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DEVELOPMENT AND CHARACTERIZATION OF *O*-METHYLATED FREE *N, N, N*-TRIMETHYLATED CHITOSAN NANOPARTICLES AS NEW CARRIERS FOR ORAL VACCINE DELIVERY IN MICE

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ABSTRACT

Chitosan (CS) has already gained considerable attentions as vehicles for mucosal immunizations due to its excellent biocompatibility, biodegradability and non-toxicity. However, poor aqueous solubility and loss of penetration-enhancing above pH 6 are major drawbacks for its use as oral vaccine carrier. The study aims to investigate the potential utility of O-methylated free trimethylated chitosan (CS-TM) nanoparticles as an effective adjuvant for oral vaccine delivery. Nanoparticles were formulated by modified coacervation method using different MW of CS consisting two different degree of quaternization (DQ) to which measles antigen was entrapped by an ionic cross-linking technique. Drug loading, encapsulation efficiency, and particle properties such as SEM, size distribution and zeta potential were evaluated. In vitro release studies showed an initial burst followed by extended release of antigen from all formulations, best fitted the Higuchi model. SDS-PAGE assay showed that CS-TM nanoparticles could effectively protect the antigen from degradation in acidic condition for at least 2 h. Cell viability was assessed using MTT assay into HT29 cell line. CS-TM nanoparticles of different formulations were orally administered to mice and immunological responses were evaluated using dried blood spot ELISA method. Obtained results showed that antigen-loaded CS nanoparticles induced strong humoral immune response and significant correlation was observed between the immune response with DQ. Protecting ability of antigen in the gastric environment, sustained release kinetics, enhancement of the systemic and mucosal immune responses and low cytotoxicity observed for the CS-TM nanoparticles demonstrated that it could be a promising platform for oral vaccine delivery.

KEY WORDS

Adjuvant, cytotoxicity, mucosal immunity, vaccine

INTRODUCTION

Oral vaccine delivery offers several attractive advantages over the traditional routes such as lower costs, ease of administration, higher patient compliance, reducing the need for trained personnel and averting vaccine-related infections correlated to the disposal and reuse of needles in systemic delivery as well as higher capacity of much immunizations [1-5]. It has been demonstrated that oral immunization induces mucosal IgA and systematic IgG antibodies responses, providing a complete immune response [6]. However, immune responses following oral administration are usually poor due to the rapidly degradation of antigen when in contact with the gastrointestinal fluids and inefficient targeting to the site of action in the gut [7]. Keeping this in mind,

several studies have been performed by researchers to show that by associating the vaccine with a number of particulate delivery systems, the degradation of the antigen in the harsh environment of the GI tract is prevented and the uptake by M-cells is enhanced by several times [8-10]. In addition, biodegradable particles allow a sustained release of the antigen, increasing the duration of the contact between antigen and immune cells thus favouring an effective immune response.

In the past decade, chitosan (CS) nanoparticles have immerged as novel carrier candidate because of their excellent stability, mucoadhesive property, and enhanced penetration capacity across mucosal barriers and good compatibility with vaccine antigen [11-14]. Additionally, CS nanoparticle can be easily constructed using tripolyphosphate (TPP) under very mild conditions without the application of harmful organic solvent at room temperature, which is a great benefit for the encapsulation of proteins, peptides and antigens [15]. Although CS nanoparticles have numerous advantages as delivery carriers for oral vaccination, solubility of CS only in acidic solution severely restricts their application to bioactive agents such as gene delivery carriers, peptide carriers and drug carriers [16]. Recently, there has been growing interest in the chemical modification of different molecular weight of CS of their functional groups (amine and hydroxyl) in order to improve their solubility and extend their applications [17, 18]. Trimethylated chitosan (CS-TM), a partially quaternized CS derivatives has been shown to have good water solubility, muco-adhesive properties and is able to open tight junctions above a certain degree of quaternization (DQ) [19]. These physicochemical properties have been major factors to its widespread evaluation as a component of oral protein and vaccine delivery purposes.

The DQ of CS-TM determines the number of positive charges available on the molecule for interactions with the negatively charged sites on the epithelial membrane and thereby influences its drug penetration-enhancing properties. The increase in solubility was also attributed to the replacement of the primary amino group on the C-2 position of CS with quaternary primary amino groups. Several studies have been performed to determine the optimal DQ with different molecular weight of CS for different types of drug delivery. CS-TM with higher DQ (>22%) were able to reduce the transepithelial resistance (TEFR) in a neutral environment and the maximum reduction in TEFR was reached with 48% DQ, and this effect did not increase further with higher DQ [20]. Since polymer chain length and extent of O-methylation affect the physicochemical as well as biological properties of CS-TM, O-methyalted free trimethylated CS may be the better choice as mucosal vaccine delivery vehicle [21].

