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

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Nasal Shigellosis vaccine is quite attractive for inducing a protective immune response. The reason is the invading effect of Shigella spp. to body through mucosal surfaces. Chitosan is a mucoadhesive polysaccharide with great potential for nasal vaccine delivery regarding to the exclusive features including biocompatibility, biodegradability, high charge density and non-toxicity. In this study, the potential of chitosan nanofibrous membrane as a novel carrier system for the nasal delivery of shigella subunit vaccine was investigated. The antigen-containing chitosan nanofibrous membrane was prepared by electrospinning of chitosan/AcOH solution. Guinea pigs were vaccinated by direct administration of antigen-containing chitosan nanofibers into the nasal cavity. The guinea pigs immunized intranasal administered N-IpaD/NFs showed higher serum and mucosal antibody responses comparing to those of other groups. All immunized animals were subsequently protected against a challenge with wild-type S. flexneri 2a in a keratoconjunctivitis Sereny test that N-IpaD/NFs group was protective.

Key words: Shigellosis – Nasal vaccine – Chitosan nanofibrous membrane – Electrospinning technique – Mucosal antibody response.

Shigellosis, an endemic disease throughout the world caused by the Gram negative bacteria of *Shigella* spp., is still a significant public health problem which affects more than 165 million cases of severe dysentery with blood and mucus in the stools, the overwhelming majority of them occur in developing countries [1]. About 1.1 million people were estimated to die from *Shigella* infection each year, 60 % of whom being children fewer than 5 years old [1, 2]. *Shigella* has no animal reservoir; therefore, infection always results from contamination with human feces and is common among travelers and military troops deployed in camp situations with less than optimal hygiene conditions [2]. Currently no vaccine against *Shigella* exists and the increased frequency of antimicrobial resistant *Shigella* strains worldwide is a source for increasing concern [3].

Three major species of *Shigella*: *S. sonnei, S. flexneri* and *S. dysenteriae*, and a minor species, *S. boydii*, are responsible for bacillary dysentery, which are commonly associated with acute diarrhoeal diseases. These species are subdivided into serotypes, according to the antigenic specificity of the O-polysaccharide portion of their LPS [1, 2]. *Shigella* invades the colonic epithelium by transcytosis through M cells and then invades the colonic epithelium cells via a type III secretory system (TTSS) that is encoded by pathogenicity islands which are located in a 220-260-kb, low copy number virulence plasmid (pINV), functionally interchangeable between species and serotypes [4]. The invasiveness and virulence of *Shigella* spp. are largely due to the expression of plasmid-encoded virulence factors, among which are the invasion plasmid antigens (Ipa proteins) [5]. Secretion of the Ipas (IpaA, -B,-C,-D) as well as other effector proteins serves to modify the host cell environment allowing for intracellular survival [4,5]. IpaD is one of most imperative *Shigella* virulence agents and is essential for *Shigella* invasion [6]. The globular and hydrophilic protein IpaD, is localized to the *Shigella* TTSA tip of needle that induces type III secretion system (TTSS) [7]. The localization of IpaD to the surface is a prerequisite for cellular invasion that is very likely necessary for the proper insertion of the IpaB/IpaC into the host cell membrane [7], in order to form a translocon pore through which other effector proteins subsequently pass [8]. Afterwards this process is followed by active depolymerisation of the actin microtubules through the action of VirA, a plasmid encoded virulence factor [4-8]. Then *Shigella* escapes into the cytoplasm and begin to disseminate to adjacent epithelial cells through polarized actin polymerization process by polar expression of VirG (IcsA) on the surface of the bacteria which provides the motive force for bacterial movement [9]. N-terminal region is most significant functional fragment of IpaD [10]. IpaD epitopes exposed on the surface of intact *Shigella* cells were identified consistently within the amino-terminal half of the protein [10, 11]. Moreover, epitopes recognized by antibodies in immune serum from infected monkeys were characterized and found to be located predominantly in the exposed amino terminal portion of IpaD [11].

