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Development and Characterization of Tetanus Toxoid Loaded Trimethylchitosan Chloride Nanoparticles for Nasal Immunization

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ABSTRACT

Chitosan is an established mucoadhesive and absorption-enhancing polymers, but it has a pKa of 5.5, thus it is only soluble in acidic environments, where most of the amino groups are protonated. Recent studies have shown that only protonated soluble chitosan opens the tight junctions, facilitating the paracellular transport of hydrophilic compounds. To overcome the above problem a chitosan derivative, N, N, N-trimethyl chitosan chloride (TMC) has been synthesized and characterized, which shows higher solubility than chitosan in a broader pH range and is proved to be non-toxic. Tetanus toxoid (model antigen) loaded nanoparticles of TMC was prepared by ion gelation technique and were characterized for their size by transmission electron microscopy (TEM), zeta potential and zeta sizer. The antigen loading efficiency was found to be 52 % in TMC nanoparticles. In vitro release of tetanus toxoid was performed in phosphate buffer saline pH 7.4 and pH 5.6. In vivo study was accessed to study the antibodies production by ELISA (enzyme linked immuno-sorbant assay) and fluorescence microscopy was done to study mucoadhesive property of tetanus toxoid loaded TMC nanoparticles.

Keywords: Trimethyl chitosan chloride, tetanus toxoid, nanoparticles, nasal delivery.

1. INTRODUCTION

The mucosal vaccine administration is one of the most attractive methods for inducing protective immune responses, as many pathogens invade the body through mucosal surfaces[1]. Despite various advances in biotechnology that have led to the availability of large numbers of protein drugs, these drugs remain difficult to deliver by routes other than parenteral delivery [2]. Recently, alternative methods of administration such as the mucosal route, including nasal, oral and vaginal routes, have been used to avoid such disadvantages [3].

Novel carrier system nanoparticles are nanosized colloidal structures composed of natural, synthetic or semisynthetic polymers. The colloidal carrier system based on biodegradable and biocompatible polymeric system have largely influenced the controlled and targeted drug delivery concepts. Nanoparticles are solid core particulate, which are nanometric in size and they contain drug embedded within the matrix or adsorbed on to surface. Chitosan is a natural product derived from the polysaccharide chitin which is an amino polysaccharide (combination of sugar and protein) an abundantly available biopolymer found in the exoskeleton of crustacean like shrimp, crabs, lobster and other shellfish. Partial deacetylation of chitin to remove acetyl group present in chitin give us chitosan. Chitosan has unique ability to attach itself to lipids or fats. Chitosan is insoluble at neutral and alkaline pH values, but form salts with inorganic and organic acids such as hydrochloric acid. Chitosan has good biocompatibility and biodegradability and it exhibit net positive charge, it has been recently introduced in the market as an aid for weight loss and as cholesterol lowering agent [4].

Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained through alkaline deacetylation of chitin. It is a compound polymer of glucosamine and N-acetyl glucosamine. The term chitosan refers to a group of polymers varying in molecular weight upward to several million Daltons [5].

Chitosan is an established mucoadhesive and absorption-enhancing polymers, but it has a pka of 5.5, thus it is only soluble in acidic environments, where most of the amino groups are protonated. Recent studies have shown that only protonated soluble chitosan opens the tight junctions, facilitating the paracellular transport of hydrophilic compounds. To overcome the above problem a chitosan derivative, N, N, N-trimethyl chitosan chloride (TMC) has been synthesized and characterized, which shows higher solubility than chitosan in a broader pH range and is proved to be non-toxic [6].

2. MATERIALS AND METHODS

2.1 Materials

Chitosan of low molecular weight (50,000-190,000), with a degree of deacetylation of 85% was purchased from sigma Aldrich Company (USA), n-methylpyrrolidinone was purchased from alpha chemika, maharashthra , India and all other chemicals are of analytical grade.