To the best of our knowledge, there is no published data whereby O-methylated free CS-TM nanoparticles are explored as vehicles for the oral administration of vaccine. In this study, we hope to develop a novel oral antigen carrier based on CS-TM nanoparticles to meet the requirement of oral vaccine. Model antigen (measles antigen) was adopted to evaluate the physicochemical properties, loading efficiency of the particles and the stability of antigen loaded nanoparticles against acidic condition. Cytotoxicity was evaluated using HT29 human epithelial cell lines for the different formulation of nanoparticles. Finally, the effects of molecular weight and DQ of CS on the ability of these nanoparticles were studied in mice model to elicit significant immune response using DBS ELISA method. Two different DQ (18 and 34) of CS HMW and two different DQ (21 and 38) of CS MMW were used to study the effect of molecular weight of native CS and DQ of CS-TM on synthesis and characterization of nanoparticle formulation. Besides the primary goal, we also expected to obtain information regarding the suitability of the nanoparticle formulation process for the preservation of measles antigen immunogenicity.

MATERIALS AND METHODS

Materials

O-methylated free CS-TM of two different DQ was obtained through two-step reaction method published by Verheul *et al* [21]. CS-TM products synthesized were analyzed by ^{1}H- Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy (Bruker, Germany). Sodium tripolyphosphate, DMEM (Dulbecco's Modified Eagle's Medium), HEPES [N-(2-hydroxyethyl piperazine-N-(2-ethanosulphonic acid)],

MTT (Methylthiazolyl diphenyl-tetrazolium bromide) reagents, goat anti-mouse IgG peroxidise conjugate and TMB/H_2O_2 substrate for ELISA were procured from Sigma (USA). Anti measles monoclonal antibody was purchased from Millipore. BCA kit was purchased from Pierce (USA). Measles antigen was obtained from Serum Institute of India Ltd (Pune, India). 96-well flat bottom polystyrene plate was purchased from Nunc (Denmark). All other reagents were of analytical grade. Ultrapure water (MilliQ, Waters, USA) was used to prepare all solutions and freshly prepared solutions were used in all experiments.

Human intestinal epithelial cell (HT 29) were cultured and maintained in DMEM with supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). Cells were maintained in 5% CO_2 at 37 ^{0}C humidified incubator for 10 days.

Specific pathogen-free male BALB/c mice (6 weeks old, 22-25 g) from the animal house of Gupta College of Technological Sciences, India were used. They were kept into individual cages with autoclaved food and water *ad libitum*. All animals were housed in an aseptic room with 12 h light/dark cycle and temperature of 20 ± 2 ^{o}C. Mice were acclimated to the new environments for 1 week before being used for immunization.

Preparation of CS-TM nanoparticles

The formulation of CS-TM nanoparticles and loading of measles antigen was done by ionic gelation process technique using TPP as a cross linking agent under stirring [22]. Briefly, an aqueous solution of 2 mg/ml of CS-TM polymer containing 1% (w/v) Tween 80 and 1 mg/ml sodium tripolyphosphate were separately prepared. Subsequently, TPP solution (pH 8) was slowly added drop-wise to the CS-TM solution (pH 6) while stirring (450 rpm) at room temperature for 1 h, yielding a final pH of around 7. The nanoparticle suspensions were then concentrated by centrifugation at 10,000 × g for 15 min. The supernatants were discarded and the pellets were re-suspended in phosphate buffered saline (PBS).

6 mg of CS-TM was dissolved in 3 ml of PBS containing 600 mg measles antigen and 0.5% (w/w) Tween 80. Thereafter, 1.5 ml of an aqueous TPP solution (1 mg/ml) was added drop wise to the CS-TM measles antigen solution while stirring. For association of measles antigen with CS nanoparticle, 600 µg of measles antigen was incorporated in the TPP solution. Cryoprotectant mannitol was added to the resulting nanoparticle suspension and frozen at -70 ^{o}C for 48 h and then lyophilized in freeze dryer (FDU-S603, Operon, Korea) [24]. Freeze-dried CS nanoparticles were redispersed in ultrapure water for further use.

Loading efficiency and loading capacity of nanoparticles

Loading efficiency (LE) and loading capacity (LC) of measles antigen on CS nanoparticles were detected in an indirect way by determining the measles antigen remained in the aqueous phase using the BCA kit. The supernatant of blank CS nanoparticles was adopted as the blank to correct the absorbance reading value of the measles antigen loaded nanoparticles.

$$\mathrm{LE}(\%) = \frac{\text{total amount of antigen} - \text{free antigen}}{\text{total amount of antigen}} \times 100$$

$$\mathrm{LC}(\%) = \frac{\text{total amount of antigen} - \text{free antigen}}{\text{dried nanoparticle weight}} \times 100$$

Morphological characterization, size and surface charge

The morphological characteristics of nanoparticles were examined by scanning electron microscopy (JEOL JSM 5200, Japan). The particle size distribution was measured by laser diffraction and zeta potential of the particle was examined by zeta analyser using Zetasizer (Ver. 6.00, Malvern Instruments Ltd, UK) with ultrapure water as solvent (pH 7, 25 ^{o}C). These measurements were run at least three times with independent particle batches.

