak of TT in processed sample was observed at 7.56 min with additional peak at Rt of around 12 min, which may be attributed to sodium CMC, which was used as dispersing agent.

Both SDS-PAGE and HPLC observations complement each other and proves that there are no conformational changes or breakdowns of the TT molecule during the particle size reduction processing. Indicating that antigenicity of the vaccines shall be maintained after the entire entrapment and size reduction processing.

Release Study of TT in 0.1 N HCl and Phosphate Buffered Saline (pH 7.4)

In order to ensure that encapsulated toxoids are protected from the external luminal conditions and they are delivered in the form of microparticles up to the PP's present in lower intestine, it is essential that microparticles should remain intact in gastric acidic medium as well as in intestinal alkaline medium. As the gastric emptying time is up to 2 h, the study was carried for 2 h in gastric conditions simulated by use of 0.1 N HCl. There was only 2.4% release of the TT from loaded chitosan microparticles, in 0.1 N HCl. This amount can be attributed to the surface bound toxoids.

Study in PBS (pH 7.4) showed no release of TT and TT up to 2 h. The reason may be that optimized formulation conditions had ensured there is complete complexation of chitosan and STPP giving maximum crosslinking and crosslinking density leading to maximum entrapment of toxoids. Further, the ionic interaction between the TT and CS might have prevented their release in PBS.

The stability of chitosan microparticles in gastric pH (0.1 N HCl) and intestinal medium pH [PBS (pH 7.4)] is important from the delivery point of view of entrapped TT to PP's, as the soluble TT is incapable of formation of memory cells. This observation is line with observations reported by van der Lubben who observed there was only very negligible, i.e., only 2% release of the TT in 0.1 N HCl and no release in PBS (pH 7.4) buffer from TT adsorbed on preformed chitosan microparticles (Lubben, 2003).

Stability Study

Stability of the Batch containing encapsulated TT was studied at 40 and 250°C for three months. Samples were withdrawn after every 15 days and analyzed for leached TT using ELISA in the supernatant. Encapsulation of TT in chitosan is important from the absorption and induction of immunity point of view. The structural integrity of the suspended particles is an

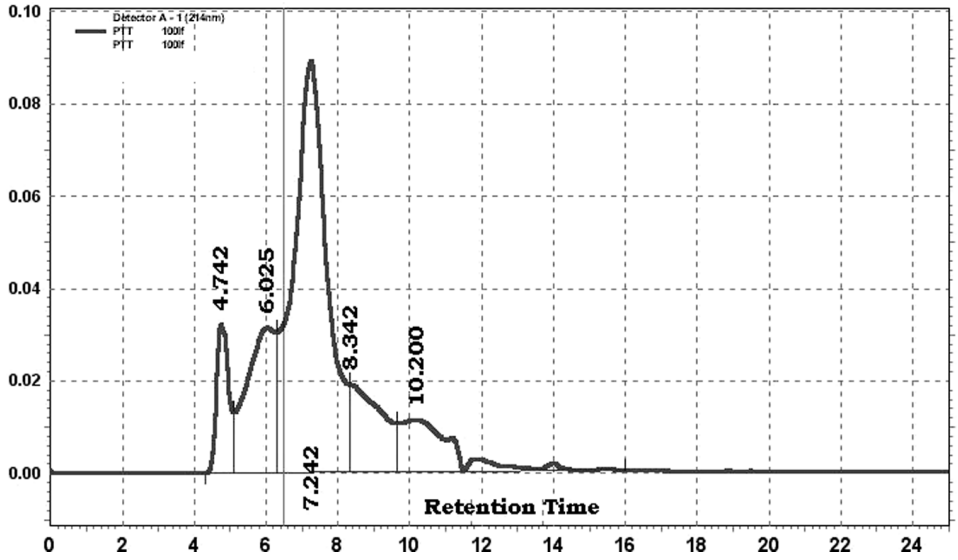

FIGURE 5. HPLC chromatogram of TT-control sample.

