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Development and characterization of alginate coated low molecular weight chitosan nanoparticles as new carriers for oral vaccine delivery in mice

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ABSTRACT

In the present study, nanoparticles of low MW chitosan (CS) were formulated in which measles antigen was entrapped and subsequently coated with sodium alginate. The size and surface properties of the nanoparticle can be tuned with different MW of CS. *In vitro* release studies showed initial burst release followed by extended release, best fitted in the Makoid–Banakar model ($R^2 > 0.98$). SDS-PAGE assay revealed that alginate coating could effectively protect antigen in acidic condition for at least 2 h. Cell viability was assessed using MTT assay into HT 29 cell line. Formulations were orally administered to mice and immunological responses were evaluated using ELISA method. Obtained results showed that measles antigen-loaded CS nanoparticles induced strong immune response and significant correlation was observed between the immune response with CS MW. Protecting ability of antigen in gastric environment, sustained release kinetics, systemic and mucosal immune responses and low cytotoxicity observed for the alginate coated nanoparticles demonstrated that LMW CS could be promising platform for oral vaccine delivery.

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1. Introduction

It is well established that protection against pathogenic organisms occurs better with the presence of mucosal antibody than with serum antibody (Baumann, 2008). Compared to traditional routes of administration, oral vaccine delivery systems offer several attractive advantages such as lower cost, ease of administration, higher patient compliance, reducing the need for trained personnel, averting vaccine-related infections correlated to the disposal and reuse of needles in systemic delivery as well as higher capacity for mass immunizations (Fooks, 2004; Kim, Lee, & Jang, 2012; Kostrzak et al., 2009; Zhu & Berzofsky, 2013). It has also been demonstrated that orally administered antigens can induce both local and systemic immune responses, providing a complete immune response (Neutra & Kozlowski, 2006). However, immune responses following oral administration of vaccines are usually poor due to rapid degradation of antigen in the harsh gut environment and their poor uptake by appropriate target sites, namely M cells located in the Peyer's patches (Czerkinsky & Holmgren, 2009). Keeping this in mind, several studies have been performed 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 (Delie & Blanco-Prieto, 2005; Eyles, Sharp, Williamson, Spiers, & Alpar, 1998; van der Merwe, Verhoef, Verheijden, Kotze, & Junginger, 2004). 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 decades, chitosan (CS) has been extensively used as a carrier candidate because of its excellent stability, mucoadhesive property, enhanced penetration capacity across the mucosal barriers and good compatibility with vaccine antigen (Borges et al., 2007; Lee et al., 2011; Yoo et al., 2010; Zaharoff, Rogers, Hance, Schlom, & Greiner, 2007). The formation of CS nanoparticles is a process based on the complexation of oppositely charged molecule

Abbreviations: PAGE, polyacrylamide gel electrophoresis; GALT, gut associated lymphoid tissue.

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by electrostatic interactions (Csaba, Koping-Hoggard, & Alonso, 2009). Nanoparticles can be easily constructed using an ionic gelation process that involves sodium tripolyphosphate as a negatively charged molecule and CS as polycation (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997; Oliveira et al., 2012). The mild technique involves the mixing of two aqueous solutions at ambient temperature while stirring without using sonication or organic solvents. Proteins with low isoelectric point, such as BSA and measles antigen, were better associated with the nanoparticles when dissolved in the alkaline sodium TPP solution (Li et al., 2008). Although CS nanoparticles have numerous advantages as delivery carriers for oral vaccination, their limited ability to control the release of encapsulated antigens and easy solubility in acidic medium largely hamper their development (Li et al., 2008). These obstacles may be overcome by coating those antigen loaded particles with an acid-resistant polymer, like sodium alginate (Borges et al., 2006).

