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RESEARCH PAPER

Tetanus Toxoids Loaded Glucomannosylated Chitosan Based Nanohoming Vaccine Adjuvant with Improved Oral Stability and Immunostimulatory Response

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ABSTRACT

Purpose The present report embarks on rational designing of stable and functionalized chitosan nanoparticles for oral mucosal immunization.

Methods Stable glucomannosylated sCh-GM-NPs were prepared by tandem cross linking method followed by lyophilization. The *in vitro* stability of antigen and formulation, cellular uptake and immunostimulatory response were assessed by suitable experimental protocol.

Results Stability testing ensured the chemical and conformation permanency of encapsulated TT as well as robustness of sCh-GM-NPs in simulated biological media. The antigen release from sCh-GM-NPs followed initial burst followed by controlled Weibull's type of release profile. The higher intracellular uptake of sCh-GM-NPs in Raw 264.7 and Caco-2 was concentration and time dependent which mainly attributed to Clathrin and receptor mediated endocytosis via mannose and glucose receptor. The *in vivo* evaluation in animals revealed that sCh-GM-NPs posed significantly ($p < 0.001$) higher humoral, mucosal and cellular immune response than other counterparts. More importantly, commercial TT vaccine administered through oral or intramuscular route was unable to elicit all type of immune response.

Conclusion The sCh-GM-NPs could be considered as promising vaccine adjuvant for oral tetanus immunization. Additionally, this technology expected to benefit the design and development of stable peroral formulation for administration of protein, peptides and variety of other antigens.

KEY WORDS chitosan nanoparticles · glucomannosylation · mucosal immunity · stability · vaccine

INTRODUCTION

Global organization such as WHO, UNICEF, GAVI, Bill & Melinda Gates Foundation and PATH are implementing necessary interventions to save our children. In 2012, WHO endorsed "Global Vaccine Action Plan (GVAP)" to prevent millions of deaths by 2020 through more equitable access to existing vaccines for people in all communities (1). The major goal of GVAP decade plan is to develop new and improved vaccine technologies which meet vaccine coverage and global elimination targets to reduce child mortality. Conventional strategies comprising of alum adsorbed vaccines failed to GVAP challenges owing to their instability to freezing/drying and inconsistency in producing all type of immune response. Conventional vaccines also require cold storage facility to intensify the vaccination coverage which may pose huge burden in immunization campaign (1,2). Oral vaccination can become a foremost strategy of GVAP which can be affordable, accessible and acceptable. Oral vaccination improves patient compliance due to avoidance of pain and trauma. It is also cheaper due to elimination of sterile manufacturing processes and trained medical personnel for vaccine administration. Further it is free from the risk of needle borne infections (3). Most importantly, it has been well explored that it also induces mucosal immune response (secretory IgA (sIgA)) to combat pathogen at entry site. However, degradation of the antigen in the harsh gastric milieu and poor permeability through GIT membrane are the major challenges to make oral vaccination successful.

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Tetanus toxoid vaccine which was developed in 1890 to fight against the dominance of tetanus, however failed to eradicate the tetanus completely. Nearly 22.6 million infants were not immunized by DTP3 in 2012 which may be ascribed to the poor vaccine coverage (less than 80%) in low-income countries like India (72%), Nigeria (41%), Indonesia (64%), etc. (4). It suggests that GVAP is also mandatory to fight against the menace of tetanus.

Novel drug delivery approaches are widely preferred for the efficient delivery of proteins, peptides and vaccine antigens. Amongst, nanoparticles are vital delivery vehicles owing to their ability to offer protection in harsh gastrointestinal environment, permeation through M cells of Peyer's patches and presentation of encapsulated antigens to antigen presenting cells (APCs) (5,6). Therefore, nanoparticles can act as immunopotentiator for tetanus toxoid (TT) capable of eliciting humoral (IgG), cellular (interleukin and interferon) and mucosal (sIgA) immune response more effectively than conventional aluminium based adjuvants. Among, chitosan nanoparticles (Ch-NPs) have tremendous potential as delivery vehicle due to intrinsic biocompatibility, biodegradability and nontoxicity. However, conventional Ch-NPS have demonstrated instability in biological milieu after peroral administration as well as poor storage stability, which is mainly observed due to acid solubility of chitosan and poor mechanical strength of Ch-NPs (7). To troubleshoot this problem, few researchers have tried the delivery of Ch-NPs by oral route by appropriate surface modification. It includes chitosan-polyglutamic acid (γ-PGA) nanoparticles encapsulated in enteric coated gelatin capsules (8), chitosan-hydroxyl propyl methylcellulose phthalate (HPMCP) nanocapsules (9), encapsulation within alginate microcapsules (10). Unfortunately, higher particle size upto several micron and complex method of preparation further limit their use. Therefore, there is strong prerequisite of improved technology using chitosan nanoformulations for oral administration.

Herein, we report preparation of TT loaded stable glucomannosylated chitosan nanoparticles (sCh-GM-NPs) by tandem crosslinking method which dealt with ionic gelation using TPP followed by covalent crosslinking using glutaraldehyde. This simplest troubleshooting strategy was assumed to improve the poor mechanical strength in biological milieu, poor storage stability and burst drug release. Further, glucomannan (GM) was supposed to fortify the efficacy by providing additional stability to chitosan nanoparticles as well as facilitating their uptake by APCs because of higher density of mannose and glucose receptors on their surface (11). The formulation was freeze dried to obtain fluffy and easily dispersible lyophilized cake as well as to remove the traces of glutaraldehyde. Lastly, the efficacy was evaluated in mice to study the immunostimulatory response as well as antigen inhibition assay following their oral administration.

MATERIALS AND METHOD

Materials

Materials and Reagents

Chitosan (medium molecular weight, 190–300 kDa; deacetylation degree, 87%), pentasodium tripolyphosphate (TPP), bicinchoninic acid (BCA), sucrose, bovine serum albumin–fluorescein isothiocyanate conjugate (BSA-FITC), Concanavalin A (Con-A), sodium dodecyl sulphate (SDS), acrylamide, bisacrylamide, ammonium persulpahte (APS), Tetramethylethylenediamine (TEMED), and pilocarpine were procured from Sigma, Missouri, USA. Copper sulphate (pentavalent), panacreatin, pepsin, and glutaraldehyde (25% w/v) were purchased from Loba chemie, Mumbai, India. Sodium dihydrogen phosphate, and dipotassium hydrogen phosphate were acquired from Central Drug House, New Delhi, India. Acetic acid was obtained from Fisher Scientific, India. Bromophenol Blue, Coomassie Brilliant Blue G, Glycine, β-mercaptoethanol, and Tris Buffer were obtained from Himedia, Mumbai, India. Novex Sharp pre-stained protein standard (3.5–260 kD) was obtained from Invitrogen, California, USA. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from PAA laboratories, Pasching, Austria. Konjac Glucomannan (GM) was procured from Megazyme, Wicklow, Ireland. All other chemicals and reagents used were of HPLC or analytical grade. Ultrapure water (Labostar, ultrapure water system, Germany) was prepared in house and used throughout the experimentation.