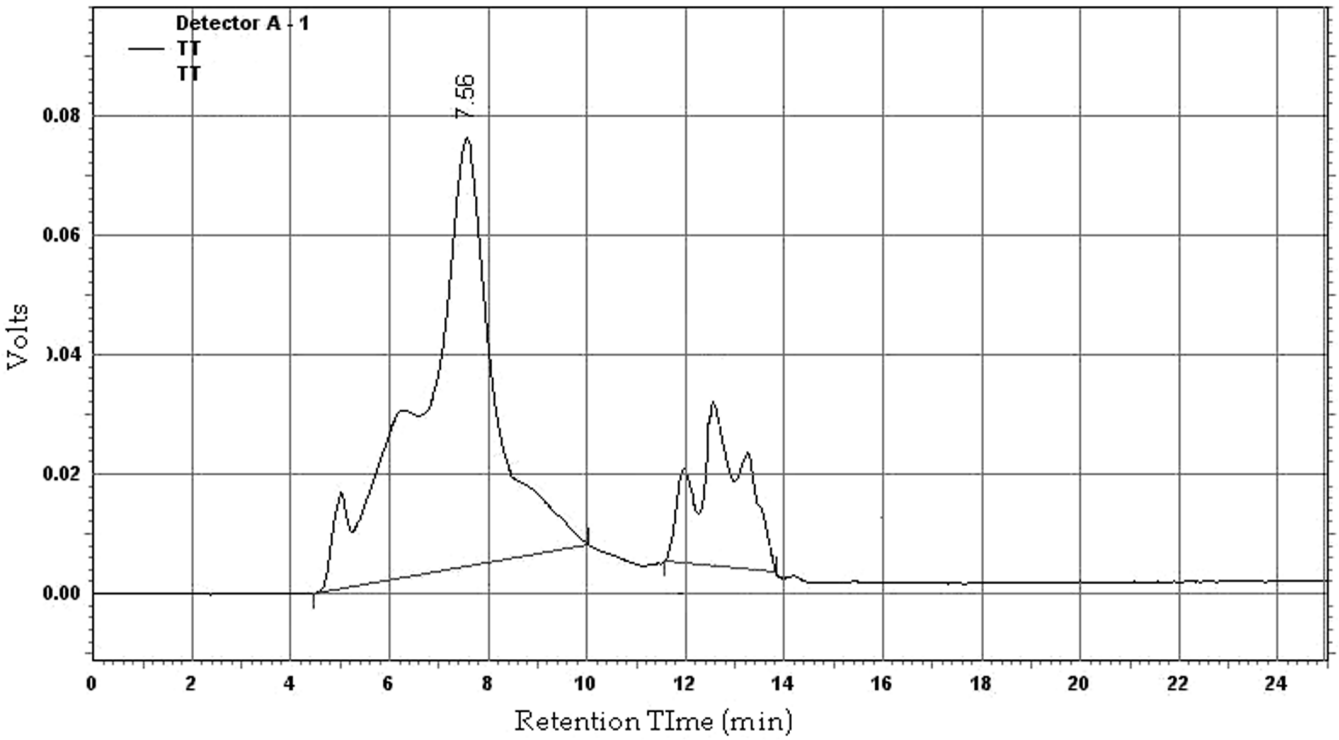

FIGURE 6. HPLC chromatogram of sample of TT, processed using Emulsiflex C-5 and Silverson type homogenizer.

important parameter during the storage period therefore the amount of leached out TT was estimated using ELISA. There was very negligible leaching of TT in both conditions. (Table 3)

This study shows that the product is stable at 250°C/Room temperature (RT) up to 3 months. This study needs to be further substantiated by carrying out invivo studies of the stored samples.

This is of great significance from the transportation and administration point of view. As it is a oral delivery system it can be self-administered by the patients thus eliminating the need of medical supervision and storage in refrigerator conditions. This will be of great help in increasing immunization coverage and avoiding the lapses in booster dose administration,

TABLE 3
Stability of TT Batch

	TT (% leached)	
Time (Days)	4°C	25°C
30	0.752 ± 0.014	0.512 ± 0.009
60	1.216 ± 0.025	1.726 ± 0.083
90	1.568 ± 0.072	1.962 ± 0.098

especially in far flung rural areas where as appropriate storage facilities (refrigerator, cold chain) are not available.