2.2 Chitosan modification for preparation of trimethyl chitosan chloride

Chitosan was modified according to the method by Sieval et al. Briefly, 2 g of chitosan was dissolved in 80 ml of n-methyl pyrrolidinone and stirred in a 2-necked flask in a constant temperature on water bath at 60^{o}C, the flask was connected to a condensation column. 11 ml of 15% sodium hydroxide solution was added to the flask and it was followed by addition of 11.5 ml of methyl iodide and 4.8 g of sodium iodide. The mixture was stirred for 75 minutes and precipitated with addition of 200 ml of 90% ethanol, centrifuged and washed with acetone on a sintered glass filter and then dried.

In the second stage, 80 ml of N-methylpyrrolidinone was added to the precipitate (mainly dimethyl chitosan iodide) and the mixture was stirred at 60^{o}C. Then 11 ml of 15% NaOH, 7 ml of methyl iodide (CH_3I) and 4.8 g NaI were added successively and the mixture was stirred for 30 min. An additional 2 ml of methyl iodide and 0.6 g pellets of NaOH were added and stirring was continued for 1 hour. This mixture was precipitated with 200 ml of 90% ethanol, centrifuged and the solid substance was filtered on a sintered glass filter and washed with acetone to obtain a powdery substance, which is chitosan iodide. To exchange iodide with chloride, trimethyl chitosan iodide was dissolved in 40 ml solution of 10% sodium chloride (NaCl). The solution was precipitated with 200 ml of ethanol. Excess ethanol should be avoided because it will also precipitate NaCl. The mixture was centrifuged and the supernatant containing the excess NaCl was removed. The precipitate was filtered and washed with acetone, dried and milled to obtain an off-white water-soluble powder [7].

2.3 Preparation of TMC Nanoparticles

The nanoparticles of TMC were prepared by ion gelation of TMC with tripolyphosphate (TPP) anions using 10mg/ml solution of TMC was prepared by stirring on magnetic stirrer at room temperature by drop wise adding tripolyphosphate 2 ml (0.1-0.9 % w/v), yielding a final pH around 7. Tetanus toxoid loaded nanoparticles were prepared by dissolving the tetanus toxoid in TMC before adding the TPP solution [8].

2.4 Characterization of modified chitosan

The product was characterized for degree of quaternization by ^{1}H NMR spectra measured in D_2O at 80^{o}C using a Bruker, advance II 400 NMR spectrometer [9]. The degree of quaternization (DQ) and degree of methylation (DM) was calculated according to a previously described method using the following equations:

$$DQ = [\{CH3\}3] / [H] \times 1/ 9] \times 100$$

$$DM = [\{CH3\}2] / [H] \times 1/ 6] \times 100$$

2.5 Morphology and size of nanoparticles

2.5.1 *Transmission Electron microscopy (TEM)*

Formulation was characterized for their size using transmission electron microscopy, in this study sample of nanoparticulates were mounted on metal stubs, gold coated under vacuum and then examined in a Zeta Nano ZS (Malvern Instruments Ltd., Malvern, UK).

2.5.2. Zeta potential and size distribution analysis

The zeta potential of the modified polymer particles was deduced from the electrophoretic mobility of the particles by Doppler Electrophoresis (Zetasizer nano series Malvern Instrument Ltd. Worcestershire, UK) in HEPES buffer pH 7.4 after suitable dilution (1/ 200 v/v) of the different nanoparticles suspension. The hydrodynamic mean diameter and the size distribution of the

nanoparticles were determined at 250C by quasielastic light scattering using a Zeta Nanosizer (Malvern instrument, UK).

2.6 Determination of loading efficiency of nanoparticles for tetanus toxoid

The amount of protein entrapped in the nanoparticles was calculated from the difference between the total amount added to the solution and the amount of non-entrapped protein remaining in the supernatant. TT concentrations in the supernatant were measured by the bicinchoninic acid (BCA) protein assay. Aliquots of the resulting nanoparticles suspension were then separated by centrifuged for 20 min. at 18000×g and 10 ^{o}C and the supernatant were then separated from the nanoparticles. The amount of non-entrapped protein remaining in the supernatant was measured by the micro BCA protein assay (Pierce, USA). A non-loaded nanoparticles suspension was used as a blank to correct the interference by TMC.