Antigen, Antibodies and ELISA Kits

Tetanus toxoid (TT) and standard tetanus antitoxin was a generous gift from Panacea Biotech Ltd., India. Commercial TT vaccine IP, Serum Institute, Pune, India was purchased from local pharmacy. Anti-mouse IgA (α-chain specific) peroxidase conjugate, anti-mouse IgG (γ-chain specific) peroxidase conjugate, 3,3′,5,5′-tetramethylbenzidine (TMB) and Nunc Immuno™ Maxisorb F96 well solid plates were obtained from Sigma, Missouri, USA. Mouse IL-2 and IFN-γ Legend ELISA MAX™ Deluxe kits were obtained from Biolegend Inc., California, USA.

Animals

Male Balb/c mice (20–25 g weight and 5–6 week old) were obtained from central animal facility of National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, India and housed in a temperature-controlled environment with a 12-h light/dark cycle and fed a standard diet

with water *ad libitum*. All animal protocols were approved by the Institutional Animal Ethics Committee and Institutional Biosafety Committee of NIPER, SAS Nagar, India.

Preparation of Chitosan Nanoformulations

Herein, chitosan nanoformulations refers to chitosan nanoparticles (Ch-NPs), stabilized chitosan nanoparticles (sCh-NPs), mannosylated chitosan nanoparticles (Ch-GM-NPs) and stabilized mannosylated chitosan nanoparticles (sCh-GM-NPs).

Chitosan nanoparticles were prepared by ionotropic gelation method using polyanionic crosslinking agent, TPP (12–14). The chitosan was dissolved in acetic acid (0.1% w/v; pH 3.5) and pH was adjusted to 6.0 with the help of NaOH (1 N). The crosslinking solution was prepared by dissolving TPP (0.1% w/v) along with TT (100 Lf/mL) in distilled water. A 1 mL of crosslinking solution was added dropwise to 4 mL of the chitosan solution at 1,500 rpm for 15 min. All the operations were carried out at room temperature.

For glucomannosylation, 1 mL aqueous solution of GM (0.1% w/v) was added to 4 mL chitosan solution at pH 6.0 keeping all other formulation and process parameters constant. The amount of GM associated with Ch-NPs was calculated by sulphuric acid–phenol (SAP) colorimetric method, while confirmation of formation of GM as a ligand on the surface of chitosan nanoformulations was demonstrated by Con-A agglutination assay (11) (see supplementary materials).

The stabilization of TT loaded Ch-GM-NPs or Ch-NPs was accomplished using glutaraldehyde as surface crosslinking agent. Briefly, 1 mL of (0.05% w/v) glutaraldehyde was added to 6 mL of nanoparticle dispersion (Ch-GM-NPs or Ch-NPs) at 500 rpm for 15 min.

To obtain final stable dispensable form and to separate the unreacted glutaraldehyde, the chitosan nanoformulations were lyophilized using 5% w/v sucrose, previously optimized and patented lyophilization cycle (Vir Tis, Wizard 2.0, New York, USA) (15). The traces of residual glutaraldehyde in formulation was determined by gas chromatography (see supplementary materials). The lyophilized TT loaded chitosan nanoformulations were also examined for appearance of the cake, redispersibility index (ratio of initial to final size), and reconstitution time.

Characterization of Chitosan Nanoformulations

The average particle size and polydispersity index (PDI) were measured by photon correlation spectroscopy (Zetasizer, Nano ZS, Malvern Instruments Corp, UK) at 25°C in disposable polystyrene cuvettes. Zeta potential was also measured by using Zetasizer. All Measurements were performed in distilled water adjusted after proper dilution of nanoparticles. To calculate the entrapment efficiency (%EE), chitosan nanoformulations were separated by centrifugation at 41,000 g for 30 min (High speed centrifuge, 3 K30, Sigma, USA). The supernatant containing unentrapped TT was collected and amount of TT was measured using validated microBCA colorimetric assay at 561 nm (PowerWave XS2, BioTek Instruments Inc., Vermont, USA) (16).

The shape and morphology of the chitosan nanoformulations was analyzed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). A drop of chitosan nanoformulations was placed on cover slip adhered on metal slab, allowed to air dry and gold coating was done with the help of gold sputter. The coated nanoformulations were observed under SEM (S-3400N, Hitachi, Japan) at 15 kV. For TEM, a drop of TT loaded chitosan nanoformulations was placed on formvar coated grid, dried and stained with 1% w/v phosphotungstic acid. The excess of phosphotungstic acid was removed by washing with distilled water and observed under TEM (FEI, Technai G2 F20, USA) at 80 kV.

Stability Studies

In Process Stability

The chemical integrity of TT was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (17), while conformation stability was confirmed by far UV circular dichroism (CD) spectroscopy (3). The TT samples for process stability were prepared by incubating lyophilized TT loaded chitosan nanoformulations with phosphate buffer saline (pH 7.4) for 24 h followed by separation of released TT by centrifugation. A part of sample was used for SDS-PAGE and remaining was analyzed using CD. Equivalent concentration of standard TT was used as control. For SDS-PAGE, standard TT solution and TT obtained from chitosan nanoformulations (equivalent concentration) were loaded into individual wells of 10% polyacrylamide gel and electrophoresed using a mini-PROTEAN tetra cell electrophoresis unit (Bio-Rad Laboratories, California, USA) at constant voltage (200 V). A Novex Sharp pre-stained protein standard (3.5–260 kD) was used to estimate molecular weights. The protein bands in the gel were visualized by staining with coomasie blue dye.

For CD analysis, an equivalent amount of standard TT and TT extracted from various chitosan nanoformulations were subjected to CD analysis in the far UV region of 250–190 nm at 0.5 nm data pitch and 50 nm/min scanning speed (J-815, JASCO, Tokyo, Japan). Average of three accumulations for each sample was taken and baseline correction with distilled water was carried out to obtain CD spectrum.

Flocculation Test

Antigenic bioactivity and purity of TT encapsulated in chitosan nanoformulations was determined by flocculation test. A constant amount of TT extracted from nanoformulations was mixed to varying amount tetanus antitoxin in transparent glass tube at 37°C. The time required for the immunoprecipitation was recorded visually which termed as lime of flocculation (Lf) (18).

Stability in Simulated Biological Media

The stability of chitosan nanoformulations was tested at different pH and enzymatic conditions viz. phosphate buffer saline (PBS; pH 7.4); simulated gastric fluid (SGF)-USP XVIII (comprised of 320 mg pepsin in 700 µL concentrated HCl, 200 mg sodium chloride in 100 mL at pH 1.2); and simulated intestinal fluid (SIF)-USP XVIII (contained 680 mg monobasic potassium phosphate, 7.7 mL of 0.2 M NaOH and 1 g pancreatin in 100 mL water at pH 6.8). Briefly, NPs were suitability diluted with simulated biological media and incubated for 2 h (in SGF) or 4 h (in PBS and SIF) at 200 rpm and 37 ± 1°C. The stability of nanoformulations was determined on the basis of change in particle size, %EE, PDI and zeta potential (11).

In Vitro Release

The release of TT from chitosan nanoformulations was determined by direct incubation method. A 200 µL of TT loaded chitosan nanoformulation was incubated in 1,000 µL of PBS (pH 7.4) in microcentrifuge tube (n = 78) and placed in shaker bath at 80 rpm at 37 ± 1°C. At each time interval, total aliquot present in microcentrifuge tube (n = 6) was centrifuged at 41,000 g for 30 min (High speed centrifuge, 3K30, Sigma, USA). The amount of released TT in supernatant was measured by validated microBCA method at 561 nm (16,19).