Oral Vaccination Studies

Assessment of Dose Response Relationship

The aim of the in vivo study was to determine a dose-response relationship following oral vaccination with TT. After estimating the IgG levels, different dilution of hyperimmunized mice serum was used to prepare standard curve, which was used for the estimation of TT specific IgG in experimental mice using ELISA (Xing, 1996).

As evident from Figure 7, dose-dependent response could be induced at 3 weeks after oral vaccination with TT loaded chitosan microparticles. All mice vaccinated with CS-TT showed a strong and significant enhancement in IgG titres against TT. Only minor immune responses were observed in the groups vaccinated with TT in PBS. Mice vaccinated with 20 Lf TT entrapped in chitosan microparticles were found to have a significantly lower systemic immune response than the groups vaccinated with 40 or 60 Lf TT entrapped in chitosan microparticles. The response obtained for 60 Lf was found to be lower than 40 Lf TT entrapped in chitosan.

The systemic immune response of the mice vaccinated with 40 Lf TT entrapped in chitosan microparticles was less as compared to that of mice intramuscularly vaccinated with 10 Lf TT adsorbed on Aluminium Phosphate. This indicates that for oral vaccination, approximately four times higher dose would be required to get similar immune response as that of positive control.

The IgG levels were compared statistically using Student's *t* test, which were significantly lower in case of CS-TT 20Lf ($p < 0.02$) and CS-TT 60Lf ($p < 0.05$) when compared with IgG levels after i.m. TT 10 Lf. But the IgG levels after CS-TT 40 Lf were not significantly different as compared to mean IgG levels after i.m. TT 10 Lf. The mean IgG levels after CS-TT 20, 40, and 60 Lf were significantly higher than TT-PBS 20Lf ($p < 0.05$), TT-PBS 40 Lf ($p < 0.002$), and TT-PBS 60 Lf ($p < 0.002$), respectively.

From this study, it was concluded that 40 Lf oral dose of TT could elicit a systemic immune response. However this study

