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## ORIGINAL ARTICLE

# IpaD-loaded N-trimethyl Chitosan Nanoparticles Can Efficiently Protect Guinea Pigs against *Shigella Flexneri*

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## ABSTRACT

**Background:** *Shigella flexneri* is a pathogen responsible for shigellosis around the world, especially in developing countries. Many immunogenic antigens have been introduced as candidate vaccines against *Shigella*, including N-terminal region of IpaD antigen (NIpaD). **Objective**: To evaluate the efficiency of O-metylated free trimethyl chitosan nanoparticles (TMC NPs) in the oral delivery of NIpaD. **Methods:** TMC was synthesized by a two-step method from high molecular weight chitosan. The recombinant NIpaD protein was used as the immunogen. The protein was overexpressed in *E. coli* BL21 (DE3) and characterized by gel electrophoresis. The NIpaD-loaded TMC NPs were synthesized by ionic gelation method and were characterized by electron microscopy. NPs were orally administered to guinea pigs and specific humoral and mucosal immune responses were assessed by serum IgG and secretory IgA, respectively. The protectivity of the formulation was assessed by keratoconjunctivitis (Sereny) test. **Results:** The immunized guinea pigs showed a significant raise in rNIpaD-specific serum IgG and faecal IgA titers. Specific secretory IgA was detected in eye-washes. Sereny test results showed that immunized animals vaccinated with IpaD-loaded TMC NPS tolerated the wild type of *Shigella flexneri* 2a in Sereny test. However, in the group immunized with NIpaD antigen and non-immunized group, no increase was observed in antibody titer against NIpaD. These animals were infected following the challenge with *Shigella flexneri* 2a ($p<0.0152$). **Conclusion:** The recombinant rNIpaD formulated with TMC obtained from high molecular weight chitosan, can be considered as a mucosal vaccine against *Shigella flexneri* through oral route.

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**Keywords: Nanoparticles, Oral Delivery, O-methylated free Trimethyl Chitosan, Sereny Test, Shigellosis**

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## INTRODUCTION

*Shigellae* are pathogenic bacteria cause diseases in the large intestine of humans and other primates, known as 'bacillary dysentery' or shigellosis. The disease is most likely to be severe, with a high risk of death, especially among infants and elderly people (1). *S. flexneri* (Serogroup B) is the most frequently isolated species of *Shigella* in developing countries (2). The entry rout of the bacterium is mouth, and it colonizes and infects the host intestine. The human intestine, the site of *Shigella* invasion, plays a crucial role in host defense via the mucosal immune response. So far, there has been no approved vaccine to stimulate a mucosal immune response by the host to prevent shigellosis (3,4), hence the importance of , developing an efficient vaccine to control antibiotic resistant strains (5). Oral delivery of vaccines allows for the elicitation of immune responses at mucosal surfaces and can prevent orally-transmitted infections more efficiently. Mucosal immune system and enteric epithelial barrier is first line of protection against the disease. However, due to the harsh conditions of gastrointestinal tract as well as the short time period of the vaccine-mucus interaction, there is a great need for an efficient vaccine delivery conduit. Owing to such interesting properties as proper size, easy fabrication, biocompatibility, biodegradability, and mucoadhesivity, to name a few, nanoparticles (NPs) are increasingly exploited for the delivery of vaccine and drug materials (6-8). Chitosan, a natural polymer, and its derivatives, such as trimethyl chitosan (TMC), have been extensively used for the entrapment of biomaterials, including proteins, DNA, RNA and drugs, and their delivery onto desired sites (9-10); the results have been astonishing mainly due to the charge of the polymer. The positive charge of the molecule seems to be important for cellular uptake enhancement (11). The recombinant DNA technology allows for the expression of different antigens, including recombinant vaccine candidates. In previous studies, a novel subunit vaccine candidate was reported from the N-terminal region of the IpaD protein (12-13). The Nasal administration of this protein, formulated with chitosan nanofibers, is immunogenic in guinea pigs (14). In the present study, we used NIpaD-loaded TMC-NPs to immunize guinea pigs through oral rout.

## MATERIALS AND METHODS

**Materials.** High molecular weight chitosan (310,000-375,000 Da) with a residual degree of 15-25% acetylation was purchased from Sigma-Aldrich Chemical Co., USA. N-methyl-2-pyrolidone (NMP), formaldehyde, formic acid, iodomethane, Sodium dodecyl sulfate (SDS), sodium acetate, acetic acid, tween 80, sodium hydroxide and hydrochloric acid were prepared from Merck, Germany. All chemicals were of analytical grade. Hank's balanced salt solution (HBSS) was obtained from Invitrogen. The HRP Conjugated Rabbit anti-Guinea Pig IgG was purchased from ABNOVA (Taiwan) and HRP Conjugated Sheep anti-Guinea Pig IgA was prepared from Immunology Consultants Laboratory, Inc. (Portland, USA).

**Bacterial Strains and Growth Condition.** The wild strain of *Shigella flexneri* serotype 2a was applied to challenge guinea pigs via keratoconjunctivitis (Sereny) test. The virulent strains included in this study were obtained from hospital samples.