Cellular Uptake

Antigen Presenting Cells (APCs) Uptake

BSA-FITC was selected as model tracer for imagining of cells following endocytosis. The BSA-FITC loaded chitosan nanoformulations were prepared and characterized by ionic gelation method as discussed in "Preparation of Chitosan Nanoformulations" section (see supplementary materials). Murine macrophage cell line (RAW 264.7; procured from NCCS, India) were grown in RPMI-1640 containing 20% FBS 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells (passage # 12) were counted and suitably diluted to obtain 5×10^4 cells/well followed by overnight incubation for cell attachment. Cultured cells were incubated with BSA-FITC loaded chitosan nanoformulations (10–100 µg/mL) and free BSA-FITC for 1, 2 and 3 h. The mechanistic understanding of receptor based endocytosis was also studied after GM or mannose pre-treatment. For pretreatment, 50 µL of GM (0.01% w/v) or mannose (0.01% w/v) was added to cells per well for 1 h followed by washing with PBS and reincubation with chitosan nanoformulations (10–100 µg/mL) for 1, 2 and 3 h. Finally, cells were thoroughly washed with PBS and analyzed by two-dimensional confocal laser scanning microscopy (CLSM; Olympus FluoView 1000, Tokyo, Japan). The cells were later lysed using 200 µL of 0.1% Triton X-100 and fluorescence was measured at excitation/emission wavelength of 490/525 nm (20–22).

Caco-2 Cells Uptake

The Caco-2 cells (procured from ATCC, Manassas, USA) were cultured in DMEM supplemented with 20% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The 5×10^4 cells/cm^2 (passage #11) were seeded overnight in black 96-well plates with transparent bottom for quantitative uptake experiments and 6-well plates for CLSM (23). For quantitative and qualitative analysis, protocol described in "Antigen Presenting Cells (APCs) Uptake" section was followed.

Immunization Experiments

Immunization

Animals were divided into six groups containing 5 animals each. First and second group received commercial TT vaccine (Serum Institute, Pune, India) by intramuscular (im) and oral route, respectively; Remaining groups received TT loaded Ch-NPs, Ch-GM-NPs, sCh-NPs, and sCh-GM-NPs, respectively by peroral route using oral gavage (G22 X 38 mm). Each group received formulation dose equivalent to 5 Lf TT on day 0 (primary immunization) and day 21 (booster immunization).

Sample Collection

The blood samples were collected from the retro-orbital plexus of mice on day 0 (pre-immune), 7, 14, 21 (before booster), 28 and 35 (Post booster). The serum was separated from blood samples by coagulation followed by centrifugation at 8,000 g for 8 min (High speed centrifuge, 3K30, Sigma, USA). All serum samples were stored at −20°C until analyzed for IgG levels using ELISA kits (22,24).

Saliva, intestinal fluids and fecal matter were collected on day 35 for sIgA estimation. Mice were injected 0.2 mL pilocarpine (80 mg/kg) intraperitoneally and began to salivate within a couple of minutes which was collected and pooled. Mice were then sacrificed, intestine was excised and incubated

in PBS (pH 7.4) at 4°C for 24 h. The supernatant containing intestinal secretions was collected by centrifugation. Fecal matter was also collected from each group, vortexed and incubated overnight in cold PBS at 4°C. The mixture was centrifuged at 8,000 g for 8 min and supernatant was collected. All mucosal secretions were stored at −20°C until analyzed for IgA titer (3).

The endogenous levels of interleukin 2 (IL-2) and interferon γ (IFN-γ) were estimated in spleen homogenate. Spleens were excised and homogenized in RPMI-1640 at 2,000 rpm using tissue homogenizer (PT-MR-4000, Polytron, Fisher Scientific, New Hampshire, USA). The spleen homogenate (1% w/v) were seeded in 24 well plate and restimulated with 100 µL TT (1 Lf/mL) at 37°C followed by incubation for 48 h. The plate was centrifuged and supernatant was collected for cytokines determination by ELISA kits.

Measurement of IgG Titer

A well-established ELISA protocol was adopted for estimation of IgG titer in serum (3,11). A 100 µL of TT (1 Lf/mL) in carbonate buffer (pH 9.5) was added to 96 well plates (Nunc Maxisorb, Sigma, USA) and incubated overnight at 4°C. Following incubation, each well was washed three times with PBS containing 0.05% w/v Tween 20 (PBST). Consequently, serial dilutions of serum samples were done with PBS and vortexed. The 100 µL of serum samples were added in each well and incubated at 37°C for 2 h. The plate was washed 3 times with PBST and 100 µL of Rabbit antimouse IgG peroxidase conjugate (1:15,000) in PBS was added in each well and incubated for 1 h at 37°C. Later, plate was washed 3 times with PBST and 100 µL of TMB substrate was added in each well. The plate was incubated for 30 min at room temperature. A 100 µL of stop solution (H_2SO_4, 2N) was added in each well and developed color was measured at 450 nm on a UV-microplate reader (BioTek, USA) (3). Antibody titer was represented as 'end point titer' which was obtained after maximum dilution of the post immunized sera till the optical density was matched with pre-immune sample at 450 nm.

Measurement of sIgA Titer

The sIgA titer was estimated in different mucosal secretions viz. saliva, intestinal content and fecal matter by ELISA. The ELISA protocol used for the sIgA estimation was similar as that of ELISA used for IgG estimation, only 100 µL of Rabbit antimouse IgA peroxidise conjugate (1:10,000) in PBS was used instead of Rabbit antimouse IgG peroxidase conjugate.

Estimation of Cytokines Levels

Commercially available Legend ELISA MAX™ Deluxe kits (Biolegend Inc., USA) were used for measurement of cytokine (IL-2 and IFN-γ) levels in spleen homogenates. Protocol provided by manufacturer was strictly followed for estimation of cytokines (16,22).

Modified TT Inhibition Assay

Toxin neutralization capability of the generated antibodies following immunization was determined by modified TT inhibition assay (25,26). Briefly, 'serum-TT blend' was produced by mixing 100 µL of serum (1:100 dilution) with varying concentration of TT (100 µL; 0.01–10 Lf/mL) in 96 well plate (plate 1). This mixture was incubated overnight at 4°C and unbound fraction (non-inhibited anti-tetanus antibody) was used for further analysis. Simultaneously the coating 100 µL of TT in carbonated buffer (5 Lf/mL) in each well and incubated overnight at 4°C (plate 2). The plate was washed with PBST and processed 'serum-TT blend' from plate 1 was mixed in coated plate 2 followed by incubation at 37°C for 2 h. After incubation, plate was again washed with PBST and 100 µL of rabbit antimouse IgG peroxidase conjugate was added in each well and further incubated for 1 h at 37°C. The plate was washed again followed by addition of 100 µL of TMB substrate and incubation for 30 min at room temperature. Finally, enzymatic reaction was stopped by 100 µL of H_2SO_4 (2N). The intensity of developed colour (optical density; OD) was measured at 450 nm using UV spectrophotometer. The percent TT inhibition or binding was calculated from following equation. The terms OD_{Test}, OD_{Max} and OD_{Blank} represent the OD of test sample, OD in absence of competing TT and OD in absence of serum, respectively at 450 nm.

$$\%\,\text{Toxoid inhibition} = 100 - \left[\frac{(OD_{Test} - OD_{Blank})}{(OD_{Max} - OD_{Blank})} * 100\right]$$

The protective levels of anti-TT antibody was also calculated from TT inhibition curve by considering >90% TT inhibition by serum components.