2.7 Study of in vitro release profile

Aliquots of 1ml TT-loaded TMC nanoparticles were centrifuge at 10000 RPM on a glycerol bed for 15min. the supernatant was decanted and the pellet was re-suspended in 1 ml of phosphate buffer saline (PBS, 0.1 M, pH 7.4) and buffer of pH 5.6. The tubes were incubated at 37 ^{o}C, under agitation (50 rpm), for 3 hours, then tube was taken and centrifuge (at 18000 rpm). The released tetanus toxoid in the supernatant was determined by micro BCA protein assay. A sample consisting only nanoparticles resuspended in PBS was used as blank. The experiment was performed in triplicate and plots a graph.

2.8 Determination of IgG and IgA titre by ELISA assay

For in vivo study BALB/c mice (n=6) of either sex of 7-9 weeks age were used in all experiments. Animals were housed in groups (n=6) with free access of water and food. They were withdrawn of any food intake 3h before immunization. The study protocol as approved by Institutional Animal Ethical Committee of Dr. H. S. Gour University, Sagar as followed. To evoke an immune response, 10µg of antigen was inoculated intranasally in small drops. Nasal dosing was performed by inserting a small piece of polyethylene tubing (sterile), attached to micropipette (5-50 µl), 0.2 cm into nostril (10µg TT formulation/nostrils) (individual dose of 2 µg at 2minute interval) of non-anesthetized animal (supine position) and ejecting into the nasal cavity. Care was taken that a new drop was only given when the former had been entirely inspired. Secondary immunization was done after 4 week with the same dose of formulation. Group-1 control, Group-2 received marketed TT (intramuscular), Group-3 received TT-PBS solution (intranasal) and Group-4 received TT loaded nanoparticles (intranasal), Each group consist 6 animals (n=6).

3. RESULTS AND DISCUSSION

The derivative of chitosan, trimethyl chitosan chloride (TMC) was successfully synthesized by two step synthesis and analyzed by ^{1}H NMR spectroscopy. The degree of methylation obtained is about 70 %. The nanoparticles of TMC were successfully prepared by ion crosslinking method using TPP as a crosslinker. Formula for nanoparticles preparation obtained after optimizing the particle size and entrapment efficiency by TPP concentration, Stirring time, Stirring speed. The degree of the quaternization and dimethylation, in mole percentage of free amine; $(CH_3)_3$ and $(CH_3)_2$ are the integrals of the chemical shift of the hydrogens of the trimethyl amino group at 3.3 ppm and the dimethylated amino group at 3.1 ppm, respectively; [H] is the integral of the H-1 peaks between 4.7 and 5.7 ppm, related to hydrogen atoms bound to carbon 1 of the chitosan molecule, which is taken as the reference signal (Figure 1).