**Recombinant Protein Expression and Purification.** Codon-optimized *ipaD* sequence (Accession Number: MH051892.1) was synthesized chemically in pET 28a (+)

expression vector. The synthetic construct was transferred to *E. coli* BL21 (DE3) pLysS competent cells. The rNIpaD protein (containing a histidine-tag at N-terminus) was produced according to the previously reported method (13). The recombinant protein was overexpressed in *E. coli* BL21 (DE3) and purified via Ni-affinity column as described in QIAGEN handbook regarding high-level expression and purification of 6xHis-tagged proteins (15). Protein concentrations were determined using Bradford assay, and protein expression was analyzed on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Preparation of TMC Nanoparticle.** N-trimethyl chitosan was synthesized through a two-step approach as described by Verheul *et al.* (16). The synthesized TMC was characterized using NMR technique. TMC nanoparticles were fabricated by ionic gelation method employing TPP (sodium tripolyphosphate) as the cross linking agent under stirring in a 10 ml vial (17). Briefly, 3 ml of aqueous solution of TMC polymer containing 0.25% (w/v) Tween 80 and 1.5 ml TPP was separately prepared. After that, TPP solution (pH 8) was slowly added drop-wise to the TMC solution (pH 6). The mixture was allowed to be stirred (500 rpm) for 1 h at room temperature until the solution became slightly opalescent. The suspension was then concentrated by centrifugation at 18,000 ×g for 30 min at 4$^{o}$C. The pellets were suspended in HBSS pH 7.4 for further analysis. The effects of TMC polymer and TPP concentrations on the characteristics of the fabricated nanoparticles were studied in order to access the optimum concentrations of TMC and TPP and synthesize the desired size of the nanoparticles.

In order to fabricate TMC-NPs containing rNIpaD, a 10 ml HBSS solution containing 2 mg/ml of TMC (pH 6) was prepared, passed through a 0.2 µm filter, and placed on a stirrer (500 rpm) at room temperature. 5 ml TPP solution (0.7 mg/ml) in HBSS, pH 8) containing 0.5 mg/ml rNIpaD was prepared and added drop-wise to TMC. The mixture was allowed to be stirred at 500 rpm for 1 h at room temperature. Finally, fabricated nanoparticles were precipitated by centrifugation at 18,000 ×g for 30 min at 4$^{o}$C. The supernatant was removed and the pellets were resuspended in phosphate buffered saline (PBS). The remaining antigen in the aqueous phase was used to specify the loading efficiency (LE) and loading capacity (LC) of antigen in the nanoparticles (Equations 3 and 4). Antigen concentration was determined by bicinchoninic acid (BCA) assay, and the supernatants of bare nanoparticles were used as the blank (18). To investigate the degradation of the entrapped proteins, nanoparticles containing rNIpaD were destroyed through adding 200 µl of 10% (w/v) NaCl. Following 30 min of incubation, released protein was electrophoresed on a 12% SDS-PAGE.

Equation 3: $$LE\% = \frac{total\ amount\ of\ protein - amount\ of\ free\ protein}{total\ amount\ of\ protein} \times 100$$

Equation 4: $$LC\% = \frac{total\ amount\ of\ protein - amount\ of\ free\ protein}{weight\ of\ nanoparticles} \times 100$$

**Characterization of the Synthesized Nps.** Dynamic light scattering and electron microscopy techniques were exploited to investigate the characteristics of NPs. The particle size distribution and Zeta-potential of the fabricated NPs were measured by use of a Zetasizer (Malvern Instruments, Malvern, UK) in HBSS pH 7.4 at 25°C. The

morphology of the nanoparticles was investigated via field emission scanning electron microscopy (FESEM, 7500F; JEOL, Tokyo, Japan) and transmission electron microscopy (TEM, 80 KV, Zeiss, EM900).

***In vitro* release study.** In order to study the release profile of the protein from the NPs, nanoparticles containing protein were suspended in 1 ml of HCl solution (pH 2, simulate gastric acid) in Eppendorf tubes and placed in a shaker at 100 rpm at 37°C. At specific intervals (30, 45, 60, 90, 120 min), 200 µl of the suspension was removed and the reaction was stopped and neutralized by 0.5 ml of NaOH solution (0.1 M). The tubes were centrifuged at 14000 ×g for 30 min and the protein released into the supernatants was measured by Bradford protein assay and was expressed as a distributive percentage of total encapsulated antigens. Intestinal simulation was done in neutral pH. The nanoparticles were suspended in 3 ml of PBS, pH 7.4, and shaked at 100 rpm at 37°C. At specific intervals (0.5, 0.75, 1, 2, 4, 8, 12, 24, 48, 96 h), 200 µl of suspension was removed and centrifuged at 14000 ×g for 30 min. The release of the protein was determined by Bradford protein assay method.

**Animals and Immunization Studies.** For oral rout studies, male Hartley guinea pigs (190-220 g, Pasteur Institute of Iran) were divided into groups of 6 animals. All immunizations were carried out on days 0, 14 and 28. The animals were administrated orally using oral gavage needles. They were fasted for 24 hours while access to water was granted in an optional manner except for two hours before each administration. Each group of animals was housed in a cage of two (n=2) animals for one week before the experiments were conducted. Negative control animals (group A) did not receive any prescriptions. Test animals (group B) received TMC-NPs containing 500 µg of rNIpaD via oral rout. Other test animals (group C) were immunized orally with 500 µg of bare IpaD. Finally, group D only received unloaded TMC NPs as the negative control (Table 1). The animals were returned to their cages, given sterile water, and after six hours, fed. These experiments were approved by the Institutional Animal Care and Use Committee at Imam Hossain Comprehensive University, Tehran, Iran.