Sodium alginate, an anionic polysaccharide with favourable biological properties, can be easily interacted with cationic CS nanoparticles to form an electrolyte complex *via* electrostatic interactions (Du et al., 2013; Kim et al., 2002). Alginate-CS polyionic complexes form through ionic gelation *via* interactions between the cationic amine groups of CS and the anionic carboxyl groups of alginate (Gazori et al., 2009). This coating procedure was performed at relatively mild conditions without using any organic solvent at room temperature, enabling antigens to be incorporated into the CS/alginate matrices with retention of biological activity (Wee & Gombotz, 1998). Calcium ion is used as crosslinking agent to strengthen and stabilize the particles (Borges et al., 2006). Recently, Liu et al. (2013) demonstrated that alginate-coated CS nanoparticles containing DNA are effectively taken up and expressed by the Payer's patches. Release profiles of vaccines from these delivery systems showed that burst release of loaded BSA in pH 1.2 (simulated gastric fluid) was largely prevented by its entrapment in alginate-coated CS particles (Anal, Bhopatkar, Tokura, Tamura, & Stevens, 2003). Moreover, Borges et al. (2006) demonstrated that sodium-alginate coated nanoparticles were readily able to be taken up by rat Peyer's patches which is one of the essential features to internalize, deliver and target the intact antigen to specialized immune cells from gut associated lymphoid tissue (GALT). *In vivo* studies have shown higher antibody titre for alginate coated CS nanoparticles compared to plain CS nanoparticles (Malik, Goyal, Zakir, & Vyas, 2011). This delivery system also acted as an effective adjuvant for hepatitis B surface antigen when subcutaneously administered in a mouse model (Borges et al., 2008). It has been demonstrated that transfection efficiency is closely related to the MW of polymer. CS of 10–150 kDa with a specific degree of deacetylation allows maximum transgenic expression *in vitro* and CS nanoparticles less than 500 nm in diameter are transported through an endocytotic mechanism (Godbey, Wu, & Mikos, 1999; Lavertu, Methot, Tran-Khanh, & Buschmann, 2006; Liu et al., 2013).

Recently, there has been growing interest in low molecular weight CS (CS-LMW) as a new mucosal delivery vehicle due to its greater solubility in neutral aqueous solvents with low viscosity and faster degradation than higher molecular weight CS counterparts (Amoozgar, Park, Lin, & Yeo, 2012; Fernandes et al., 2012; Liu et al., 2013). Vila et al. (2004) reported that nanoparticles made of CS-LMW with tetanus toxoid (TT) as a model protein could be produced by an ionic-cross linking technique and proving to be promising carrier for nasal vaccine delivery. Following intranasal administration, TT-loaded nanoparticles elicited an increasing and long-lasting humoral response as compared to the soluble antigen. Plasma or serum samples have traditionally been used for generation of serological data due to the difficulties associated with handling whole blood. However, this approach requires relatively large volumes (200–1000 μl) of blood in order to produce the required volume of matrix for bioanalysis, which makes it difficult to generate serial profiles in mice test models. Composite profiles are therefore necessary, which can result in lower quality data and requires the use of a greater number of animals. Conventional ELISA are formatted to be used only for determining whether a given sample contains antibody at a concentration above or below a set threshold value, although quantification of antibody response is prerequisite to monitor the progress after immunization. In recent years, dried blood spot (DBS) assays have received increasing attention as an alternative to venous blood sampling and can be adopted in preclinical serological studies.

To the best of our knowledge, there is no published data whereby alginate coated CS-LMW nanoparticles are explored as vehicles for the oral administration of vaccine. In this study, we have tried to develop a novel oral vaccine carrier based on alginate coated CS-LMW nanoparticles to meet the requirement of oral vaccine. So the objectives of the studies are to develop the nanoparticle based oral vaccine delivery system and the delivery system will be adopted to evaluate the physicochemical properties, loading efficiency of the particles and the stability of antigen loaded nanoparticles against acidic condition. Cytotoxicity will be evaluated using HT 29 human epithelial cell lines for the different formulation of nanoparticles. Finally, the effects of molecular weight of CS (42, 74 and 106 kDa) on the ability of these nanoparticles will be studied in a mouse model to elicit significant immune response as measured using DBS ELISA method.

2. Experimental

2.1. Materials

CS-LMW of three different molecular weight (42, 74 and 106 kDa) was obtained through oxidation–reduction reaction of high molecular weight CS (MW 375 kDa) (Sigma Aldrich, USA) using $NaNO_2$ as described by Mao et al. (2004). Briefly, varying quantities of 0.1 M $NaNO_2$ were added to the CS solution at 25 °C under magnetic stirring, and the reaction was left overnight to assure completion of the degradation. The depolymerisation process of CS has been previously simulated based on Box–Benkhen design experiments. With this approach, the required amount of $NaNO_2$ could be predicted in order to obtain approximate molecular weight of 42, 76 and 106 kDa. The molecular weight of the CS was determined using high pressure size exclusion chromatography (HPSEC).

Sodium tripolyphosphate (TPP), sodium alginate, $CaCl_2$, 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 kindly donated by 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 cells (HT 29) were cultured and maintained in DMEM with supplemented with 10% foetal bovine serum and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA). Cells were maintained in 5% CO_2 at 37 °C humidified incubator for 10 days.

Immunogenicity of the antigen was evaluated in 6 weeks old specific pathogen-free male BALB/c mice. Animal care and handling were maintained according to the guidelines established by the Institutional Animal Ethical Committee (IAEC) of Gupta College of Technological Sciences, India. The experiment protocols were

approved by IAEC and the experimental procedures were in accordance with IAEC.