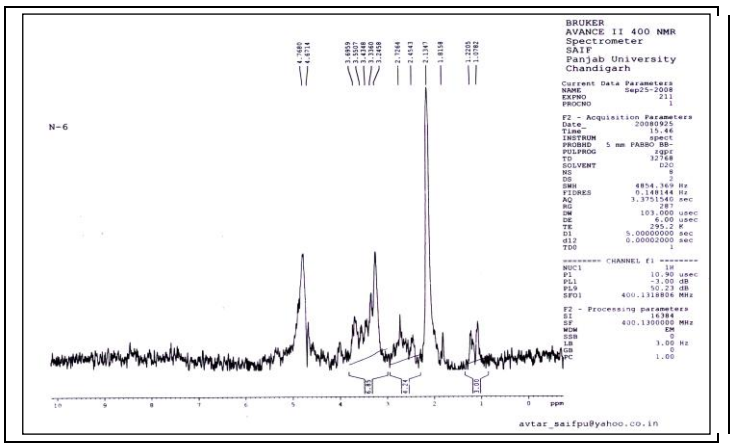

Figure 1. H[1] NMR spectra of modified chitosan

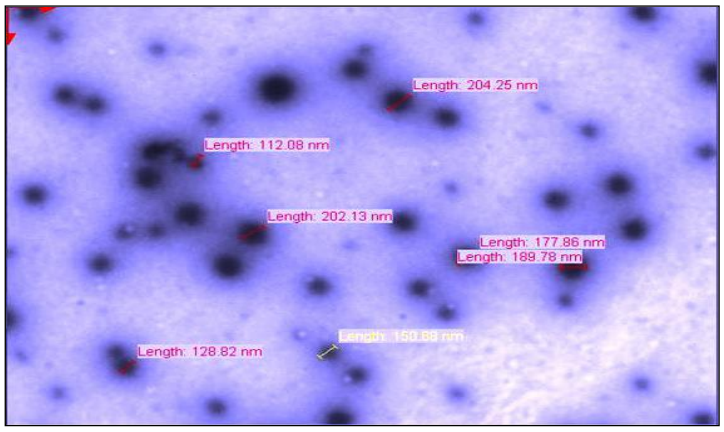

Figure 2. Transmission Electron Microscopy photograph of TMC nanoparticles

Table No.1 Anti- TT IgG levels with formulation (OD at 450nm)

Dose (10µg)	IgG levels in serum				
	0 days	7th days	14th days	28th day	42nd day
G-Control	0.0210 ±0.0101	0.0194 ±0.011	0.0221 ±0.012	0.0197 ±0.0110	0.0231 ±0.107
G-2 IM	0.0315 ±0.0120	0.198 ±0.011	0.275 ±0.0103	0.673 ±0.0121	0.854 ±0.121
G-3 Intranasal	0.0256 ±0.101	0.108 ±0.210	0.204 ±0.103	0.646 ± 0.110	0.716 ±0.102
G-4 Intranasal	0.0216 ±0.223	0.166 ±0.129	0.198 ±0.142	0.562 ±0.0120	0.649 ±0.221

Table No-2 Anti- TT IgA levels with formulation (OD at 450 nm)

Frmulation/ Dose/Route	IgA levels in salivary secretion after	
	28 days	42 days
G-1 Control	0.0197±0.0110	0.0231±0.0120
G-2 Intramuscular	0.0642±0.0127	0.0845±0.0121
G-3 Intranasal	0.011±0.0134	0.016±0.0112
G-4 Intranasal	0.0512±0.0117	0.0779±0.0118

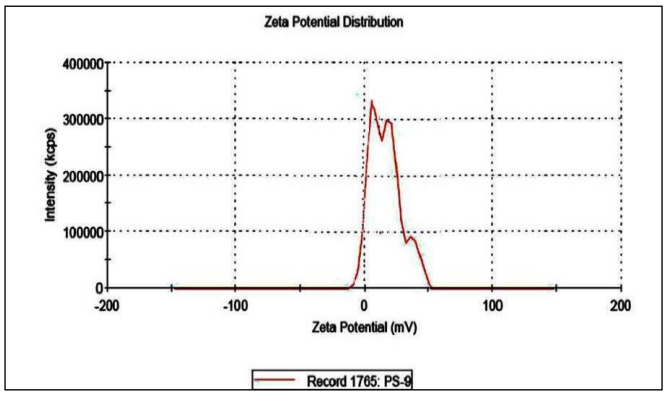

Figure 3. Zeta Potential distribution graph

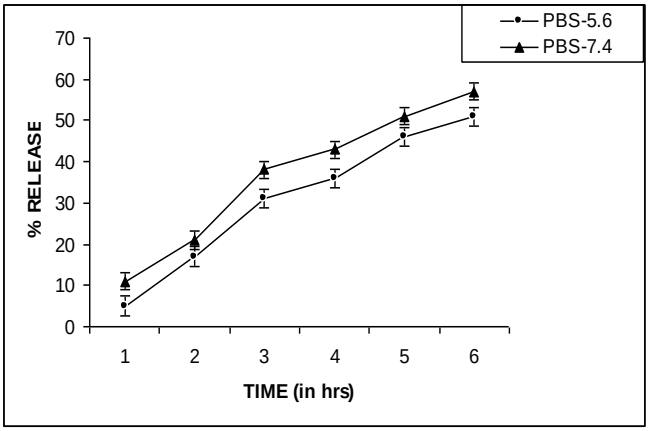

Figure 4. In vitro release curve of TT loaded nanoparticles (Time vs % Release)