**Table 1. Administration design.**

| Designation | Route of administration | Formulation (prescriptions) | Buffer | Total Volume | pH |
|---|---|---|---|---|---|
| **A** | Oral | - | HBSS | 2 ml | 7.2 |
| **B** | Oral | 2 mg nanoparticles containing 0.5 mg rN-IpaD | HBSS | 2 ml | 7.2 |
| **C** | Oral | 500 µg rN-IpaD | HBSS | 2 ml | 7.2 |
| **D** | Oral | 2 mg unloaded nanoparticles | HBSS | 2 ml | 7.2 |

**Collection of Serum and Other Body Fluids.** Blood samples, eye-washes and stool pellets were collected one week after each administration in order to analyze humoral and mucosal immune responses. Blood samples were taken from the Tarsal Vein of animals and were allowed to clot for 1h at 37°C, a procedure done without anesthesia. Blood serums were stored at -20°C for further analysis. Faecal samples were collected

from the cage bed and 100 mg of pellets was suspended in a 0.5 mL of cold (4°C) buffer (30 mM disodium EDTA in PBS, pH 7.6). Samples were homogenized and centrifuged at 5000 × g for 15 min at 4°C. The supernatant was removed, sodium azide was added (0.025% w/v), and the samples were stored at -20°C for future analysis. The prepared samples (dilution 1:2) were applied for IgA analysis in feces (19). Eye-washes were gathered from the anesthetized guinea pigs through instilling 30 µl of PBS-Tween in each eye. The guinea pigs were anesthetized with intramuscular injection of 1 mL of anesthetizing agent (ketamine 30 mg/mL, xylazine 6 mg/mL and atropine 0.1 mg/mL in physiological saline).

**Measurement of Antibody Titers.** Serum IgG and secretory IgA antibodies elicited against N-terminal region of rIpaD were measured in serums, stools and eye-washes via enzyme-linked immunosorbent assay (ELISA) (14). IgG and IgA antibody titers were determined using peroxidase labeled rabbit anti-guinea pig IgG (3000×, Abnova, Taiwan) and anti-guinea pig IgA (100× from IgA ELISA kit, Abnova, Taiwan). Endpoint titers were determined for each serum sample by taking the reciprocal of the dilution. For immunogenicity analysis, the geometric mean titers of each group of guinea pigs were calculated.

**Challenge Assay.** Guinea pigs were challenged by virulent *S. flexneri* 2a two weeks following the final administration (day 42). Control animals were used to verify the virulence of the organisms (the age of control animals was the same as the age of test animals). *S. flexneri* strains were spread from the freeze beads onto BHI agar plates containing 0.01% Congo red dye. The plates were incubated overnight at 37°C, and colonies which bound the dye (positive colonies) were then spread on BHI agar plates and grown overnight at 37°C. Next, bacterial cells were harvested with 5 ml of trypton soy broth, and the suspension was used to challenge guinea pigs. Sereny (keratoconjunctivitis) test was done by administering virulent strains into the conjunctiva sac of one eye. Approximately 30-50 µl of the suspension containing $8\times10^8$ colony-forming units (CFU) was inoculated slowly into the eye, and the lids were gently massaged to distribute organisms over the entire eye. Animals were daily inspected for the development of keratoconjunctivitis.

**Statistical Analysis.** Data analysis was carried out using GraphPad Perism 6 (GraphPad Software version 6.07, Inc., La Jolla, CA). The antibodies induced within groups were statistically analyzed by one-way ANOVA and Tukey's adjustment for multiple comparisons. Values of $p<0.05$ were considered to be statistically significant. The differences among groups regarding antibody responses were determined using Two-way ANOVA with Sidak correction. To compare responses at single time points between a specific group and control, unpaired t test with a 95% confidence interval was used. The protection data were compared in guinea pigs using two tailed Fisher's exact test.

## RESULTS

### Expression and purification of the protein.

The purification and expression of the recombinant protein is shown in Figure 1 where high expression levels of the protein are seen. Following the purification of the protein by nickel column, a pure band with no extra band was obtained.

**Synthesis and characterization of DMC and TMC.**

Figure 2 shows the 1H NMR spectra of DMC and TMC. The signal at 4.67 ppm is related to D2O (the solvent), and the signals of the protons of methyl groups shifted at

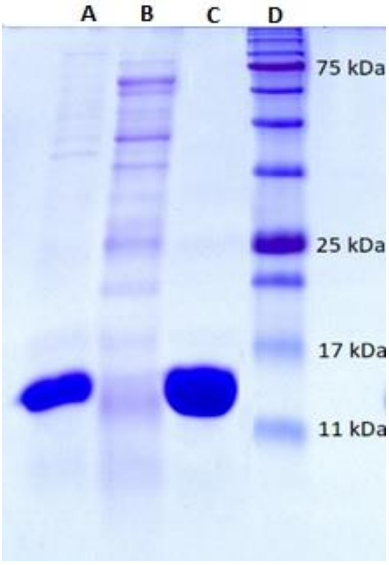


**Figure 1. IpaD expression and purification.** A: IPTG-induced *E. coli* cells; B: Un-induced *E. coli* cells; C: Purified protein (≈14 kDa); D: Molecular weight marker (Prestained protein ladder, SinaClon, Iran).