2.2. Preparation of CS nanoparticles

CS nanoparticles were prepared by an ionotropic gelation process technique using TPP as a crosslinking agent under stirring (Li et al., 2008). Briefly, 0.2% (w/v) CS solution was prepared in 25 mM sodium acetate buffer (pH 5.2) and TPP was dissolved in ultra-pure water in order to obtain 0.84 mg/ml solutions. Nanoparticles were formed when 10 ml TPP solution was added drop wise to a 10 ml CS solution and magnetically stirred at 300 rpm during 30 min. For association of measles antigen with CS nanoparticle, 600 μg of measles antigen was incorporated in the TPP solution. Cryoprotectant mannitol (1:1) was added to the resulting nanoparticle suspension, frozen at −70 °C for 48 h, and then lyophilized in a freeze dryer (FDU-S603, Operon, Korea). Freeze-dried CS nanoparticles were redispersed in ultrapure water for further use.

2.3. Preparation of alginate coated CS nanoparticles

Antigen loaded CS nanoparticle suspension (20 ml, pH 5.1) was added drop-wise to sodium alginate solution (20 ml, pH 7.0) at concentration of 1 mg/ml under mild stirring for 30 min at room temperature. Alginate coated CS nanoparticles were redispersed into aqueous solution of $CaCl_2$ (pH 7.0, 0.5 mmol/l) to crosslink the alginate layer on the surface of antigen loaded CS nanoparticles. Cryoprotectant mannitol (1:1) was added to the resulting suspension and stored at −70 °C for 48 h before freeze-drying.

2.4. Loading capacity of antigen in coated nanoparticles

Loading capacity (LC) of measles antigen loaded CS nanoparticles was calculated in an indirect way by determining the measles antigen remaining in the aqueous phase using the BCA kit (Borges et al., 2006). Aliquots of the resulting nanoparticle suspension were centrifuged for 20 min at 18,000 × *g* and the supernatants were then separated from the nanoparticles. The supernatant of blank CS nanoparticles was adopted as the blank to correct the absorbance reading value of the measles antigen loaded nanoparticles. The LC values of antigen loaded nanoparticles were calculated from the following equations:

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

2.5. 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 °C). These measurements were run at least three times (replicates) and were presented as mean ± SD.

2.6. In vitro release studies

Approximately 20 mg dried nanoparticle samples (CS or alginate-coated CS) were incubated in 3 ml of phosphate buffered saline (PBS, pH 7.4) in an Eppendorf tube, and then placed in an air shaker at 100 rpm at 37 °C. At predetermined time intervals, the samples were centrifuged at 13,200 rpm for 20 min and the supernatant was replaced with fresh release medium to reach the original volume and resuspended. The amount of antigen released was determined by BCA reagents as described in Section 2.4. According to protocol, the amount of antigen released was expressed as percentage of total antigen encapsulated in CS nanoparticles as calculated from the LC value. A sample consisting of only non-loaded nanoparticles re-suspended in PBS was used as a blank. *In vitro* release experiments were repeated three times.

2.7. Stability study in simulated gastric fluid

Different formulations of CS nanoparticles (antigen loaded CS nanoparticles and alginate coated antigen loaded nanoparticles) were incubated with 0.5 ml of HCl (0.01 M) in a shaker bath at 37 °C for 2 h. Then, acid was neutralized by 0.5 ml of aqueous NaOH (0.01 M) solution. These mixtures were allowed to stand for another 24 h, then diluted with PBS (pH 7.4) to a final volume of 4 ml. 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 2-mercaptoethanol) and heated for 5 min at 95 °C. The pure antigen was used as control. After electrophoresis, the protein bands were visualized by staining with Coomassie Blue.

2.8. In vitro cytotoxicity assay

To investigate the potential cytotoxicity of uncoated and alginate coated CS-antigen nanoparticles, the cell viability was analyzed using HT 29 cell lines in a MTT assay. The HT 29 human epithelial cell line was selected in the present study as a convenient *in vitro* model to assess polymer toxicity (Sergent, Paget, & Chevillard, 2012). The assay was performed according to the method described by Mosmann (1983). 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 the cells were incubated for 6 h with 50 μl nanoparticle in HBSS (Nanoparticle concentrations were 0.25, 1.0 and 4.0 mg/ml). SDS (10 mg/ml) was used as positive control and HBSS as reference for 100% cell viability. After incubation for 6 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 °C for another 6 h. Subsequently, the wells were emptied, 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). The MTT assay was repeated three times.

2.9. Immunization studies

The immunogenicity of the antigen was assessed in BALB/c mice following oral immunization. Mice were housed in groups of six ($n = 6$) animals for 1 week before the experiments were conducted. 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-guage 1 ml hypodermic needle (Song et al., 2005). Subcutaneous immunization was also carried out with measles vaccine to serve as positive control. One group of mice was treated with alginate coated blank beads to serve as negative control and measles antigen was administered to another control group. All animals were conscious during the administration.