Statistical Analysis

All *in-vitro* results are presented as the mean ± standard deviation (SD) while *in-vivo* results are represented as mean ± standard error mean. Statistical analysis was performed with Sigma Stat (Version 2.03) using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. $p < 0.05$ was considered as statistically significant.

RESULTS

Preparation and Characterization of TT Loaded Chitosan Nanoformulations

The stable and functionalized chitosan nanoformulations were prepared by tandem crosslinking technique and formulation attributes are represented in Table I. The glucomannosylation of Ch-NPs resulted in insignificant ($p>0.05$) increase in particle size and lowering of zeta potential. In contrast, particle size was significantly reduced ($p<0.05$) after glutaraldehyde crosslinking. In all cases, the %EE (~95%) and bioactivity of TT (~14 Lf/mL) remained unaffected by formulation processes and parameters. The presence of GM as a ligand on the surface of chitosan nanoparticles was confirmed by Con-A agglutination assay on the basis of change in absorbance of nanoparticles after incubation with Con-A (see supplementary materials) (16).

The elegant dry lyophilized formulations were obtained using sucrose as cryoprotectant. The lyophilized powder was easy to reconstitute with acceptable redispersibility index (~1.2) (Table II). The residual glutaraldehyde content in final lyophilized product was non-detectable as measured by gas chromatography.

Figure 1 shows the SEM and TEM images of TT loaded chitosan nanoformulations. Both the microscopic techniques revealed the particle size in the range of 100–200 nm which was in good agreement with that observed using Zetasizer. It is evident from SEM images that non-stabilized nanoformulations had distorted morphology, while stabilized nanoformulations demonstrated smooth, spherical and particulate structure. Similarly, TEM analysis also revealed smooth and spherical structure of nanoformulations.

In Process Stability of TT

Figure 2 shows the results of in process stability as determined by CD and SDS-PAGE. The same migration pattern of standard TT and TT extracted from chitosan nanoformulations in SDS-PAGE confirmed no chemical degradation of TT throughout the process (Fig. 2a). Figure 2b illustrates the CD spectra of TT obtained from chitosan formulations which coincide with standard TT solution.

Stability in Simulated Biological Media

The effect of simulated biological fluids on quality attributes of chitosan nanoformulations is presented in Table III. Biological media had detrimental effect on characteristics of conventional non-stabilized chitosan nanoformulations which showed significant increase in particle size and loss of %EE in PBS and SIF, while complete solubilization in SGF. These results were in agreement with previous literatures (7,27). On contrary, stabilized chitosan nanoformulations were highly stable in biological media. The particle size and PDI were not affected ($p>0.05$), although notable reduction in %EE (upto 40%) was observed. However the loss was significantly less in comparison with non-stabilized counterparts which showed upto 70% loss of antigen content in PBS and SIF.

In Vitro Release

The release profile of TT from chitosan nanoformulations is shown in Fig. 3. All chitosan nanoformulations exhibited a nonlinear burst followed by controlled release in 24 h. Approximately 65% TT was released from non-stabilized chitosan nanoformulations (Ch-NPs and Ch-GM-NPs) in comparison to only 40% release from stabilized chitosan nanoformulations (sCh-NPs and sCh-GM-NPs) in 2 h (Fig. 3a). No notable ($p>0.05$) effect on release pattern was observed after glucomannosylation of formulations. Further it is evident from Fig. 3b that all formulations followed Weibull's release patterns ($r2=0.990$, $n=0.53$). DDsolver was also used to establish characteristic difference between chitosan nanoformulations using univariate ANOVA and the results confirmed the existence of a significant difference ($p<0.05$) between stabilized and non-stabilized version of chitosan nanoformulations at most of time points. DD solver further identified the significant difference in release profiles of stabilized and non-stabilized chitosan nanoformulations on the basis of similarity factor [$f2 \in (50, 100)$] and difference factor [$f1 \in (0, 15)$], the value of which were found to be ~40 and ~25, respectively.

Table I Formulation Attributes of TT Loaded Chitosan Nanoformulations

Nanoformulations	Particle size (nm)	PDI	Zeta potential (mV)	%EE	Bioactivity of TT (Lf)
Ch-NPs	155 ± 6	0.237 ± 0.04	12.70 ± 0.36	95.20 ± 0.75	14.06 ± 0.58
sCh-NPs	116 ± 7	0.231 ± 0.030	7.37 ± 0.78	93.81 ± 4.15	13.57 ± 0.32
Ch-GM-NPs	169 ± 9	0.253 ± 0.028	10.54 ± 0.79	95.55 ± 0.73	13.94 ± 0.64
sCh-GM-NPs	123 ± 5	0.266 ± 0.014	6.30 ± 0.20	94.11 ± 3.24	13.82 ± 0.44

All values are represented as mean ± SD ($n=6$)

Table II Critical Quality Attributes of Chitosan Nanoformulations After Lyophilization

Sucrose (5% w/v)	Ch-NPs			Ch-GM-NPs			sCh-NPs			sCh-GM-NPs		
	PS	Ri	RT	PS	Ri	RT	PS	Ri	RT	PS	Ri	RT
Initial	155 ± 6	–	–	169 ± 9	–	–	116 ± 7	–	–	123 ± 5	–	–
Final	188 ± 9	1.21 ± 0.06	< 1 min	196 ± 11	1.16 ± 0.06	< 1 min	135 ± 10	1.17 ± 0.09	< 1 min	141 ± 10	1.15 ± 0.08	< 1 min

PS Particle size, *Ri* Redispersibility index, *RT* Reconstitution time. Data is represented as mean ± SD ($n = 6$)

Cellular Uptake

APCs Uptake

The uptake of BSA-FITC loaded chitosan nanoformulations in RAW 264.7 cells is shown in Fig. 4. CLSM analysis demonstrated green fluorescence owing to higher uptake of chitosan nanoformulations in comparison to free BSA-FITC (Fig. 4a–g). Both concentration and time dependent uptake was observed upon spectrofluorometric analysis (Fig. 4h). The glucomannosylated chitosan nanoformulations showed significantly higher ($p < 0.001$) uptake which was 2 and 13 fold higher in comparison with non-glucomannosylated chitosan nanoformulations and free BSA-FITC, respectively. The pretreatment of cells with free ligand such as mannose or GM significantly reduced the uptake of sCh-GM-NPs.

Caco-2 Cells Uptake

Figure 5 showed an enhanced uptake of chitosan nanoformulations than free antigen (BSA-FITC) in Caco-2 cells. CLSM images displayed higher green fluorescence in case of chitosan nanoformulations as compared to free antigen (Fig. 5a–e). Quantitatively, the uptake was time (1–6 h) and concentration (10–100 μg/mL) dependent (Fig. 5f) (23). The sCh-GM-NPs showed 4.3 fold higher ($p < 0.001$) uptake as compared to free antigen.