60°C (spectrum at 25°C not shown). The spectrum of DMC (Figure 2) shows signals at 3.31 ppm, associated with the hydrogen atoms of dimethyl amino groups (N-(CH3)2). In the spectrum of TMC, the peaks at 3.35 and 3.60 correspond to dimethyl amino group (N+-(CH3)2) and trimethyl ammonium group (N+-(CH3)3), respectively. The average degree of dimethylation and quaternization (DQ) of DMC and TMC was calculated as 99% and 74%, respectively. The FTIR spectra of chitosan (CHT), dimethyl chitosan (DMC) and trimethyl chitosan (TMC) show that N-methylation process occurred efficiently.

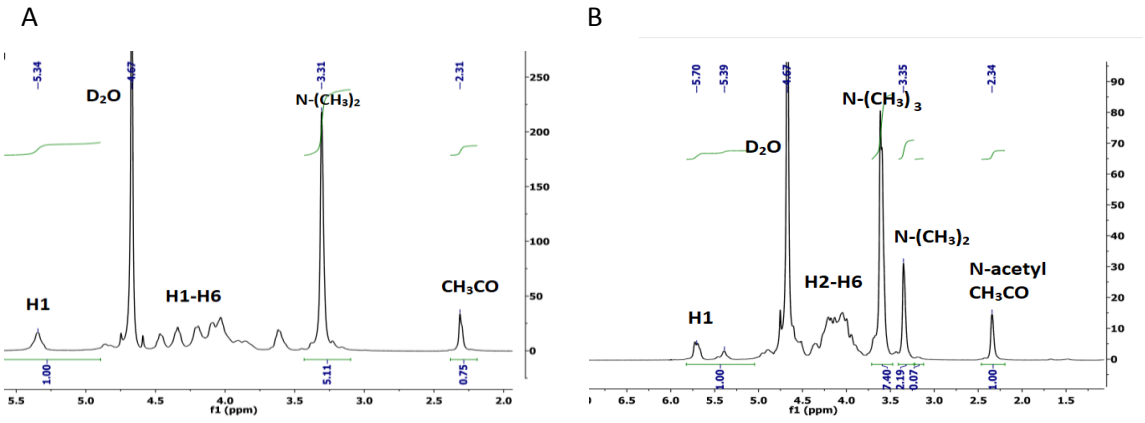


**Figure 2. HNMR spectra of DMC (left) and TMC (right).**

The peaks at 1570 $cm^{-1}$ related to chitosan, 1565 $cm^{-1}$ to DMC and 1559 $cm^{-1}$ to TMC were assigned to the bending vibration of N-H bonds of R-NH2 group. The new peaks appearing at 1473 $cm^{-1}$ regarding DMC and 1477 $cm^{-1}$ concerning TMC were attributed to the asymmetric angular deformation of C–H bonds of -CH3 groups. Regarding TMC, the peak at around 1415 $cm^{-1}$ was assigned to the characteristic absorption of N–CH3 (Figure 3).

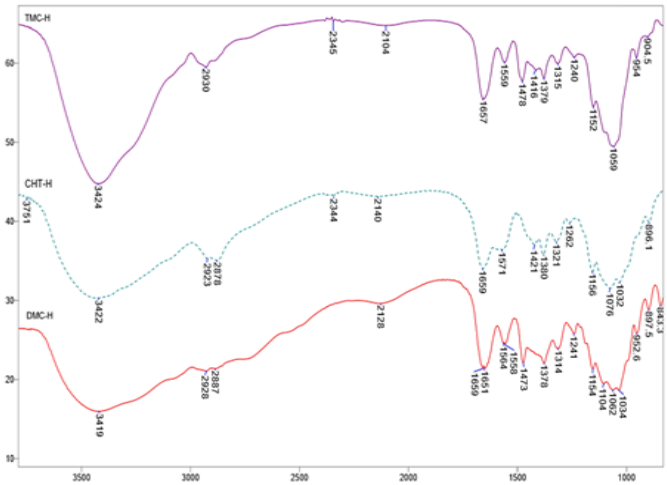


**Figure 3. FTIR spectra of high molecular weight TMC (TMC-H), DMC (DMC-H) and chitosan (CHT-H).**

**Nanoparticles synthesis and characterization**.

As shown in Table 2, to prepare the optimized unloaded nanoparticles (without protein), different concentrations of TMC and TPP were assayed. Table 3 shows the loading efficiency (LE) and loading capacity (LC) of nanoparticles at the optimized rNIpaD concentrations of 0.5 mg/ml protein. DLS showed that the average particle sizes of the optimized bare and antigen-loaded nanoparticles were approximately 159 nm and 274 nm, respectively.