Table 1
Measles antigen loaded vaccine formulation for immunization study.

Formulation code	Description	Group
AL-CS-42-MA	Measles antigen loaded alginate coated CS (MW 42 kDa) nanoparticles	I
AL-CS-74-MA	Measles antigen loaded alginate coated CS (MW 74 kDa) nanoparticles	II
AL-CS-106-MA	Measles antigen loaded alginate coated CS (MW 106 kDa) nanoparticles	III
AL-CS-42	Alginate coated CS (MW 42 kDa) nanoparticles, used as negative control	IV
MA-SC	Measles antigen given subcutaneously, used as positive control	V
MA-ORAL	Measles antigen given orally	VI

2.10. Sampling of body fluids

Blood samples were drawn from the tail-vein on day 7, 14, 28, 56 post administration using a lancet (Hoff, 2000). 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, then 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 °C until analyzed.

2.11. ELISA

IgG antibody levels in each sample were determined using a DBS ELISA test. Discs of approximately 3 mm diameter were punched from DBS cards, 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 °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 °C in a humid container and the plate were washed three times with PBST. Eluted samples were serially diluted 5-fold starting at either a 1:10 or 1:100 dilution and incubated 2 h with a known concentration of antigen, then were added to the wells and the plates were incubated for 2 h at 37 °C in a humid container and washed. Then, 100 μl of anti-mouse IgG peroxidase conjugate, diluted 1:2000 in PBSTM buffer, was added into each well and plates were incubated for another 1 h at 37 °C. The plates were washed, 50 μl of substrate was added to each well, and the reaction was allowed to proceed in the dark for 15 min at room temperature. 100 μl of stopping solution (1 N sulphuric acid) was added to each well 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 × *g*, 60 min, 4 °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. Reciprocal dilutions corresponding to endpoint titres were determined and were presented as mean $\log_{10}$titre ± SEM per group.

2.12. 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 on the cell viability and adjuvant capacity was assessed with the aid of SPSS 16.0 (SPSS Inc., Chicago, USA) using one way ANOVA with the significance level set at $p < 0.05$. Modelling and comparison of dissolution profile and release kinetics were evaluated using DDSolver.

3. Results and discussion

3.1. Formulation and characterization of CS LMW nanoparticles

To achieve the nanoparticle formulation for measles antigen with CS LMW, systematic preformulation studies were carried out and the composition of this formulation was chosen as CS/TPP ratio of 2:0.84 and CS/sodium alginate ratio 2:1. Antigen loaded nanoparticles were systematically characterized to evaluate morphology, drug loading and releasing efficiency. The study of scanning electron microscopic images revealed that antigen-loaded alginate-coated CS nanoparticles have irregular shape with slight aggregation (Fig. 1). The particle sizes and zeta potentials of antigen loaded alginate uncoated and coated CS nanoparticles of three different MW are shown in Table 2. Decreasing the MW of CS resulted in a decrease in nanoparticle size and increasing loading efficiency (Lee, Lee, & Jon, 2010). However, very low MW CS may have poor loading efficiency and may not form stable nanoparticles (Fernandes et al., 2012). Vaccine carried with alginate coated CS nanoparticles exhibits similar trends in increasing particle size compared with CS nanoparticles in all three cases. 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 (Gan, Wang, Cochrane, & McCarron, 2005). CS molecular weight effect on encapsulation is largely the consequence of how the cationic CS chain interacts with protein molecules which have a more elaborate and complex 3-D structure as polypeptide chains contain both positive and negatively charged functional groups, and also

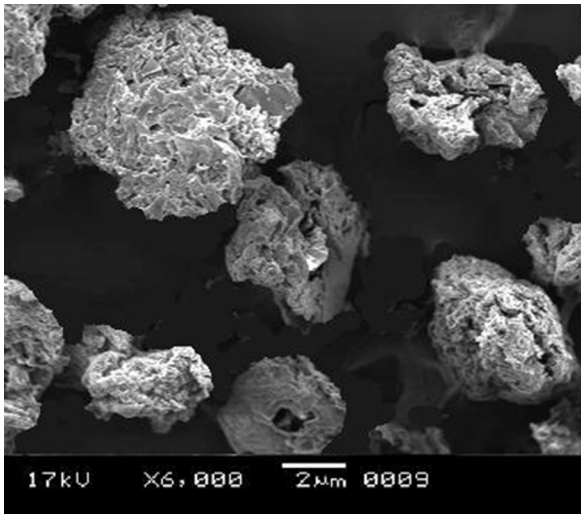

Fig. 1. Scanning electron microscopy image of antigen loaded alginate coated CS (MW 106 kDa) nanoparticle after freeze-drying process, magnification 6000×.