Peroral Immunization

The mean IgG titer following the administration of TT loaded chitosan nanoformulations are represented in Fig. 6a. It was observed that serum IgG levels in case of commercial TT vaccine (im) and stable TT loaded chitosan nanoformulations were significantly ($p < 0.001$) higher than orally administered commercial TT vaccine and non-stabilized counterparts. The sCh-GM-NPs produced significantly ($p < 0.001$) higher IgG titer in comparison with all other orally administered formulations, and interestingly it was equivalent to intramuscularly injected commercial TT vaccine ($p > 0.05$). Of note, immune response was decreased after 21 days following primary dose, but it shoot up again ($p < 0.001$) and nearly doubled following booster dose after 3 weeks of primary immunization.

Figure 6b–d depicts mucosal immune response i.e. sIgA titer in saliva, intestinal secretions and fecal content. Commercial TT vaccine (oral and im) as well as non-stabilized chitosan nanoformulations did not elicit significant sIgA titer in either of the above mucosal fluids, which was elicited by peroral administration of sCh-GM-NPs ($p < 0.001$).

Endogenous cytokines levels namely IL-2 and IFN-γ in spleen homogenates after 35 days is shown in Fig. 6e–f. Stabilized chitosan nanoformulations demonstrated

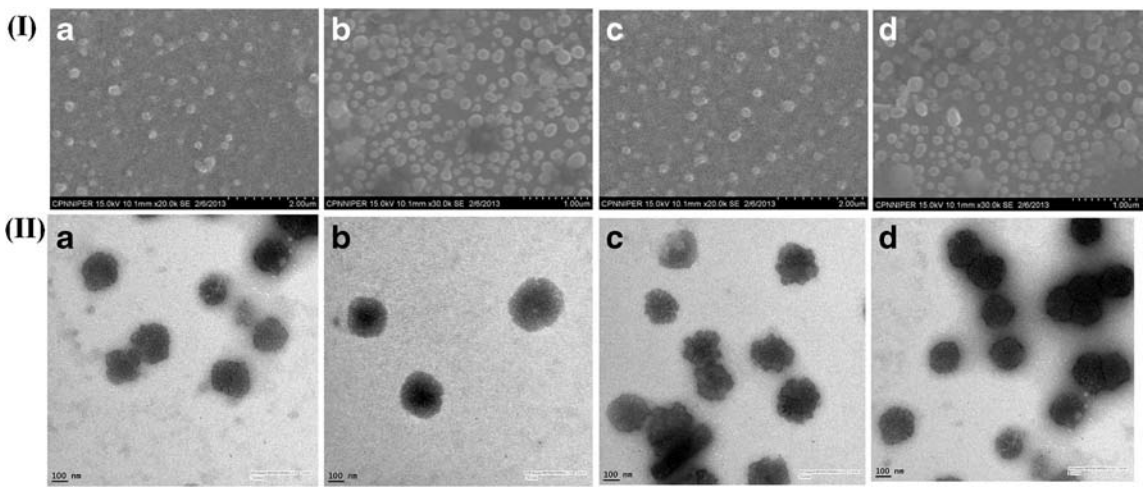

Fig. 1 Morphology of chitosan nanoformulations viz. (**a**) Ch-NPs, (**b**) sCh-NPs, (**c**) Ch-GM-NPs, and (**d**) sCh-GM-NPs, using (*1*) SEM and (2) TEM.

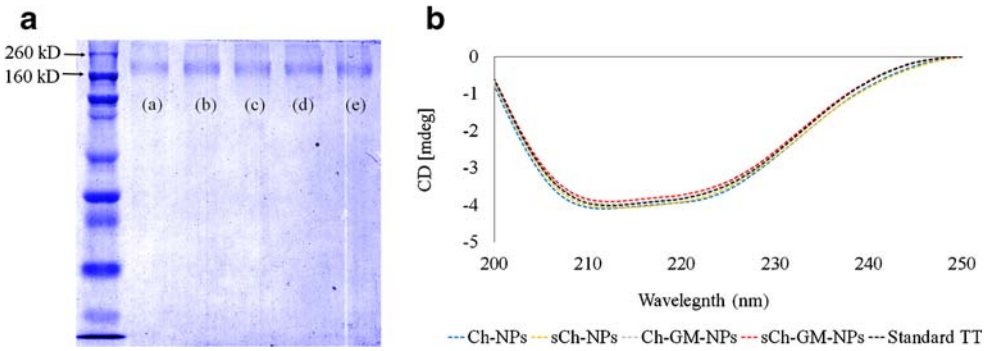

Fig. 2 In process stability of TT extracted from lyophilized chitosan nanoformulations viz. (*b*) Ch-NPs, (*c*) sCh-NPs, (*d*) Ch-GM-NPs, (*e*) sCh-GM-NPs using (**a**) SDS-PAGE and (**b**) CD.

significantly higher ($p<0.001$) levels of both IL-2 and IFN-γ as compared to TT vaccine administered by both im and oral routes and non-stabilized nanoformulations. It is worthy to note that sCh-GM-NPs resulted in significantly higher ($p<0.001$) cytokines levels in comparison with its counterparts.

The toxoid inhibition by serum anti-TT IgG elicited by formulations is represented in Fig. 7. The TT inhibition curve evidenced that the amount of TT required to neutralize the minimum 50% of anti-TT IgG elicted by formulation followed the same order as that of IgG titers: TT (im) > stabilized chitosan nanoformulations (sCh-GM-NPs > sCh-NPs) > non-stabilized chitosan nanoformualtions (Ch-GM-NPs > Ch-NPs). Commercial TT vaccine administered by peroral route required <0.01 Lf/mL TT concentration to counterbalance the anti-TT IgG titer, whereas ~0.08 Lf/mL TT was required to neutralize anti-TT IgG titer in the serum of sCh-GM-NPs treated animals.

DISCUSSION

The main "state of art" of present formulation strategy was stabilization of chitosan nanoparticles by tandem cross linking technique using dual cross linking agents namely TPP and glutaraldehyde which could protected the antigen from harsh gastric milieu and made formulation suitable for oral administration. Further, functionalization using GM made it superior for presenting of antigen to APCs of oral immune system.