**Table 2. Nanoparticle preparation and size optimization (n=3, mean ± SD).**

| TMC | TPP | Mean particle |
|---|---|---|
| mg/ml | mg/ml | Size (nm) |
| 1 | 0.5 | 76.4 ± 5.32 |
| 2 | 0.75 | 159.8 ± 9.91 |
| 3 | 1 | 456.7 ± 19.86 |

The PDIs of nanoparticles were 0.239 for blank nanoparticles and 0.350 for antigen-

loaded nanoparticles, indicating a narrow particle size distribution. The zeta potential was reduced from 20 to 15 due to protein loading (Table 3). The rNIpaD-loaded nanoparticles showed about 78% loading efficiency and 13% loading capacity. The high loading efficiency is likely due to the cationic nature of TMC and negative charge of rNIpaD (pI 6.22). The rNIpaD loaded nanoparticles were stored for 48 hours in the HBSS buffer at 37°C, and the STM image shows an irregular margin and an increase in size (Figure 4). Spherical nanoparticles with a smooth surface were found in the image of a FESEM electron microscope. In Figure 5, SEM image displays the unloaded rNIpaD nanoparticles.

**Table 3. Nanoparticle characteristic (n=3, mean ± SD).**

| TMC NPs | Mean particle Size (nm) | Polydispersity index (PDI) | Zeta potential (mV) | Encapsulation Efficiency % | Loading Capacity % |
|---|---|---|---|---|---|
| Unloaded NPs | 159.8 | 0.239 ± 0.13 | 21.2 | NA | NA |
| Protein-loaded NPs | 274 | 0.35 ± 0.15 | 14.0 | 74 ± 3 | 13 ± 1.5 |

***In vitro* release profile**

The release profiles of rNIpaD fromTMC nanoparticles into SIF and SGF buffers are shown in Figure 6. A burst release can be seen in SGF in the early time. After 60 min, 70 % of rNIpaD was released slowly. However no significant release was observed after 1 h. The release of the protein in SIF shows a slow release of rNIpaD from the NPs (26 % after 4 h).

**Assessment of immune response.**

On day 0, as it was expected, no specific antibody titers were detected against rNIpaD in serum, fecal and eye-wash samples. The serum IgG response to the oral-vaccinated group B exhibited significant specific serum IgG titers of $2 \times 10^2$ EU $ml^{-1}$ on day 14, increasing significantly to a titer of $6 \times 10^2$ EU $ml^{-1}$ on day 28 (Figure 7a) ($p<0.0001$).

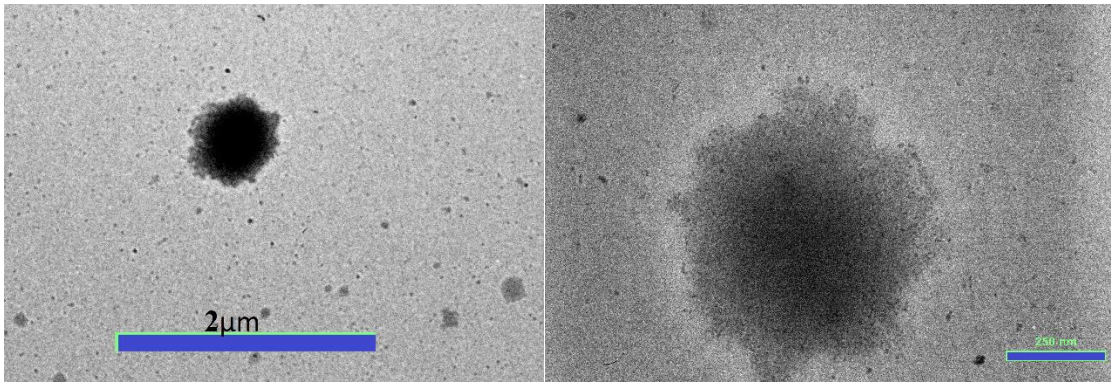


**Figure 4. TEM image of rNIpaD-loaded nanoparticles (Left image bar 2 µm, Right image bar 250 nm).**

These results show that the serum IgG response to the orally-administered antigen was delayed. The two-way ANOVA showed significant differences between groups A and B ($p<0.0001$). The mucosal immune response was further assessed by IpaD-specific secretory IgA titration in stools and eye-washes. Moreover, the orally-immunized animals of group B exhibited specific IgA titers of 10 EU $ml^{-1}$ by day 14 and 40 EU/$ml^{-1}$ by day 28 in stools (Figure 7b).

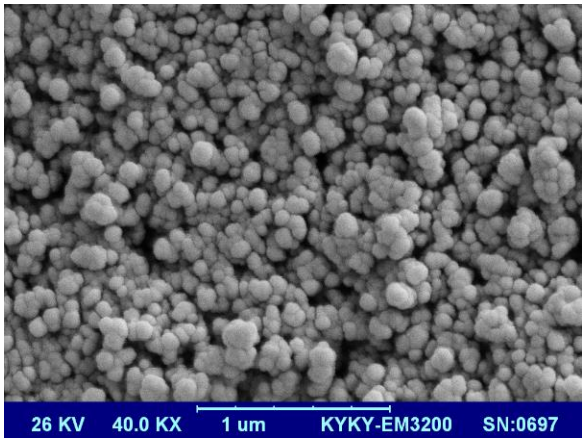


**Figure 5. SEM image of unloaded nanoparticles in dry form on glass slide.**

Stool IgA responses obtained in group B were significantly higher than those of group A ($p<0.0001$). The specific secretory IgA responses in the eye-washes of group B was not detected until day 28. Nevertheless, on day 42, an unexpected increase was observed in secretory IgA (16 EU $ml^{-1}$) (Figure 7c). Statistical analysis further showed a significant difference between groups A and B ($p<0.0001$). No specific immunoglobulin responses were observed in groups C and D.