Table 2
Particle size, zeta potential and loading capacity of antigen loaded three different molecular weight of CS-LMW nanoparticles without or with alginate coated nanoparticles ($n = 3$, mean ± SD).

S. No.	Composition	Mean particle size (nm)	Zeta potential (mV)	PDI	Loading capacity (%)
1	LMW-CS (42 kDa)-Antigen	284 ± 36	+31.6 ± 1.4	0.310 ± 0.08	2.87 ± 0.46
2	Alginate/LMW-CS (42 kDa)-Antigen	432 ± 52	+1.65 ± 0.4	0.368 ± 0.06	1.56 ± 0.14
3	LMW-CS (74 kDa)-Antigen	507 ± 37	+21.4 ± 1.2	0.412 ± 0.04	3.41 ± 0.88
4	Alginate/LMW-CS (74 kDa)-Antigen	651 ± 28	−10.1 ± 0.6	0.438 ± 0.08	2.10 ± 0.26
5	LMW-CS (106 kDa)-Antigen	852 ± 76	+14.2 ± 0.6	0.472 ± 0.05	2.50 ± 0.32
6	Alginate/LMW-CS (106 kDa)-Antigen	1003 ± 45	−24.1 ± 0.9	0.484 ± 0.03	1.23 ± 0.18

hydrophobic regions which tend to fold inside the 3-D structure in an aqueous environment (Gan et al., 2005). However, encapsulation efficiency of a protein, tetanus toxoid, was high, irrespective of the CS molecular weight and indicated that the entanglement of the protein within the CS chains is not affected by their molecular weight within the range studied (Vila et al., 2004).

3.2. *In vitro release studies*

The release profiles of antigen from uncoated and alginate coated CS nanoparticles in phosphate buffer are shown in Fig. 2. CS nanoparticle formulations exhibited two release phases, a rapid release over the first 3 h followed by a slow release for up to 120 h. The burst release might be attributed to the fact that antigen was loosely bound onto CS nanoparticles by ionic interactions which could be easily desorbed in an ionic environment (Chen, Zhang, & Huang, 2007). The second phase might correspond to those antigen molecules more efficiently entrapped and tightly bound to the CS molecules. Antigen release was relatively rapid for the first 3 h in case of lower CS MW, which can be attributed to an easier detachment of low MW CS molecules and antigen molecules located at the surface of the particles. However, alginate coated CS nanoparticles could increase the stability of CS nanoparticles in the PBS, resulting in extended release of antigen. The dissolution data were plotted according to different models and drug release profiles of formulations F2–F6 were compared with that of reference product (F1) to evaluate the effect of process using the similarity factor (f_2). The curvilinear nature of the cumulative percent antigen released *versus* time plots depicts that the release of antigen from the nanoparticles does not follow zero-order or first order kinetics (Table 3). The *in vitro* dissolution studies suggest that drug release was governed by Higuchi kinetics. However, the mathematical expression that best describes the antigen release from these nanoparticles is the Makoid–Banakar model in which the resultant R^2 values were greater than 0.98. The Korsmeyer–Peppas release exponent, n, is approximately 0.3, confirming that the release of antigen is governed by a diffusion process. So it can be confirmed that antigen release is controlled by a Fickian diffusion process that will also result in sustained release of antigen in blood. Antigen release from nanoparticles was found to be affected by antigen–polymer interaction, decreasing with higher MW of CS (Costa & Sousa Lobo, 2003).

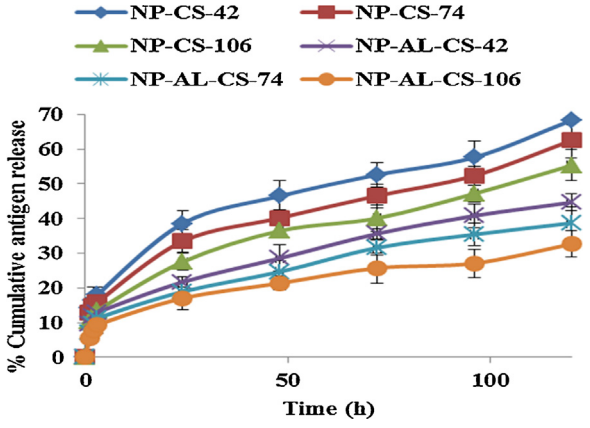

Fig. 2. *In vitro* release profiles of antigen from uncoated and alginate coated CS nanoparticles in PBS at 37 °C in the first 120 h.