***In vitro* release studies**

CS-TM nanoparticles were incubated in 3 ml of phosphate buffer saline (PBS, pH 7.4) in Eppendorf tube and were placed in air shaker at 100 rpm at 37 ^{o}C. At predetermined time interval, the samples were centrifuged at 13,200 rpm for 20 min and the supernatant was replaced with fresh release medium and resuspended. The amount of antigen released was determined by BCA reagents and expressed as a distributive percentage of total antigen encapsulated

in the nanoparticle as calculated from the LE value. *In vitro* release experiments were repeated three times.

Stability study in simulated gastric fluid

Different formulations of CS-TM nanoparticle were incubated with 0.5 ml of HCl (0.01 M) in a shaker bath at 37 ^{O}C for 2 h. Finally, the reaction was stopped and neutralized by 0.5 ml of aqueous NaOH solution. Twenty four hours later, supernatant containing released antigen was collected and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in slab gel systems employed a 7.5% running gel and a 5.0% stacking gel. The protein sample was prepared in electrophoresis loading-buffer (60 mM Tris-HCl, pH 6.8, with 25% glycerol and 2% SDS containing 0.1% Bromophenol blue solution, and h-mercaptoethanol) and heated for 5 min at 95 ^{O}C. After electrophoresis, the protein bands were visualized by staining with Coomassie Blue.

***In vitro* cytotoxicity assay**

The MTT assay was performed according to the method described by Mosmann [24]. HT-29 cells were seeded into 96-well microplates at a density of 3000 cells/cm^2. The medium was removed 24 h after plating and fresh media containing different nanoparticle formulations at concentrations of 0.25, 1.0 and 4.0 mg/ml were added. After incubation for 1 h the medium was discarded, the cells were washed twice with phosphate-buffered saline and 50 µl of 5 mg/ml MTT solution in PBS were added to each well. The plates were incubated in a humidified atmosphere of 5% CO_2 at 37 ^{0}C for 6 h and the formazan crystals were dissolved by adding 100 µl dimethyl sulfoxide (DMSO) to each well. The absorbance was measured in triplicate at 570 nm, with a background correction at 630 nm, using a microplate ELISA reader (Multiscan Ex, Thermo Scientific). Results were recorded as percentage absorbance relative to untreated control cells. The cytotoxicity assay results were used to calculate cell viability after incubation with formulation as follows:

$$\text{Cell viability [\%]} = x/x_C \times 100\%$$

Where x is the absorbance in a well containing a particular formulation concentration and x_C is the absorbance for untreated control cells. All experiments were run four times.

Immunization studies

The immunogenicity of the CS-TM formulations was assessed in BALB/c mice following oral immunization. These animal experiments were approved by the Ethical Committee of Gupta College of Technological Sciences, Asansol, India. Based on our previous study results, we have selected the lowest dose of 20 µg and studied the influence of MW and DQ on the antibody responses. Mice were housed in group of six (n=6) animals for one week before the experiments were conducted. The mice were immunized orally with various formulations (Table 1) containing 20 µg of antigen (calculated from loading capacity of the nanoparticles) in 50 µl of saline solutions on days 0, 7, 14 and 28 via a 25-gauze 1-ml hypodermic needle [25]. Subcutaneous immunization was also carried out with measles vaccine to serve as positive control. One group of mice was treated with blank bead to serve as negative control and measles antigen for another group. All animals were conscious during the administration.

Table 1: Measles antigen loaded vaccine formulation for immunization study

Formulation code	Description	Group
CS HMW TM-18-MA	Measles antigen loaded CS HMW TM (DQ 18) nanoparticles	I
CS HMW TM-34-MA	Measles antigen loaded CS HMW TM (DQ 34) nanoparticles	II
CS MMW TM-21-MA	Measles antigen loaded CS MMW TM (DQ 21) nanoparticles	III
CS MMW TM-38-MA	Measles antigen loaded CS MMW TM (DQ 38) nanoparticles	IV
MA-SC	Measles antigen given subcutaneously, used as positive control	V
MA-ORAL	Measles antigen given orally	VI
CS HMW TM-18	Blank CS-TM (DQ 18) nanoparticles	VII

Sampling of body fluids

Blood samples were drawn from the tail-vein on day 7, 14, 28, 56 post administration using lancet [26]. Three drops of blood were collected in the protein-savour dry blood spot (DBS) card (Biorad, USA). Blood spots were allowed to dry overnight at room temperature. To obtain intestinal lavage fluids, on day 28 and 56 three mice were anesthetized i.p. with pentobarbital, were necropsied. Lengths of upper small intestine from each mouse were sectioned longitudinally, and the Intestinal lavage fluids were obtained by washing the intestine three times with 0.5 ml of ice-cold saline containing the protease inhibitor. DBS card was kept in a zip-lock bag at room temperature with desiccant and gastric lavage was stored at -20 ^{0}C until analyzed.