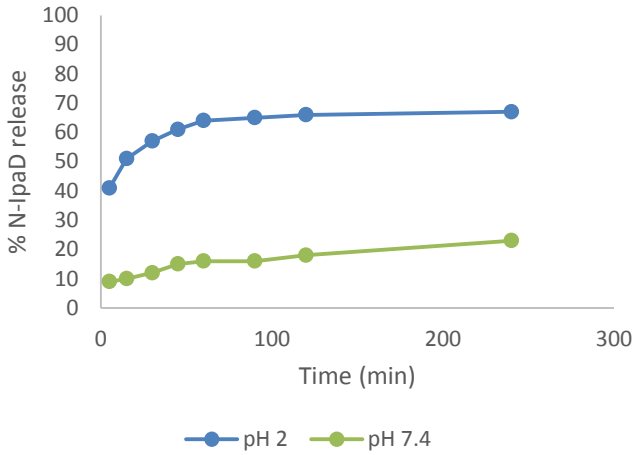


**Figure 6. Protein release profile of protein.**

**Animal challenge.**

The protective efficacy was assessed on day 42. The guinea pigs were subjected to ocular challenge with $8 \times 10^8$ CFU of *S. flexneri* 2a, and their eyes were daily monitored for 7 days after the challenge so as to develop Keratoconjunctivitis (Table 4). Keratoconjunctivitis was observed in the animals of control groups within 2-4 days after challenge with *S. flexneri* 2a.

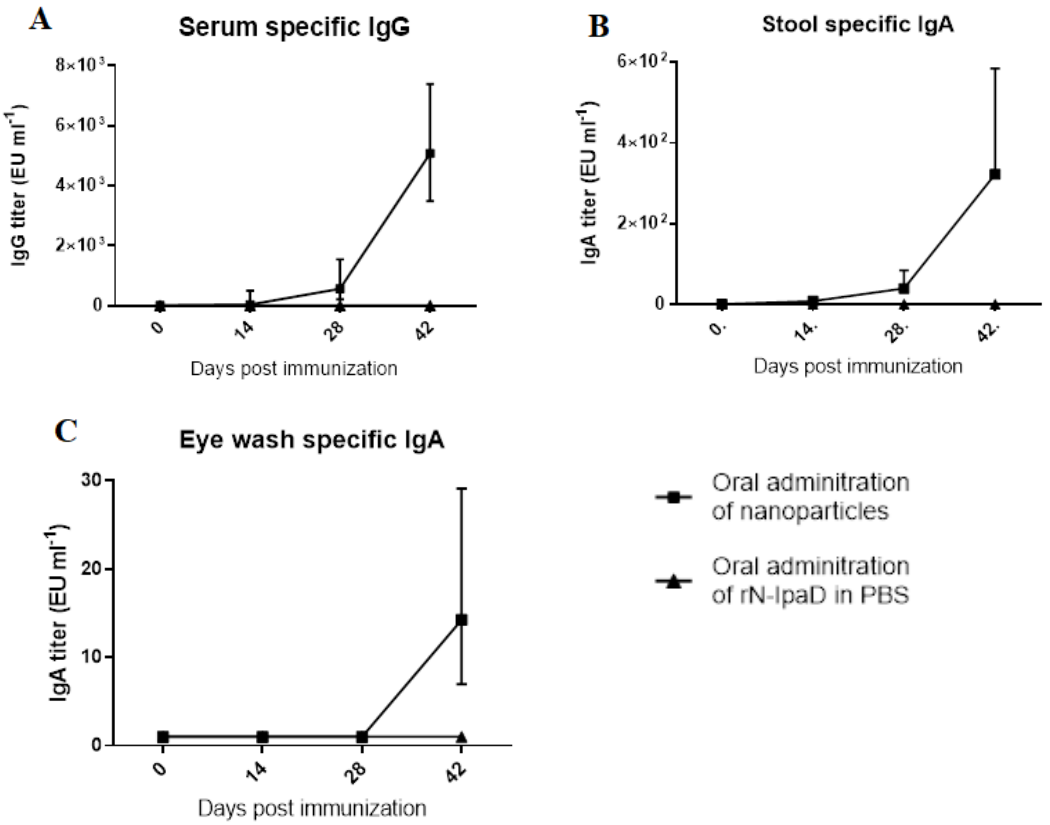


**Figure 7. Geometric mean of rN-specific antibody responses (with 95% confidence interval),** (a) rN-IpaD-specific serum IgG levels, (b) rN-IpaD-specific faecal IgA and (c) eye-wash IgA level. All samplings were done one week after each administration.