3.3. *Stability study in simulated gastric fluid.*

Antigen released from uncoated and alginate coated CS nanoparticles in an acidic medium was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. The first prerequisite for effective oral vaccine formulation is to protect antigen from the harsh conditions in the stomach after oral administration. According to Fig. 3, molecular weight marker was shown in Lane 6, and the measles antigen incubated with PBS (pH 7.4) for 24 h exhibited three clear bands at about 80, 60 and 45 kDa (Lane 5). However, measles antigen pretreated with 0.01 M HCl for 2 h had a faint smear (Lane 4) which indicated that the antigen underwent serious degradation in an acidic medium.

The SDS-PAGE data clearly indicates uncoated CS nanoparticles containing measles antigen were degraded in the presence of acid. However, antigen from alginate coated CS nanoparticles had clear bands at about 80, 60 and 45 kDa in both the lanes (Lane 1 and Lane 2), which implied that alginate coating of CS nanoparticles could effectively protect antigen from degradation in an acidic medium for at least 2 h. So, the obtained alginate coated CS nanoparticles might be an effective oral antigenic carrier for mucosal vaccine. Keeping this in mind, immunological study was performed only with alginate coated CS nanoparticles.

3.4. *In vitro cell viability studies*

The MTT test results indicated that both alginate-coated (% cell viability 86.43 ± 4.60) and uncoated (% cell viability 82.58 ± 2.80) nanoparticles of higher MW CS (MW 106) had minimal effects on HT-29 cells even at higher concentration of 4 mg/ml. No significant difference in cytotoxicity was observed among nanoparticles formulated with three different MW (Fig. 4). These results are in good agreement with other studies where MW of CS did not interfere with cell viability (Huang, Khor, & Lim, 2004; Opanasopit et al., 2007; Richardson, Kolbe, & Duncan, 1999). Cytotoxicity was observed only for higher concentration (4 mg/ml) for the formulation with CS of MW 42 kDa. Interestingly, alginate coating increases cell viability, indicating that the cytotoxicity is related to the charge density of the particle; toxicity increases with increasing nanoparticle positive charge density (Yang, He, Duan, Kui, & Li, 2007).

Table 3
Results of model fitting of antigen release of various nanoparticles in phosphate buffer pH 7.4.

Kinetic model	Parameter	Formulation code					
		NP-CS-42	NP-CS-74	NP-CS-106	NP-AL-CS-42	NP-AL-CS-74	NP-AL-CS-106
First order	R^2	0.684	0.698	0.742	0.664	0.689	0.631
	k_1	0.011	0.009	0.008	0.006	0.005	0.004
Higuchi	R^2	0.915	0.928	0.952	0.927	0.949	0.936
	k_H	6.390	5.725	5.044	4.214	3.666	3.017
Korsmeyer-Peppas	R^2	0.991	0.986	0.987	0.982	0.986	0.989
	k_{KP}	12.846	10.889	8.684	7.967	6.527	5.632
	n	0.338	0.350	0.374	0.352	0.366	0.355
Makoid-Banakar	R^2	0.994	0.995	0.996	0.997	0.995	0.994
	k_{MB}	13.809	12.454	10.123	9.526	7.631	6.225
	n	0.295	0.272	0.289	0.250	0.280	0.297
	k	−0.001	−0.002	−0.002	−0.003	−0.003	−0.002
f_2		Reference	66.05	51.14	41.07	36.28	31.83