The formulation and process variable required for strategic development of TT loaded chitosan nanoformulations were exhaustively optimized using bovine serum albumin (BSA) as model antigen by Box-Behnken design (data not shown). Cheap and easy availability are major drive for selection of BSA over TT during optimization. These optimized parameters were used for the preparation of TT loaded chitosan nanoformulations. Stepwise, the conventional chitosan nanoparticles were initially functionalized using GM which provided the higher density of mannose molecules over nanoparticle

Table III Stability Study of Chitosan Nanoformulations in Simulated Biological Media

Formulations	Biological media	Particle size (nm)	PDI	Zeta potential (mV)	%EE
Ch-NPs	Initial	155 ± 6	0.237 ± 0.036	12.70 ± 0.36	95.20 ± 0.75
	PBS, pH 7.4	476 ± 34	0.225 ± 0.016	11.87 ± 0.21	33.33 ± 3.09
	SIF, pH 6.8	507 ± 42	0.278 ± 0.019	12.27 ± 0.32	30.84 ± 2.58
	SGF, pH 1.2	ND			
sCh-NPs	Initial	116 ± 7	0.231 ± 0.030	7.37 ± 0.78	93.81 ± 4.15
	PBS, pH 7.4	120 ± 3	0.242 ± 0.026	6.26 ± 0.28	54.33 ± 1.67
	SIF, pH 6.8	130 ± 6	0.236 ± 0.020	5.05 ± 0.19	51.73 ± 0.20
	SGF, pH 1.2	137 ± 5	0.239 ± 0.012	22.60 ± 1.41	59.09 ± 1.42
Ch-GM-NPs	Initial	169 ± 9	0.253 ± 0.028	10.54 ± 0.79	95.55 ± 0.73
	PBS, pH 7.4	370 ± 28	0.284 ± 0.019	8.85 ± 0.39	35.17 ± 2.16
	SIF, pH 6.8	415 ± 34	0.292 ± 0.023	11.07 ± 0.55	31.06 ± 1.14
	SGF, pH 1.2	ND			
sCh-GM-NPs	Initial	123 ± 5	0.266 ± 0.014	6.30 ± 0.20	94.11 ± 3.24
	PBS, pH 7.4	127 ± 5	0.242 ± 0.014	6.17 ± 0.23	53.46 ± 1.14
	SIF, pH 6.8	147 ± 6	0.236 ± 0.020	3.52 ± 0.34	51.84 ± 1.46
	SGF, pH 1.2	153 ± 6	0.255 ± 0.033	24.37 ± 1.92	58.87 ± 1.79

ND not determined due to solubilization of nanoformulations. All Values are represented as mean ± SD ($n=6$)

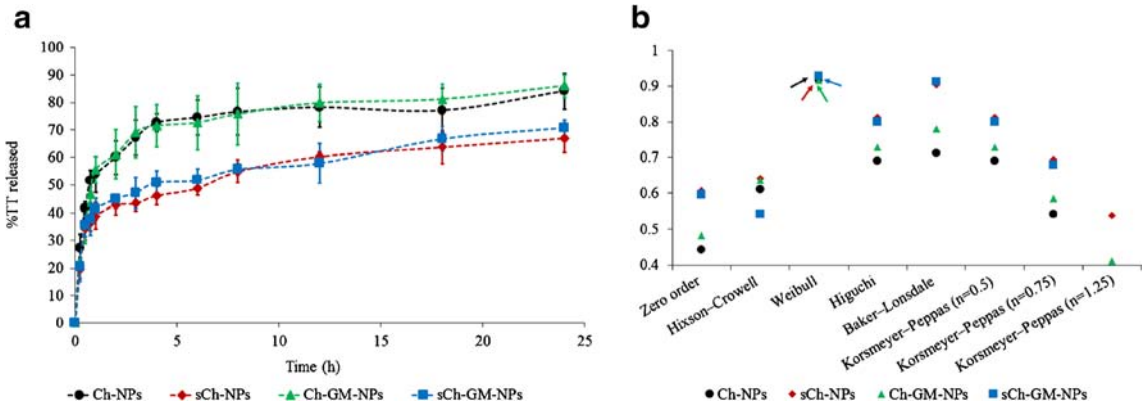

Fig. 3 A cumulative % release of TT (**a**) and mathematical modelling (**b**) representing best suitable release mechanism from chitosan nanoformulations.

surface resulting in more precise recognition and binding to mannose receptors present on APCs. This surface glucomannosylation was confirmed by Con-A agglutination assay based on increased absorbance and agglomeration by Con-A (11). It was also thought to improve the mechanical strength and stability of Ch-NPs in simulated biological

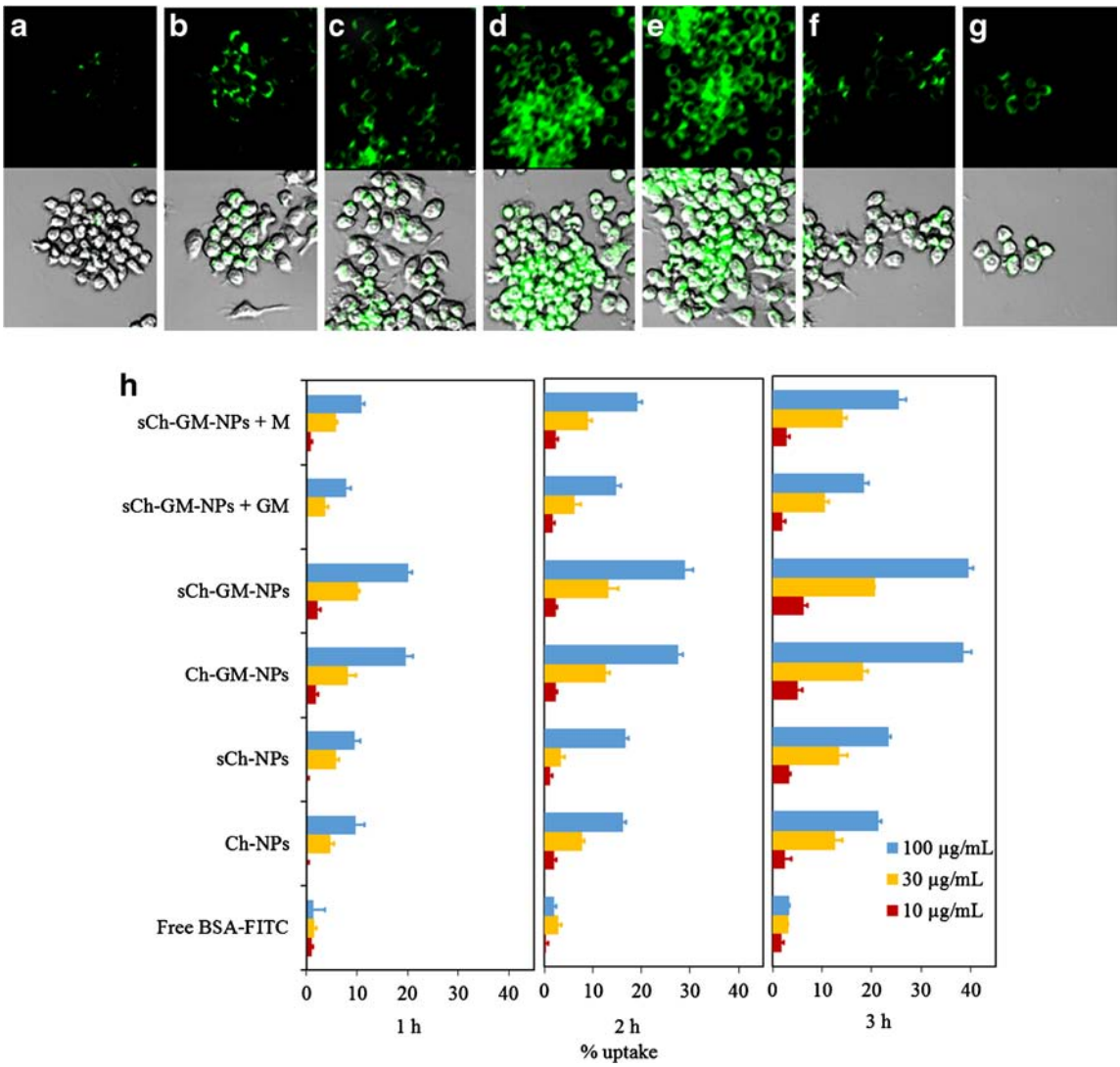

Fig. 4 Uptake by APCs: CLSM images showing uptake of (**a**) free BSA-FITC, (**b**) Ch-NPs, (**c**) sCh-NPs, (**d**) Ch-GM-NPs, (**e**) sCh-GM-NPs and after pretreatment with (**f**) glucomannan and (**g**) mannose in RAW 264.7 after 3 h of incubation. (**h**) Quantitative APC's uptake of BSA-FITC loaded chitosan nanoformulations using spectrofluorometric analysis after 1, 2, and 3 h of incubation.