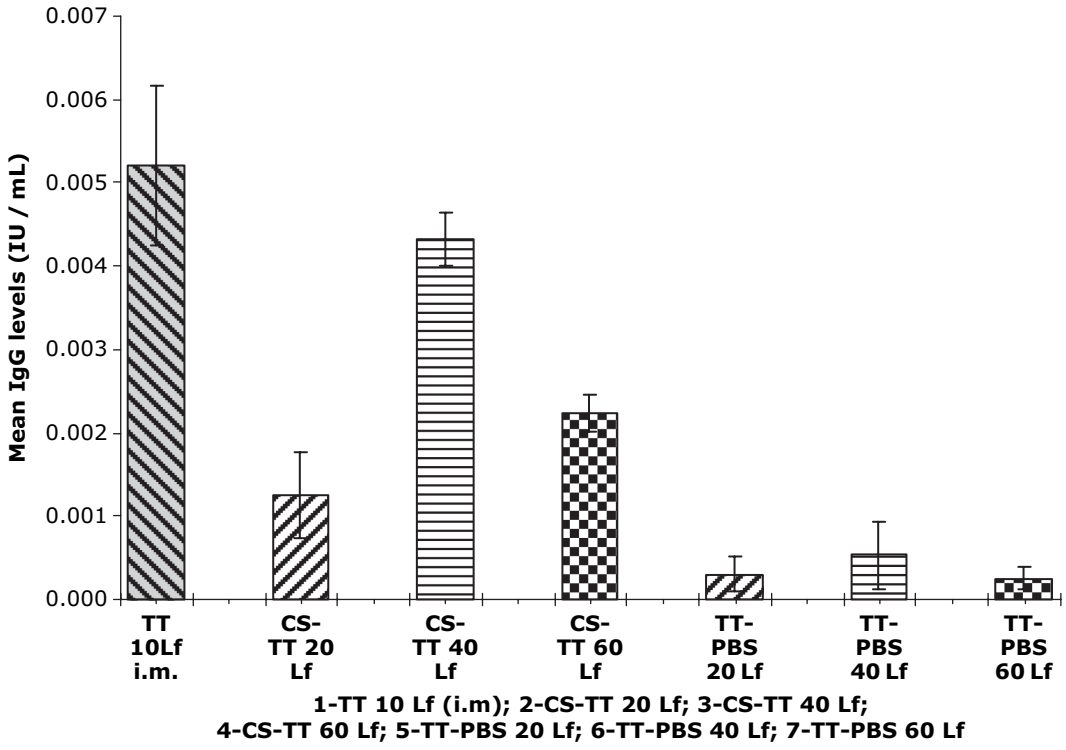

FIGURE 7. TT specific IgG titres in serum on 22nd day after oral vaccination with 20, 40, 60 Lf TT encapsulated in chitosan microparticles (CS-TT 20 Lf, CS-TT 40 Lf, CS-TT 60 Lf) and 20, 40, 60 Lf TT in phosphate buffered saline (TT-PBS 20 Lf, TT-PBS 40 Lf, TT-PBS 60 Lf). IgG levels are expressed as mean ± S.E.M.

was done at only one time point, i.e., on 22nd day. To properly understand the kinetics of the immune response, the second experiment was carried out.

Assessment of Systemic Kinetic Immune Response

Main drawback of the in vivo studies is the biological variation which can seldom be controlled, but can be minimized by increasing the number of animals. Therefore in this study, 10 animals per group were used for the evaluation of the immune response to chitosan encapsulated TT.

On all sampling days, as shown in Figure 8, no measurable TT specific IgG titres could be detected in groups orally fed with non-loaded chitosan microparticles alone. Very minor immune response could be detected after 14 days of oral vaccination with TT in PBS, which was enhanced to some extent after booster dose on 22nd day but the level was very low on 29th day. Following oral administration of TT loaded microparticles, measurable titres were found on 14th day which increased very substantially on 22nd day, after booster dose. On 29th day the IgG level was low as compared to 22nd day but still the level was very high as compared to TT in PBS on 29th day. Highly elevated TT specific IgG titres could be found in CS-TT as compared to TT-PBS on all days of sampling.

The mean TT specific IgG level after oral vaccination with CS-TT 40 Lf on 14th, 22nd, and 29th day were 10.1, 11.08, and 31.5 times higher than the level obtained after TT-PBS 40 Lf. The titres with CS-TT were significantly higher than that of CS-PBS and TT-PBS, at the significance level ($p < 0.01$) on all sampling days, when compared statistically using Student's *t* test.

From this in vivo experiment, it was concluded that the systemic immune response against TT was strongly enhanced after entrapment of vaccine in chitosan microparticles, whereas TT in PBS only induced minor immune responses. It was also observed that immune response was measurable on 14th day and increased after booster dose, indicating the formation of memory cells after priming. Highly elevated IgG titres could be found in CS-TT as compared to TT-PBS on all days of sampling indicating that the antigenicity of the antigens was retained after the entire loading and particle size reduction operation and that it was capable of delivering the vaccines orally in a safe and effective form.

Oral Vaccination Studies: Assessment of Local Immune Response in Faeces

Figure 9 shows IgA levels in the faeces. The local immune response in the gastrointestinal tract started after 4 days and reached a maximum in about 22 days. There was increase in IgA level after booster dose in the CS-TT but such trend was not observed for TT-PBS. IgA levels were not significantly different on 4th day but were significantly higher ($p < 0.01$) on 8th, 14th, and 22nd day sample in CS-TT as compared to TT in PBS on respective days, when compared statistically using Student's *t* test.

The study in mice showed that the systemic immune response was strongly enhanced after incorporating the vaccine into chitosan microparticles and this immune response was dose dependent. In addition, significantly higher levels of specific anti-TT IgA in the gastrointestinal tract were detected.