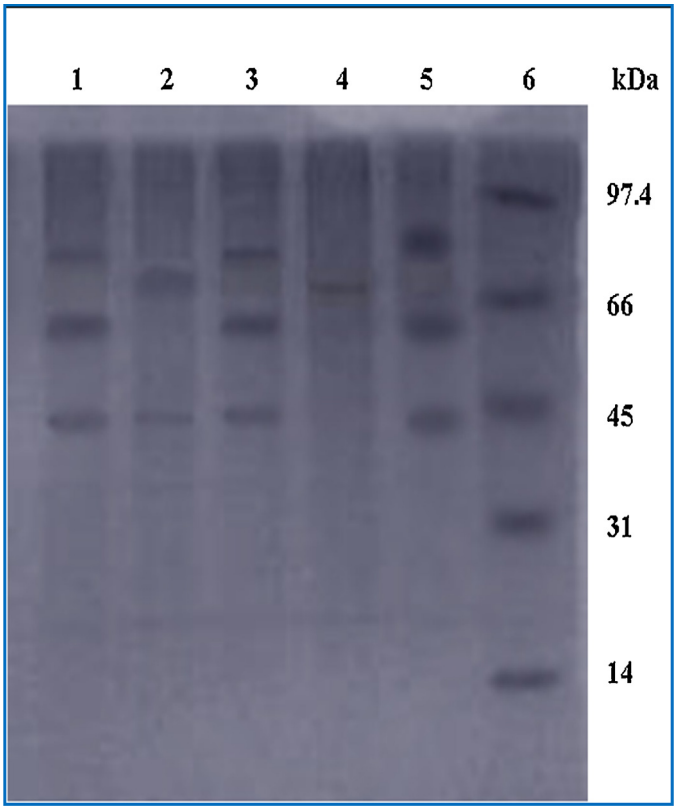

Fig. 3. Gel electrophoresis analysis, obtained under conditions of alginate coated (Lane 1) and uncoated (Lane 2) CS (MW 106 kDa) nanoparticles, alginate coated (Lane 3) CS (MW 42 kDa) nanoparticles containing antigen pre-treated with 0.01 M HCl for 2 h, then sustained release in PBS for 24 h. Measles antigen pre-treated with 0.01 M HCl for 2 h, then incubation with PBS for another 24 h (Lane 4), measles antigen incubation with PBS for 24 h (Lane 5), molecular weight marker (Lane 6).

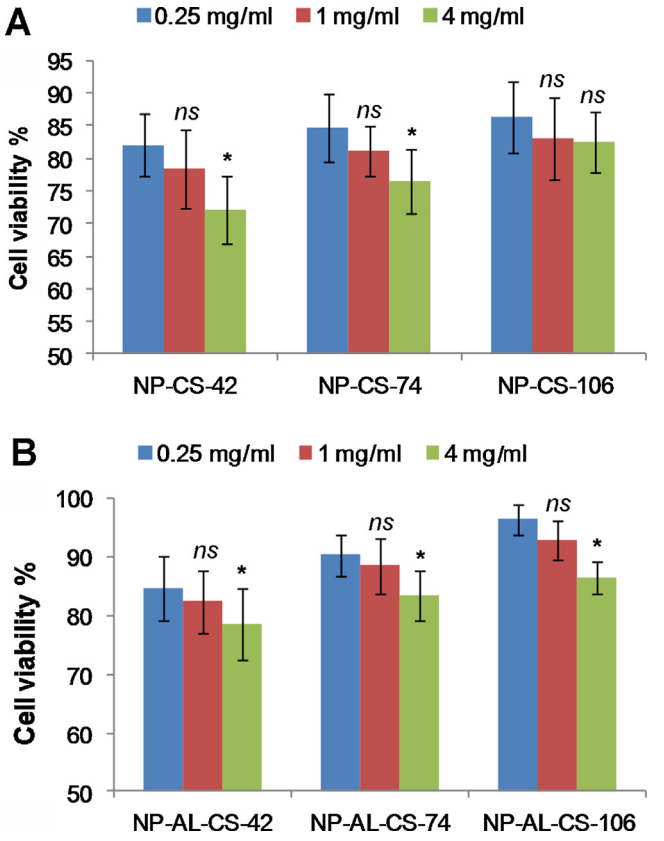

Fig. 4. Effect of (A) uncoated and (B) alginate coated CS nanoparticles at different concentrations on the viability of HT 29 cells determined by MTT assay. Indicated values were mean ± SD ($n = 6$). Statistical difference between the control groups and formulations are reported as $^*p < 0.05$, no significant difference, *ns* $p > 0.05$.

3.5. Immunological response

In the present study, we investigated the immunological response after administration of the measles antigen vaccine entrapped in alginate-coated CS LMW nanoparticles. The humoral immune response was accompanied by the presence of measles specific IgG and IgA in the serum and on the intestinal mucosa, respectively. Sample collection for a preclinical DBS assay involves spotting a small volume of blood (10–20 μl per spot) onto the special protein saver card and drying subsequently to prepare DBS specimens. These cards consist of a specialized matrix that lyses cells on contact, denatures proteins, and inactivates bacteria and viruses. When required, a disc of 3 mm diameter is punched from this card and the analytes are isolated by an elution buffer.

The measles antigen dose (equivalent to 20 μg) was selected for the immunization study, calculated from the loading capacity of each nanoparticle formulation, aimed at elucidating whether or not the MW of CS affects systemic and mucosal responses. The protective anti-measles IgG titre value and anti-measles IgA titre value results were measured and results are plotted in Figs. 5 and 6.