Enzyme-linked Immunosorbent assay

IgG antibody levels in sample were determined using DBS ELISA test. Disc approximately 3 mm diameter were punched from DBS cards and individual paper discs were transferred to individual wells of a 96-well titre plates and analytes were eluted with PBS containing 0.05% (w/v) Tween 20 (PBST) buffer. First, measles antigen (4 µg/ml) in carbonate buffer (pH 9.6) was added to microplates and incubated at 4 ^{O}C in a humid container. The wells were washed three times with PBST. To minimize non-specific interactions, 100 µl of PBST containing 2.5% (w/v) of dried skimmed milk powder was added to the wells and incubated for 1 h at 37 ^{O}C in a humid container and the plate were washed three times with PBST. Eluted samples, after serial dilution and incubated 2 h with known concentration of antigen, were added to the wells and the plates were incubated for 2 h at 37 ^{O}C in a humid container and washed. Then, 100 µl of anti-mouse IgG peroxidase conjugate, diluted 1:2000 in PBSTM buffer, were added into each well and plates were incubated for another 1 h at 37 ^{O}C. The plates were washed and 50 µl of substrate was added to each well and the reaction was allowed to keep in the dark for 15 min at RT. 100 µl of stopping solution was added to each wells and plates were read at 450 nm on a microplate reader.

Intestinal lavages were sonicated on ice to extract immunoglobulins from mucin and centrifuged (16,000 X g, 60 min, 4 ^{O}C). Supernatants were diluted with 200 µl of PBS for ELISA assay of specific IgA content as described above for IgG. Titre was derived as the maximum dilution of intestinal lavages giving an OD of 0.2 after correction for background. The results were presented as mean $\log_{10}$titre ± SEM per group.

Statistical analysis

If another method is not explicitly stated, the data were expressed as mean values ± standard deviation (SD). The effect of the CS molecular weight and degree of quaternization on the cell viability and adjuvaniticity compared to higher MW CS was assessed with the aid of SPSS 16.0 (SPSS Inc., Chicago, USA) using one way ANOVA with the significance level set at $p<0.05$. Modeling and comparison of dissolution profile and release kinetics were evaluated using DDSolver.

RESULTS AND DISCUSSION

Development of non-toxic and effective particulate oral delivery systems has always been challenge with the application of vaccine antigens. As a result, the biocompatible and biodegradable features of CS nanoparticles have gained more attention as a carrier for oral vaccination. One major obstacle to achieving satisfactory oral vaccinations in the antigen degradation results when exposed to the acidic gastric environment and the subsequent decreased uptake via lymphoid tissue in the gastrointestinal tract [27, 28].

Formulation and characterization of nanoparticles

Ionic gelation method was used for the preparation of nanoparticles and the use of complexation between oppositely charged macromolecules is easy and simple in mild conditions without using heat and organic solvents [29]. Moreover the reversible physical cross linking by electrostatic interaction has eliminated the possible toxicity and other undesirable effects of reagents used in chemical cross-linking. During pre-formulation study, various formulations were made with different initial concentrations of CS-TM (1-4 mg/ml) and TPP solutions (0.25–2 mg/ml) to establish preparation conditions at which nanoparticles are formed. It has been observed that, the intrinsic capacity of nanoparticles to interact with

mucosal surface depends upon size distribution, particle size below 1 µm results in uptake of particles by APCs which result in an increase in the immune response [30]. The criteria size, size distribution, colloidal stability and reproducibility of nanoparticle production were used to select the best formulation parameters to prepare nanoparticles. The optimal CS-TM nanoparticles were formed when the CS-TM/TPP ratio was 4 (w/w) which is close to the optimal ratio of 3–5 for CS/TPP (w/w) [29, 31]. At very low or high CS-TM/TPP ratios either a clear solution was seen (almost no particle formation) or larger nanoparticles with a low colloidal stability were obtained, respectively.

Loading efficiency and loading capacity of CS-TM nanoparticles

Antigen loaded CS-TM nanoparticles showed more than 79% loading efficiency in all the cases (Table 2). The high loading efficiency for antigen is likely due to electrostatic interactions between the positively charged CS-TM and negatively charged measles antigen (*pI*=4.5) at pH 7. Loading efficiency and loading capacity of CS-TM nanoparticles for measles antigen was influenced by the MW of CS and DQ%. Particles prepared using CS-TM MMW with 21% DQ possessed the highest LE and LC. This may be explained by the fact that the preparation of CS-TM MMW with 21% DQ particles made use of a lower amount of TPP. As a result, an intermolecular and/or intramolecular linkage between TPP and CS-TM was less, and thus the binding site on CS-TM for reacting with antigen was higher than other CS-TM [32]. The obtained high loading efficiency and loading capacity of CS-TM nanoparticles result evince the possibility to incorporate large amounts of antigen into the nanoparticles prepared with CS-TM.