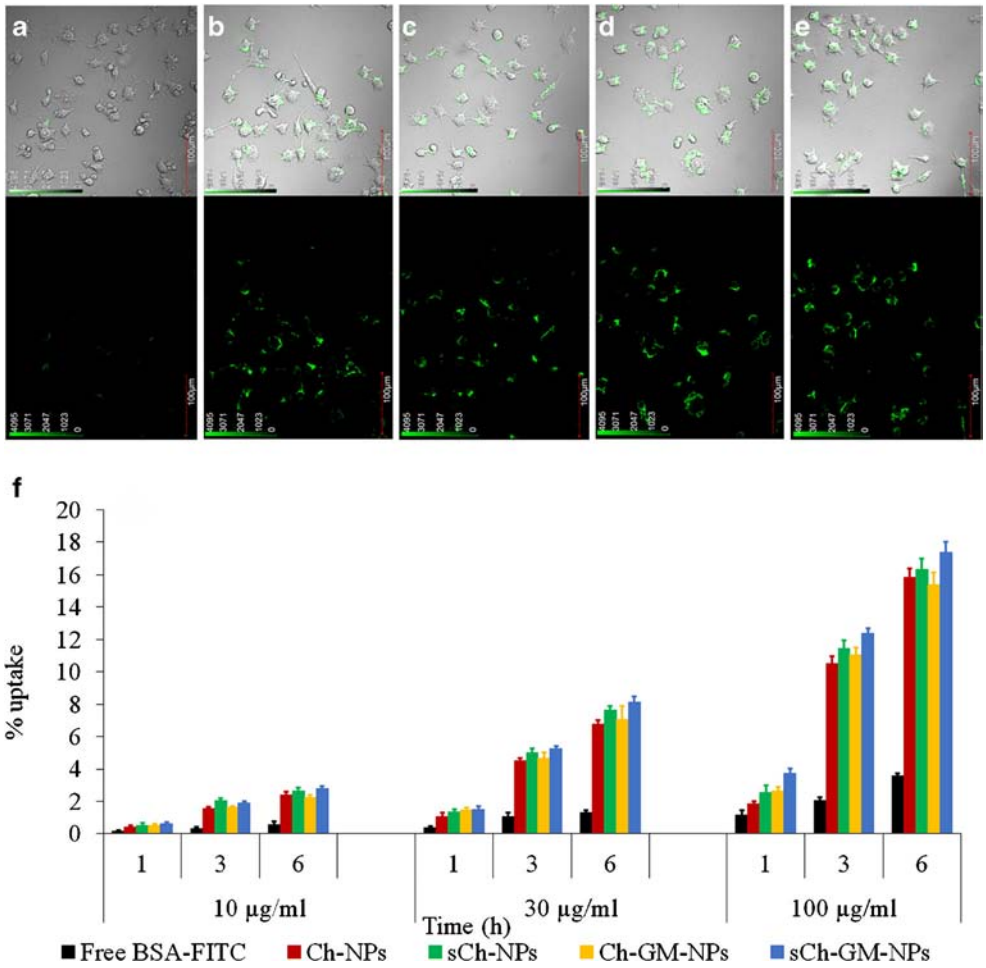

Fig. 5 Caco-2 uptake: CLSM images representing qualitative uptake of (**a**) free BSA-FITC, (**b**) Ch-NPs, (**c**) sCh-NPs, (**d**) Ch-GM-NPs and (**e**) sCh-GM-NPs. The quantitative uptake (**f**) of chitosan nanoformulations.

media. Therefore, Ch-GM-NPs were further stabilized using another cross linking agent namely glutaraldehyde. Covalent cross linking of glutaraldehyde with ionizable groups of chitosan may led to decrease in solubility potential of chitosan in acidic media. The covalent cross linking might have also reduced the swelling tendency or squeezed out the internal aquatic pores of chitosan nanoformulations, which might have resulted in shrinkage and reduction of particle size of stabilized chitosan nanoformulations. The residual glutaraldehyde which may posed toxicological issue was removed by lyophilization (28). Gas chromatography confirmed the non-detectable amount of glutaraldehyde in lyophilized product which suggested glutaraldehyde may be sublimed during the primary drying stage.

The final sCh-GM-NPs were found to be robust with high mechanical strength and stability in biological media (Table III). A higher stability of sCh-GM-NPs over other successive chitosan nanoformulations could be attributed to lesser surface ionization and thus solubilization at acidic condition. The change in zeta potential in SGF or SIF may be attributed adsorption of various oppositely charged species and ions present in media, however this phenomenon did not cause any unwanted effect on integrity of sCh-GM-NPs which was also confirmed by SEM analysis (data not shown). Any antigen when subjected to chemical and mechanical stresses may undergo chemical degradation and structural/conformational loss, which might have great impact on bioactivity or immune response (29). Therefore, chemical and conformational stability of TT entrapped within lyophilized chitosan nanoformulations was confirmed by SDS-PAGE and CD indicating the suitability of the method and formulation for delivery of antigens (Fig. 2). Surface stabilization also led to reduction in burst release of TT which was notably higher in case of conventional non-stabilized chitosan nanoformulations (Fig. 3). Dual crosslinking method increased the compactness of chitosan matrix as well as thickness of surface coating resulted in slower release over the time. Such controlled delivery of antigen may be helpful in development of 'single shot vaccine' with strong and persistent immune response (30).