3.5.1. Systemic antibody response

Mice were immunized orally with various formulations (Table 1) on days 0, 7, 14 and 28 and blood samples were drawn from the tail-vein on days 7, 14, 28, 56 post administration using a lancet. All the samples were tested in the same day with same batch of reagents

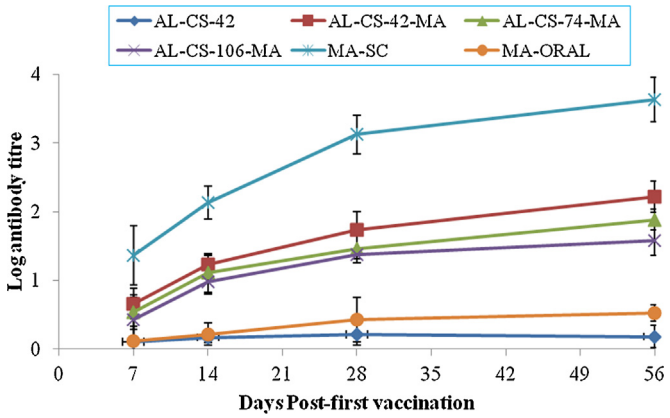

Fig. 5. Anti-measles IgG titres of mice immunized with different alginate-coated CS LMW nanoparticle oral formulations of measles vaccine. Values are expressed as antibody titres of mice on day 7, 14, 28, 56 post administration. Mean reciprocal dilutions are represented as the end point titre ($\log_{10}$) ± SD. Titres were defined as the highest dilution resulting in an absorbance value twice that of non-immune plasma.

to minimize imprecision and bias. IgG titres were measured in responder mice. The association of the measles antigen (group I–III) with the alginate coated CS nanoparticles induced a strong humoral immune response that was significantly higher ($p < 0.05$) than the mean titre found for group VI, vaccinated with measles antigen orally (Fig. 5). When comparing statistically, the immunological response elicited at each individual time point, the IgG levels for lower MW were always significantly higher than those corresponding to the higher MW. With only one boost, all the groups orally vaccinated with the antigen associated with coated nanoparticles were able to show seroconversion. The formulations were found to elicit responses that increased for 14 days and then levelled off. In fact, the group vaccinated with the uncoated measles vaccine given orally showed a very low responder number, whereas the group, with the measles antigen given subcutaneously showed higher log antibody titre.

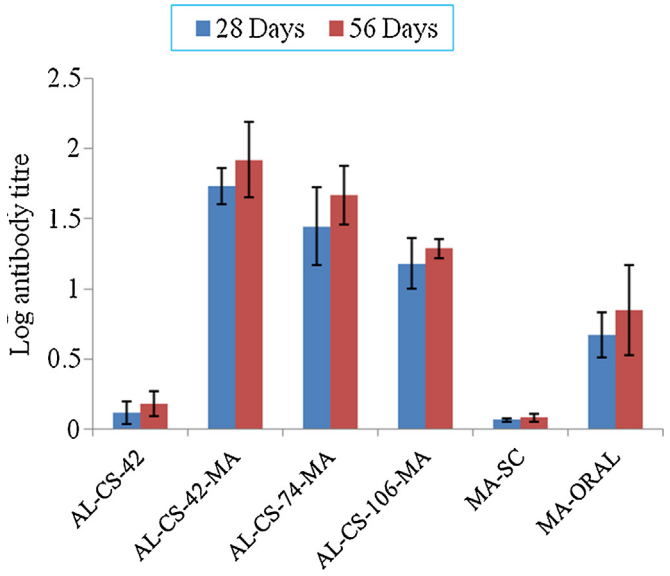

Fig. 6. Secretory anti measles sIgA profile after oral administration of various formulations in CS LMW nanoparticles detected in individual intestine washing samples of mice giving an OD of 0.1 after subtraction of background and presented as geometric mean titre per group.

3.5.2. *Mucosal anti-measles sIgA*

Mice were immunized orally with various formulations (Table 1) on days 0, 7, 14 and 28 and intestinal lavage fluids were collected on days 28 and 56 post administration. The IgA anti-measles levels in the intestinal lavages indicate that oral vaccination with alginate coated low MW CS nanoparticles induced stimulation of IgA-secreting cells of the GALT. There was a significant correlation between the IgA levels produced by the nanoparticles *vs.* CS MW, similar to the case with humoral response. In contrast, subcutaneous administration of formulation was not able to induce any IgA response, not even after several booster immunizations (Fig. 6).

4. Conclusion

An effective vaccine formulation for oral delivery maintains the antigen in a stable form, protects antigen from enzymatic degradation and harsh conditions in the stomach, adheres to the mucosal surface, ensures the antigen remains in the gastro intestinal tract region long enough for the antigen to interact with the lymphatic system, and efficiently stimulates innate responses, and evokes adaptive immune responses that are appropriate for the target pathogen. The vaccine-loaded alginate coated CS LMW 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. Moreover, alginate coating onto the surface of nanoparticles could modulate the release behaviour of the antigen and could effectively protect antigen from degradation against acidic medium (pH 2) *in vitro* for at least 2 h. Both systemic and local immune responses can be induced in a dose- and time-dependent manner through vaccine-loaded CS nanoparticles. The data presented in this study suggest that alginate-coated LMW CS nanoparticles can be used widely for the oral delivery of therapeutics.

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