Table 2: Particle size, zeta potential, loading efficiency and loading capacity of antigen loaded nanoparticles of two different molecular weight of CS-TM derivatives with two diferrent DQ (n=3, mean±SD)

S. No.	Composition	Mean particle size (nm)	Zeta potential (mV)	Loading efficiency (%)	Loading capacity (%)
1	CS HMW TM (18 %)-Antigen	612 ± 42	+22.8 ± 2.8	82.20 ± 2.68	3.34 ± 0.26
2	CS HMW TM (34 %)-Antigen	528 ± 38	+27.9 ± 3.2	79.34 ± 3.34	2.55 ± 0.32
3	CS MMW TM (21 %)-Antigen	352 ± 32	+26.4 ± 3.2	88.63 ± 2.16	5.46 ± 0.29
4	CS MMW TM (38 %)-Antigen	268 ± 21	+32.8 ± 1.8	84.18 ± 3.68	4.12 ± 0.38

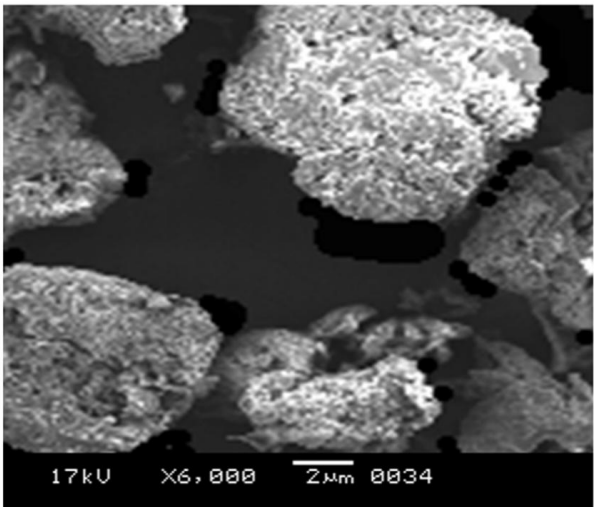
Morphological characterization, size and surface charges of CS-TM nanoparticles

The nanoparticle size and enhanced antigen loading efficiency might play a major role in predetermining the choice of a biodegradable polymer as carrier for effective oral vaccine delivery. A statistical significant correlation was observed between molecular weight of native CS and DQ% with particle size. The size of the nanoparticle increases with increased molecular weight of CS. CS HMW of 18 % DQ produced nanoparticles of mean diameter of 612 ± 42 nm, almost spherical with less aggregation and slight rough surface. Whereas, CS MMW of 20% DQ produced nanoparticles of mean diameter of 352 ± 32 nm, similar spherical shape fine free flowing powder with slightly more aggregation and decreased surface roughness. CS-TM with higher DQ produced comparatively smaller particles, and can be explained by a higher surface activity.

Surface charge (zeta potential) was clearly influenced by the DQ of CS-TM. The particle size, zeta potential and loading efficiency of antigen loaded CS-TM nanoparticles of two different MW are shown in **Table 2**. The zeta potential of CS nanoparticles is an

important factor for the stability and mucoadhesiveness of nanoparticles. Generally the higher zeta potential of particles led to more stability, whereas the lower zeta potentials of the particles induce agglomeration.

Figure 1: Scanning electron microscopy image of CS-TM nanoparticle (CS HMW DQ 34%) after freeze-drying process, magnification 6000X



***In vitro* release studies**

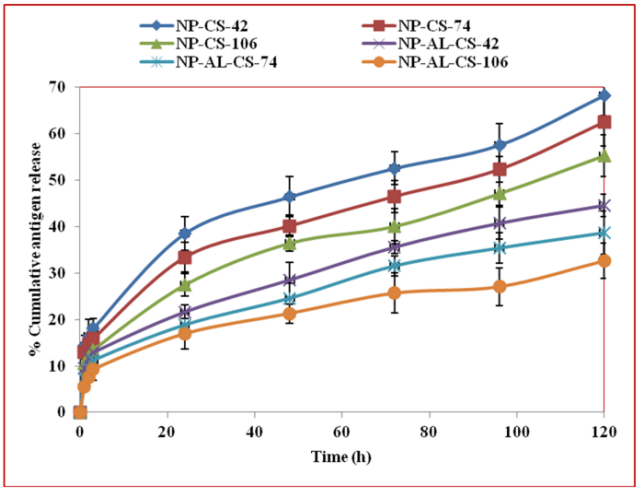
In order to investigate antigen release behaviour in physiological pH of CS-TM nanoparticles, they were incubated in release media of phosphate buffer pH at 7.4 and assessed by BCA kit. **Figure 2** demonstrates antigen release profiles up to 120 h of incubation period. As shown in Figure 2, all CS-TM nanoparticles showed an initial burst release of measles antigen in a period of 3 h, which was in the range of 13.3–25.4%. This initial rapid release, characterized as "burst effect", is due to the fact that some amounts of measles antigen were localized on the surface of nanoparticles by adsorption which could be released easily by diffusion. After this initial burst effect, a slower sustained and controlled release occurred throughout the incubation period and the release amount is in the range of 50.4-71.8%. Comparatively slower release rate was observed up to 50.4 % and 56.8 % were released after 120 h from CS HMW TM with 18% DQ and CS MMW TM with 21%, respectively. The results may be due to the fact that the nanoparticles in shrinking state entrapped antigen tightly and prevent it from penetrating through the compact structure. On the other hand, a continuous and fast release profile could be found from CS HMW TM with 34 % DQ and CS MMW TM with 38 % DQ, as shown in **Figure 2**, 63.4 % and 71.8 % of antigen released after 120 h, which may be due to the swelling behaviour of CS-TM nanoparticles with higher DQ%. Results conclude that measles antigen encapsulated CS-TM nanoparticles are strongly influenced on the DQ % of CS-TM and MW. CS-TM nanoparticles showed sustained release of antigen which is one of the important criteria for oral vaccine formulation.