The animals orally administrated with rNIpaD in HBSS (C group) and negative control groups exhibited the disease. The group B showed the highest level of protection (5 of 6 animals protected, 83.3%) against virulent *S. flexneri* 2a.

**Table 4. Sereny test results.**

| Designation | Formulation | Animals developing Keratoconjunctivitis (%) (2-4 days) | *p*-value | *p*-value summary |
|---|---|---|---|---|
| **A** | - | 100 | 1 | - |
| **B** | 2 mg nanoparticles containing 0.5 mg rN-IpaD | 16.7 | 0.0152 | * |
| **C** | 500 µg rN-IpaD | 100 | 1 | Ns |
| **D** | 2 mg unloaded nanoparticles | 100 | 1 | Ns |

## DISCUSSION

Shigellae is an enteric pathogen that colonizes and invades the gut. The mucosal immunity is more efficient against this pathogen, and mucosal vaccines are safer and better-tolerated compared with parenteral vaccines. In previous studies, we introduced a subunit candidate vaccine based on the N-terminal region of IpaD protein (12,13). The nasal administration of this protein and chitosan nanofibrous membrane formulation evoked immunity against shigellosis in guinea pigs (14).

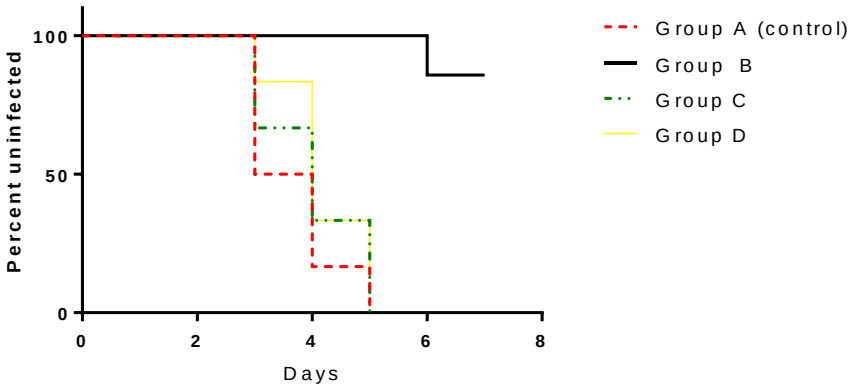


**Figure 8.** Results of the Sereny test. $8\times10^8$ CFU of virulent *S. flexneri 2a* was inoculated to the eyes of animals two weeks after the final administration and animals were monitored for 7 days.

However, studies have shown that the oral administration of IpaD in PBS cannot provide an immune response (20). Trimethylated chitosan is a mucoadhesive polymer which increases oral peptide and protein bioavailability in animal models (21). We hypothesized that oral administration of TMC nanoparticle formulations could induce immune response against rNIpaD. In aquatic media, TMC polymer is soluble in a large pH range, opening up a broad range of applications, such as delivery of biological materials to host recipients. In the present study, rNIpaD was loaded into TMC NPs and its immunogenicity was evaluated following its administration via oral rout in guinea pigs. TMC polymer was synthesized through ion gelation method. The 1HNMR and FTIR confirmed that TMC was synthesized, free of O-methylation, at high quaternary levels (74%). The loading of rNIpaD into the nanoparticles was confirmed by the increase in particle size and reduction in the zeta potential. The decrease in the charge of nanoparticles was due to the loading of the negative charged rNIpaD (pI 6.2) into the positive charge of TMC nanoparticles. The rNIpaD release profile from the TMC-NPs in SGF showed an initial burst release followed by a sustained release for 2 h, after which, no further release was observed for 5 hours. The release profile of the protein from NPs in SIF buffer showed a sustained release for 5 h, after which period, around 26 % of the protein was dissociated from the NPs. The present study demonstrated that TMC nanoparticles containing rNIpaD are immunogenic when administered to guinea pigs via the oral route. Moreover, TMC-rNIpaD formulation induces antibodies in