Higher ($p<0.001$) time and concentration dependent uptake of sCh-GM-NPs by APCs in comparison to free antigen might be attributed to selective receptor mediated endocytosis via mannose (MR) (31) and glucose (GLUT) receptors present on the surface of the macrophages (32) which was explicitly

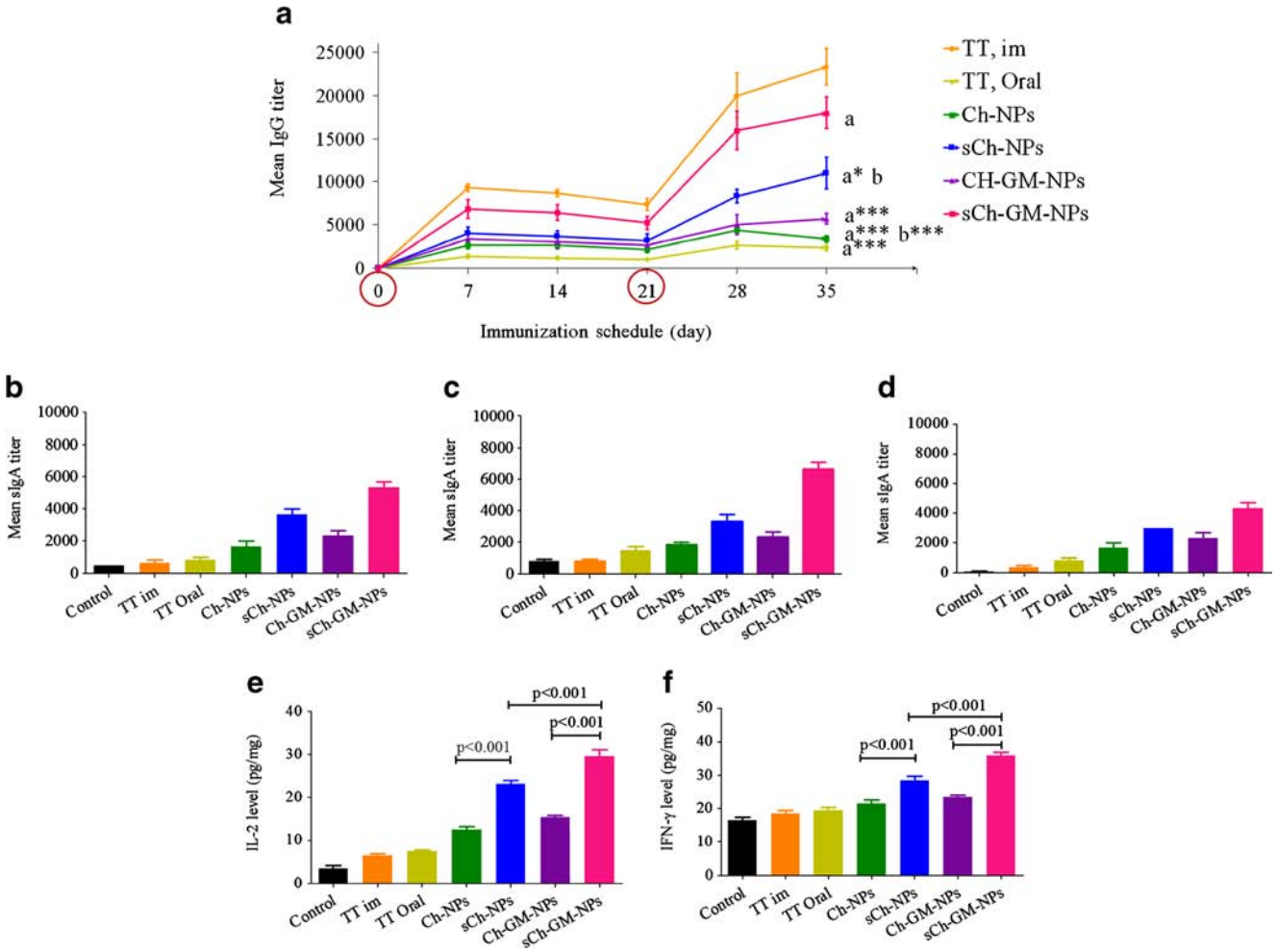

Fig. 6 Immune response following administration of TT loaded formulations: (**a**) mean serum IgG titer; mean secretory IgA titer in (**b**) saliva, (**c**) intestinal fluid and (**d**) fecal content; and cytokines levels viz. (**e**) IL-2 and (**f**) IFN-γ in spleen homogenates. Data are represented as mean ± SEM ($n = 5$).

reduced ($p < 0.05$) after incubation of mannose and glucose pretreatment. Similarly, higher Caco-2 uptake further confirmed the efficient internalization and permeation across the intestinal mucosa (33,34).

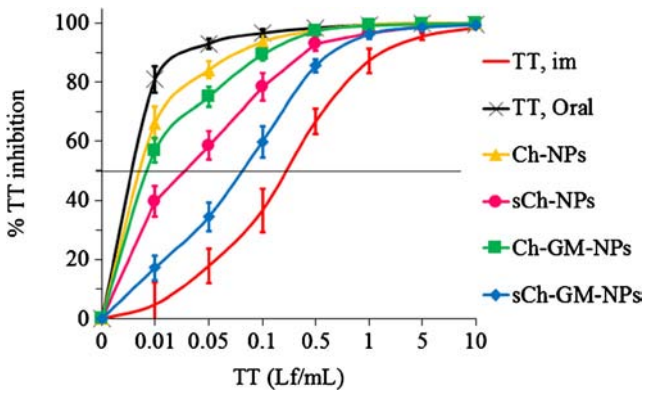

Fig. 7 Modified TT inhibition assay representing specific inhibition of anti-TT IgG antibody elicited in serum by TT loaded chitosan nanoformulations using known amount of TT.

In vivo evaluation of TT loaded chitosan nanoformulations evidenced the significant enhancement in humoral, mucosal and cell mediated responses than commercial TT vaccine (Fig. 6). Poor IgG titer after peroral administration of commercial TT vaccine could be attributed to degradation of TT in harsh GI environment, albeit higher IgG titer in case of stable and functionalized chitosan nanoformulations might be ascribed to '3P' mechanism (22) which comprises of (i) protection of TT against severe biological environment; (ii) permeation across intestinal mucosa (5,35); and subsequent presentation and processing of TT by APCs which might have stimulated the subsequent cascade mechanism include processing of TT via MHC-I and MHC-II pathways (36). Th1 and Th2 response elicited by MHC might have activated both the arms of the immune systems viz. humoral and cellular immunity. The increased levels of sIgA (mucosal immunity) in mucosal secretions may further offer the protective immunity at gastrointestinal, respiratory and genital tract (37). Negligible sIgA level was elicited by parenteral administration of TT vaccine owing to its inability to stimulate mucosal immune

system. Commercial TT vaccine might have failed to elicit all cascade mechanisms resulting in poor immune response and protection. The efficacy of chitosan nanoformulations was also established by TT inhibition using anti-TT IgG levels in serum. It confirmed that sCh-GM-NPs elicited protective immune response (>0.1 IU/mL) in comparison to other counterparts following peroral administration (38,39).

CONCLUSION

The findings of the present study suggested that sCh-GM-NPs can be a promising vaccine adjuvant for non-invasive oral immunization based on efficient elicitation of systemic, mucosal as well as cellular immune response. The novel tandem crosslinking method used for development of chitosan nanoparticles was foremost reason for higher immune response as it not only improved the mechanical strength of chitosan nanoparticles but also provided stability in simulated biological fluids without interfering the integrity and conformation of antigen. The technology is simple, economical, highly patient compliance and technically feasible to scale up which can be useful for mass immunization. However, *in vivo* challenge study and subsequent evaluation of immune response in other species may strengthen the utility of TT loaded sCh-GM-NPs. In the current scientific panorama, mass immunization is still the dream for scientific and government community such as UNICEF, WHO, GAVI, PATH, Melinda Gates Foundation, these findings, in turn are expected to benefit the design and development of stable glucomannosylated chitosan nanoparticles for variety of antigens other than TT.

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