Table 3 demonstrates correlation values (R^2) and release parameters determined from the results of model fitting of the release profiles. As seen from **Table 3**, according to correlation values, release data well fitted to the Higuchi model which indicates that antigen is released by diffusion. Moreover, the Korsmeyer-Peppas release model (high correlation values) exponent, *n*, is about 0.3, which confirms that the Fickian diffusion is the controlling factor in antigen release. Since the Kopcha model can easily be used to help quantify the contribution of diffusion and polymer relaxation, greatly improve that antigen release, occurred mainly as a result of the Fickian diffusion with regarding to the value of *A*, is far greater than the value of *B*.

Table 3 Results of model fitting of antigen release of various CS-TM nanoparticles in phosphate buffer pH 7.4

Kinetic model	Para meter	Formulation code			
		CS HMW TM (18 %)-Antigen	CS HMW TM (34 %)-Antigen	CS MMW TM (21 %)-Antigen	CS MMW TM (38 %)-Antigen
First order	R^2	0.87	0.81	0.78	0.75
Higuchi	R^2	0.97	0.94	0.92	0.91
Hixson-Crowell	R^2	0.81	0.78	0.75	0.69
Korsmeyer-Peppas	R^2	0.80	0.79	0.78	0.73
Kopcha	R^2	0.92	0.93	0.92	0.96
	A/B	43.08	36.93	36.87	37.12

Figure 2: *In vitro* release profiles of antigen from CS-TM nanoparticles in PBS at 37 ^{O}C in the first 120 h.

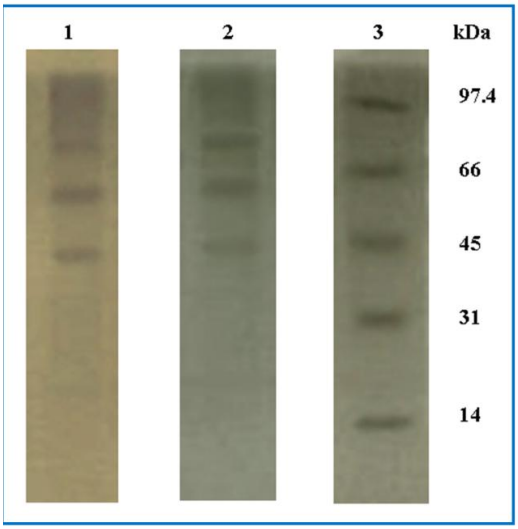


Stability study in simulated gastric fluid

The most conventional GI tract model involves incubation of particulate systems in an acidic media (pH 1-3). The residence time of drug delivery system during sequential through the GI tract is also a crucial factor that determines the viability and function of particulate systems, as is the buffering capacity of any ingested food. The survival rate of encapsulated particulate systems is expected to be higher with shorter exposure time (2 h) in simulated gastric fluid.

Antigen released from CS-TM nanoparticles under acidic medium was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. First prerequisite for effective oral vaccine formulation is to protect antigen from harsh condition in gastrointestinal after oral delivery. Gel electrophoresis analysis obtained under conditions of CS HMW TM 18% (shown in Lane 1) and CS MMW TM 21% (shown in Lane 2) nanoparticles containing antigen pre-treated with 0.1 M HCl for 2 h, then sustained release in PBS for 24 h. Antigen from CS-TM nanoparticles had clear bands at about 80, 60 and 45 kDa in both the lanes (Lane 1 and Lane 2), which implied that CS-TM nanoparticles could effectively protect antigen form degradation at acidic medium atleast for 2 h. So, the obtained CS-TM nanoparticles might be an effective oral antigenic carrier for mucosal vaccine. Lane 3 indicates molecular marker in **Figure 3**.