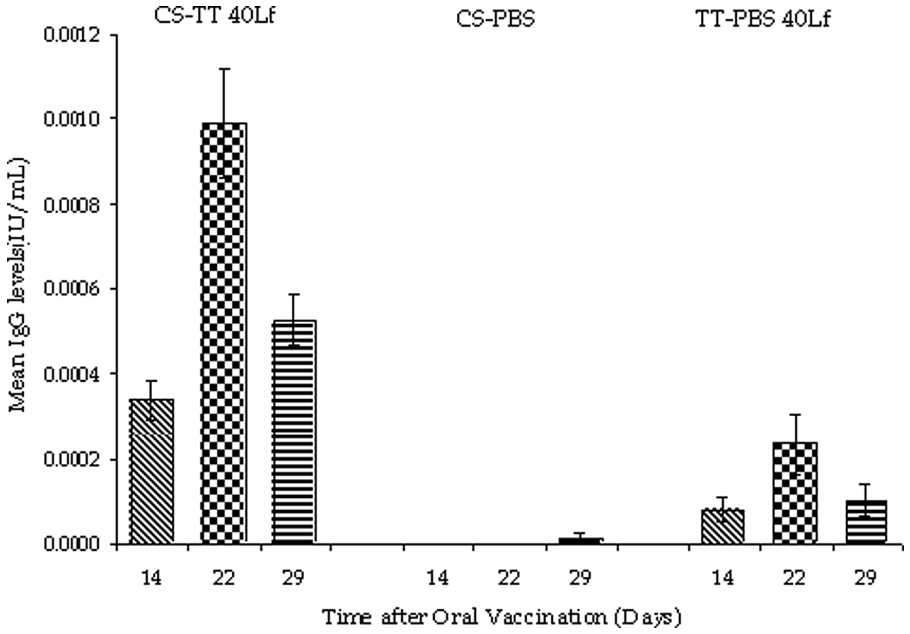

FIGURE 8. TT specific IgG titres in serum on 14th, 22nd, and 29th day after oral vaccination with 40 Lf TT encapsulated in chitosan microparticles (CS-TT 40 Lf); non-loaded chitosan microparticles in phosphate buffered saline (CS-PBS) and 40 Lf TT in PBS only(TT-PBS). IgG levels are expressed as mean ± S.E.M.

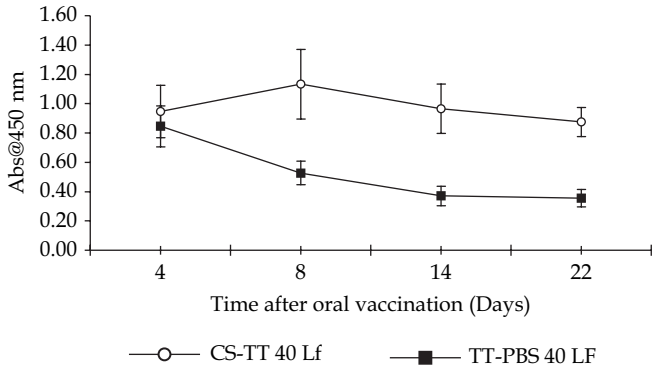

FIGURE 9. TT specific IgA levels in fecal matter on 4th, 8th, 14th, and 22nd day after oral vaccination with 40 Lf TT encapsulated in chitosan microparticles (CS-TT 40 Lf) and 40 Lf TT in phosphate buffered saline (TT-PBS 40 Lf). IgA levels are expressed as absorbance at 450 nm ± S.E.M.

Thus, these oral immunization studies clearly demonstrate that the prepared chitosan microparticles are able to enhance the local immune response as well. The systemic immune response after oral vaccination was comparable with the parenterally delivered vaccine. Besides increased patient compliance and reduction of costs, the induction of local memory cells is an important feature of chitosan microparticles as oral vaccine carriers. With formation of only IgG, as in parenteral immunization, pathogens need to be removed after infiltration into the systemic circulation. But after oral vaccination because of the production of IgA at mucosal sites, pathogens can be neutralized during the invasion through the mucosa.

CONCLUSIONS

Tetanus Toxoid was successfully encapsulated in biocompatible and biodegradable chitosan polymer, crosslinked using sodium tripolyphosphate. Formulation was prepared and processed using antigen friendly conditions. Formulation was evaluated in terms of entrapment, pH stability. It was found be stable at simulated gastric and intestinal conditions. The study shows the efficacy of chitosan microparticle suspension system, containing a high molecular protein (TT), in inducing the IgA in intestine and IgG in systemic circulation. The present investigation envisages the successful use of microparticulate system in orally administered Tetanus Toxoid vaccine.

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