serum and mucosal secretions in these animals. The highest titer of serum IgG was observed after day 42 in the group receiving rNIpad-loaded NPs, which is consistent with the findings of Abkar *et al.* (19). Secretory IgA response titers indicated that when NPs were orally administered, the specific IgA was detected in the feces of the immunized animals (Figure 7). Elevated IgA levels in the stools corroborates the hypothesis that local immune response contributes to the mucosal antibody production (22). In oral administration, the titration of secretory IgA in the eye-washes showed a delayed increase in this antibody titer on day 42. Therefore, the antigen processing in the GALT (gut-associated lymphoid tissue) resulted in antibody production in distant mucosal sites. The conjunctiva is involved in the local production of secretory IgA, the production of which was formerly believed to be exclusively located in the lacrimal gland (23,24). The results showed that the oral administration of rNIpaD in the TMC nanoparticles can actually stimulate humoral and mucosal immune responses. Regarding the challenge of guinea pigs in Sereny (keratoconjunctivitis) test, the animals that orally received the rNIpaD-TMC nanoparticles, showed higher levels of protection in comparison to those receiving bare rNIpaD (Table 4, Figure 8). This underscores the fact that mucosal immune response, indicated by IgA, is associated with protection. The results of the challenge study are in accordance with data obtained via immunoassay studies. In conclusion, evidence was provided regarding protection in animal model and a vaccine candidate was introduced based on trimethyl chitosan nanoparticles. It was further shown that the fomulation of rNIpaD in TMC nanoparticles, as oral vaccine, elicits an antigen specific mucosal and systemic immunity.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. WHO, Guidelines for the control of shigellosis, including epidemics due to Shigella dysenteriae type 1. 2005.
2. Kotloff KL, Winickoff JP, Ivanoff B, et al. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ. 1999; 77:651-666.
3. Rossi RM, Yum L, Agaisse H, Payne SM. Cardiolipin Synthesis and Outer Membrane Localization Are Required for Shigella flexneri Virulence. MBio. 2017; 8:e01199-17.
4. Hajizade A, Ebrahimi Firouz, Amani J, et al. Design and in silico analysis of pentavalent chimeric antigen against three enteropathogenic bacteria: enterotoxigenic E. coli, enterohemorragic E. coli and Shigella. Biosci. Biotech. Res. Comm. 2016; 9:225-239.
5. Avakh Majalan P, Hajizade Abbas, Nazarian S, et al. Investigating the Prevalence of Shigella Species and Their Antibiotic Resistance Pattern in Children With Acute Diarrhea Referred to Selected Hospitals in Tehran, Iran. J Appl Biotechnol Rep. 2018; 5:70-74.
6. Hajizade A, et al. Nanoparticles in vaccine development. J Appl Biotechnol Rep. 2015; 1:125-134.
7. Zhao L, Seth A, Wibowo N, Zhao CX, et al. Nanoparticle vaccines. Vaccine, 2014; 32:327-337.

8. Hajizade A, Salmanian AH, Amani J, Ebrahimi F, et al. EspA-loaded mesoporous silica nanoparticles can efficiently protect animal model against enterohaemorrhagic E. coli O157: H7. Artif Cells Nanomed Biotechnol. 2018; 46:S1067-S1075.
9. Hughes GA. Nanostructure-mediated drug delivery, in Nanomedicine in Cancer. 2017, Pan Stanford. p. 47-72.
10. Choi C, Nam JP, and Nah JW. Application of chitosan and chitosan derivatives as biomaterials. Journal of Industrial and Engineering Chemistry, 2016; 33:1-10.
11. Yue ZG, Wei W, Lv PP, et al. Surface charge affects cellular uptake and intracellular trafficking of chitosan-based nanoparticles. Biomacromolecules. 2011; 12:2440-6.
12. Hesaraki M, Saadati M, Honari H, Olad G, et al. Molecular cloning and biologically active production of IpaD N-terminal region. Biologicals. 2013; 41:269-274.
13. Saadati M, et al. Cloning and Expression of N-terminal Region of IpaD from Shigella dysenteriae in E. coli. J Paramed Sci. 2010; 1:12-17.
14. Jahantigh D, Saadati M, Fasihi Ramandi M, et al. Novel intranasal vaccine delivery system by chitosan nanofibrous membrane containing N-terminal region of IpaD antigen as a nasal Shigellosis vaccine, Studies in Guinea pigs. J. Drug Del. Sci. Tech. 2014; 24:33-39.
15. QIAexpressionist, A. "A handbook for high-level expression and purification of 6x histagged proteins." Qiagen. p1-125 (2002).
16. Verheul RJ, Amidi M, van der Wal S, et al. Synthesis, characterization and in vitro biological properties of O-methyl free N, N, N-trimethylated chitosan. Biomaterials. 2008; 29:3642-9.
17. Biswas, Subrata, et al. Development and characterization of alginate coated low molecular weight chitosan nanoparticles as new carriers for oral vaccine delivery in mice. Carbohydr Polym. 2015; 121:403-10.
18. Farhadian A, Dounighi NM, and Avadi M. Enteric trimethyl chitosan nanoparticles containing hepatitis B surface antigen for oral delivery. Hum Vaccin Immunother. 2015; 11:2811-8.
19. Abkar M, Fasihi-Ramandi M, Kooshki H, et al. Oral immunization of mice with Omp31-loaded N-trimethyl chitosan nanoparticles induces high protection against Brucella melitensis infection. Int J Nanomedicine. 2017; 12:8769-8778.
20. Heine SJ, Diaz-McNair J, Martinez-Becerra FJ, et al. Evaluation of immunogenicity and protective efficacy of orally delivered Shigella type III secretion system proteins IpaB and IpaD. Vaccine, 2013; 31:2919-2929.
21. Caramella C, Ferrari F, Bonferoni M C, et al. Chitosan and its derivatives as drug penetration enhancers. J. Drug Del. Sci. Tech. 2010; 20:5-13.
22. Kim L, Martinez CJ, Hodgson KA, et al. Systemic and mucosal immune responses following oral adenoviral delivery of influenza vaccine to the human intestine by radio controlled capsule. Sci Rep. 2016; 6:37295.
23. Knop E, Knop N. The role of eye- associated lymphoid tissue in corneal immune protection. J Anat. 2005; 206:271-85.
24. Bergmann KC, Waldman RH, Tischner H, Pohl WD. Antibody in tears, saliva and nasal secretions following oral immunization of humans with inactivated influenza virus vaccine. Int Arch Allergy Appl Immunol. 1986; 80:107-9.