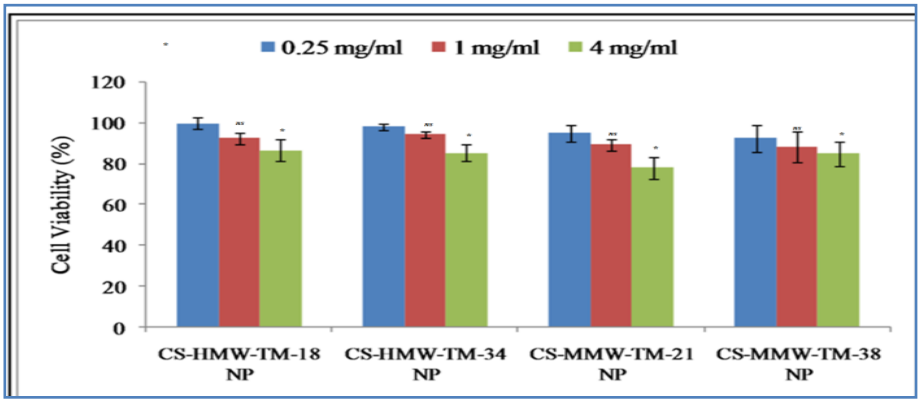
Figure 3: Gel electrophoresis analysis, obtained under conditions of CS HMW TM 18% coated (Lane 1) and CS MMW TM 21% coated (Lane 2) nanoparticles containing antigen pre-treated with 0.1 M HCl for 2 h, then sustained release in PBS for 24 h. Molecular weight marker (Lane 3).



***In vitro* cell viability studies**

The effects of CS-TM nanoparticles on HT 29 cells were investigated by testing metabolic activity via mitochondrial enzymes. For all nanoparticle formulations, cell viability percentage range between 80% and 100% in almost all the cases, in higher concentrations, considered as safe nanoparticle, except in few cases where nanoparticles were formulated with higher DQ%. Statistical analysis revealed no significant differences between different molecular weight. The membrane degrading dose-effect relation of CS MMW derivatives was more pronounced than that of CS HMW. The different behaviour of particles prepared with CS-TM of different MW and DQ with its direct correlation can be explained by the high amount of cationic moieties. Concluding from this, CS-TM with higher DQ seems to show much more amphiphilic properties than the lower DQ and hence shows a higher cytotoxicity; therefore its use as an excipient in a biological matrix should be considered carefully, and it should be replaced by lower substituted derivatives when possible.

Figure 4 Effect of antigen loaded nanoparticles, coated with CS HMW and CS MMW and at various DQ, at different concentrations, on the viability of HT 29 cells determined by MTT assay. Indicated values were mean ± SD (n=6). Statistical difference between the control groups and formulations are reported as * *p*<0.05; no significant difference, *ns p*>0.05

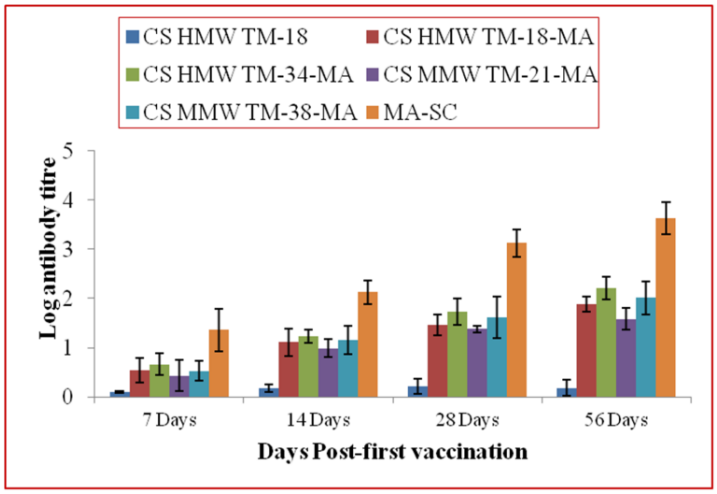


Immunological response

The mice were immunized orally with various antigen-loaded CS-TM nanoparticle formulations (Table 1) on days 0, 7, 14 and 28 and blood samples were drawn from the tail-vein on day 7, 14, 28, 56 post administration using lancet. All the samples were tested in the same day with same batch of reagents to minimize imprecision and bias. The various CS-TM formulations were compared to marketed measles formulation alone in an oral vaccination study. IgG responses were induced by all formulations containing measles antigen after prime vaccination and had increased after boost vaccination. CS-TM with DQ > 18% had strong adjuvant effect and statistically correlation was observed with DQ% that is not critically affected by DOM. All CS-TM formulations showed a higher number of responding mice and elicited significantly higher IgG titres than measles antigen alone. Additionally, no significant differences were observed among the IgG-titres of various post-days sample collection, indicating that free CS-TM does not influence total IgG titers.