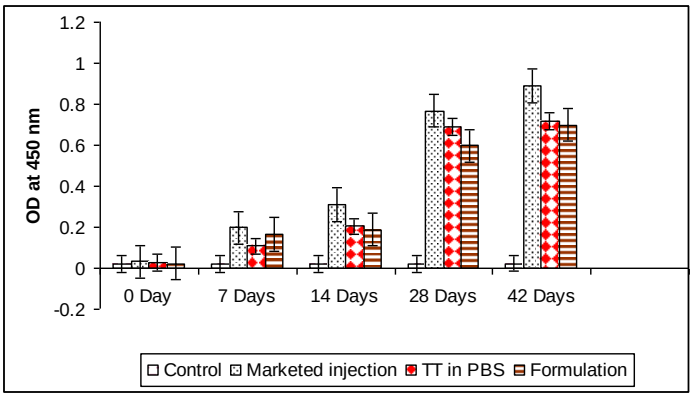

Figure 5. Anti-TT IgG levels in serum after intranasal immunization

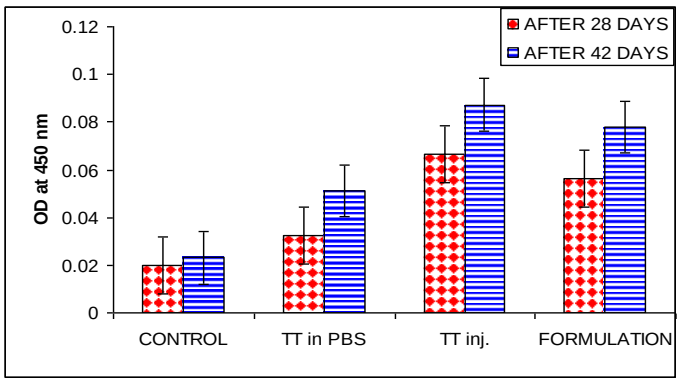

Figure 6. Anti-TT IgA level in salivary secretion after intranasal immunization

The size of the particle in the formulation was found to be of range 150-215 nm, and the average size of the nanoparticles was 183.98 nm (Figure 2). The zeta potential of the final nanoparticles formulation was found to be positive (16.2 ±0.8) and the average size of the nanoparticles was around 183 nm (Figure

3). The polydispersity index of the nanoparticles was 0.331. Loading efficiency of the nanoparticle was found to be 52% of total TT added remain associated with nanoparticles. This shows a high load of toxoid and thus good antigen properties. *In vitro* release studies shows that around 32% of the loaded protein was immediately released into PBS. The burst release might be ascribed to protein molecules that were loosely bound to TMC particle surface. The remaining 68% of loaded protein was firmly associated with the TMC nanoparticles for at least 3 hrs. The release of TTAg from the TMC nanoparticles in the PBS -5.6 and 7.4 were found to be 51% and 57% respectively in 6 hrs of total antigen loaded in the formulation. The % release obtained in PBS shows that the encapsulated TT in nanoparticles significantly delivers into the nasal mucosa (Table 1 and Table 2).

The *in vivo* study result shows that, after administration of formulation 10µg/ nostril doses in the mice of 6-7 week old, gives positive responses for production of antibodies against the tetanus toxoid antigen. In comparison intramuscular TT injection, the nanoparticulate formulations gave significant IgA response in salivary secretion, and also give IgG responses in blood serum (Figure 4). The results obtained after ELISA assays show on days 28 and 42 gives IgG and IgA production is higher (Figure 5, 6).

4. CONCLUSION

As drug delivery carriers, chitosan-based nanoparticles have the utility in controlled release and targeting studies of almost all class of bioactive molecules. Chitosan is also extensively explored in gene delivery. TT-loaded TMC nanoparticles enhanced nasal absorption of TT following nasal administration. TMC nanoparticles appear promising as a nasal delivery system for Tetanus Toxoid and potentially for other therapeutical proteins. All the results suggest good immune response of the developed formulations (TMC nanoparticles encapsulating tetanus toxoid) upon nasal immunization as both systemic and mucosal immune responses were observed in significant magnitude and hence it can be a new and successful vaccine for tetanus toxoid.

5. ACKNOWLEDGEMENT

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