Interestingly, antigen loaded HMW CS-TM induced significantly higher total IgG responses than all corresponding antigen-loaded MMW CS-TM formulations concludes MW of CS has an effect on adjuvanicity effect. The first explanation for the decreased adjuvant effect of MMW CS is a difference in interaction with cells, illustrated by a much lower *in vitro* toxicity than CS-TM HMW on various cell lines up to 40% DQ [21]. CS-TM HMW 34% DQ, however, hardly induced TEER effect or cell toxicity either but showed to be a good mucosal adjuvant. This indicates that toxicity studies, as carried out for these CS-TM nanoparticles cannot fully explain the loss of adjuvant effect by DQ. A second explanation for the comparatively poor adjuvant properties of MMW CS-TM is its enhanced gastrointestinal enzymatic degradation by lysozyme compared to HMW CS. Previous research showed that the extent of enzyme-catalyzed degradation of CS is highly dependent on the molecular weight of native CS [33]. Proteolytic enzymes are excreted in high concentrations in the stomach and causes rapid degradation of the antigen if exposed after oral administration, thereby strongly limiting its adjuvant effect on the oral vaccination. Finally, it has recently been suggested CS-TM can be recognized by specific receptors of the innate immune system and have strong adjuvant effect. However, since the type of adjuvant, animal model used, route of administration and animal model can affect the quality of immune responses, it is difficult to compare these studies to our data. Additional studies should be performed to provide mechanistical insight.

Figure 5: Anti-measles IgG titres of mice immunized with different CS-TM nanoparticles oral formulations of measles vaccine. Values are expressed as antibody titres of mice on day 7, 14, 28, 56 post administration. Titres were defined as the highest dilution resulting in an absorbance value twice that of non-immune plasma.

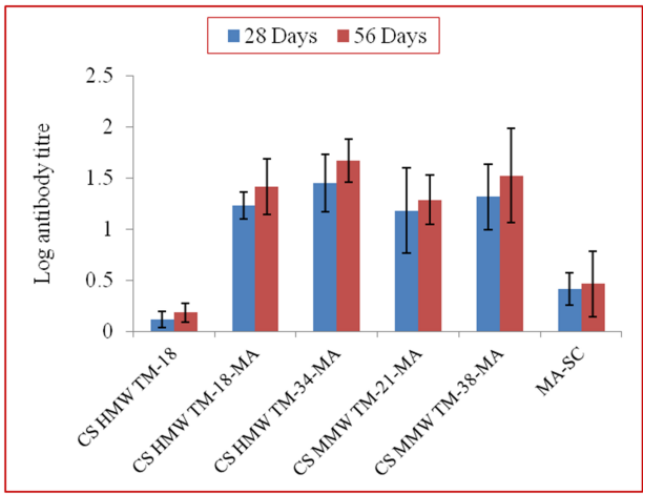


Mucosal anti-measles sIgA

The mice were immunized orally with various CS-TM formulations (**Table 1**) on days 0, 7, 14 and 28 and intestinal lavage fluids were collected on day 28 and 56 post administration. The IgA anti-measles levels in the intestinal lavages indicate, oral vaccination with CS-TM nanoparticles potentially induce stimulation of IgA-secreting cells of the GALT. Interestingly, although measles antigen subcutaneous immunization showed the highest serum IgG titres, no detectable sIgA was found in the intestinal washes of any of these mice. Altogether, the determination of sIgA in the intestinal lavages showed a relatively high variation within the formulation-groups likely due to the collection and detection methods. No significant differences of the average body weight and signs of illness were observed among all formulation-treated groups and the control group.

In the recent literature, the presence of non-responder orally or even nasally vaccinated mice is frequently reported, especially when the evaluation of the specific antibodies was performed after a single immunization. Moreover, to have a high percentage of seroconverted mice within the groups orally vaccinated, it has been always necessary to administer higher antigen concentrations with successive administration of the vaccine.

Figure 6: Secretory anti measles sIgA profile after oral administration of various formulations in CS-TM nanoparticles detected in individual intestine washing samples of mice giving an OD of 0.1 after subtraction of background and presented as geometric mean titre per group.



CONCLUSIONS

The vaccine-loaded CS-TM nanoparticles could be prepared with suitable and appropriate particle sizes, which is a very important factor in the delivery of the vaccine to the induction site of mucosa-associated lymphoid tissue for proper immune stimulation. Furthermore, both systemic and local immune responses can be induced in a dose- and time-dependent manner through vaccine-loaded CS nanoparticle. The nontoxic, highly bioavailable, mucoadhesive, and biodegradable nature of CS-TM and its particulate form are the main reason that they could become a successful oral vaccine carrier in the near future. Further research and the ability to modify chitosan may improve structural and physicochemical properties, increasing the potential of CS nanoparticle systems. However, there are many challenges including low physical and mechanical stability and low target specificity that have hindered the efficacy, practical use, and commercialization of CS nanoparticles. Thus, considering these factors, carefully designed and better functionalized CS nanoparticles could be prepared for fruitful future application.

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List of abbreviations used: PAGE- polyacrylamide gel electrophoresis, GALT-gut associated lymphoid tissue

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