Several studies have investigated different strategies for achievement to a safe and protective *Shigella* vaccine [12, 13]. Mucosal Shigellosis vaccine is really attractive for inducing a protective immune response because *Shigella* spp. invades the body through mucosal surfaces. A unique characteristic of immune responses at mucosal surfaces are the production of secretory immunoglobulin A (S-IgA) antibodies (Abs) and their transport across the intestinal epithelium [14, 15]. Prominently, mucosal vaccines can induce both systemic and mucosal immunity, resulting in two layers of host protection, which are considered as potent tools for eradicating pathogens invading via the mucosa [16, 17]. Compared with other routes of vaccine administration, intranasal vaccine delivery possesses have many advantages, such as easy and cheap to administration, highly vascularization and relatively large epithelial surface area produced by numerous microvilli, a porous endothelial membrane, low proteolytic activity and the ability to induce mucosal as well as systemic immunity [18]. Importantly, intranasal immunization was reported to be more efficient than oral immunization at inducing secretory in addition to systemic antibody responses [19]. In spite of the attractive features of nasal immunization, most nasal vaccines are poorly immunogenic when administered as solutions, which these poor immunity have been attributed to several factors such as the limited diffusion of macromolecules across the nasal barrier [20], enzymatic degradation within nasal secretions [21], and relatively rapid mucociliary clearance that may not allow sufficient

antigen uptake by antigen presenting cells in the nasal associated lymphoid tissue (NALT) [22]. Different strategies have been designed to prolong the residence time in the nasal cavity, such as the administration of antigens with mucosal adjutants, absorption enhancers and/or entrapment of antigens in biodegradable microspheres/microparticles and liposomes to protect entrapped antigens against degradation, to enhance uptake by M-cells, and to target the antigens more specifically to antigen presenting cells (APC). Chitosan, a natural polysaccharide, consisting of glucosamine and N-acetylglucosamine derived through the partial deacetylation of chitin, because of their excellent properties have been extensively studied for delivery of therapeutic proteins and antigens particularly via mucosal routes [23-26]. Chitosan is a cheap, biocompatible, biodegradable, mucoadhesive, and nontoxic natural polymer, which can interact with mucus and epithelial cells and induce a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 resulting in opening of cellular tight junctions and increasing the paracellular permeability of the epithelium [27, 28]. Chitosan-based particles loaded with proteins can be prepared by both chemical and physical methods. However major drawbacks are associated with the use of chemical crosslinking methods.

In this study, we have proposed a novel intranasal vaccine delivery system. This vaccine involves the delivery of *Shigella* antigens through the nasal cavity, via a continuous, nonwoven chitosan nanofibrous membrane. The chitosan nanofibers membranes, containing N-terminal region of IpaD *Shigella* subunit antigen, which are produced through the process of electrospinning. Electrospinning is a process in which the surface tension of a liquid polymer, formed at the tip of a needle, is overwhelmed with an electric field. This causes the droplet to form a very thin, whip-like jet that deposits solid fibers onto the collector. The fibers diameters generally range from a few nanometers to submicrons depending on the parameters used [29]. We show that the electrospinning technique allows the production of antigen-containing chitosan nanofibers membranes that are suitable for nasal administration in a Guinea pigs model and capable of inducing strong systemic and local immune responses.

I. MATERIALS AND METHODS

1. Materials

Chitosan powder with 75-85 % of deacetylation (Mw = 1.095×10^6 g/mol) from Aldrich [30, 31], glacial acetic acid (AcOH) from Merck.

2. Antigens and antibodies

In this study, we use *Shigella* dysenteriae recombinant antigen, rN-terminal region of IpaD, which had been produced in our Laboratory, Department of Biology, Imam Hussein Comprehensive University, previously [32]. Both HRP Conjugated Goat anti-Guinea Pig IgG and HRP Conjugated Sheep anti-Guinea Pig IgA were purchased from Immunology Consultants Laboratory, Inc. (Portland, USA).

3. Preparation of stock solutions for electrospinning

In order to hydrolyze chitosan polymer chains, mixtures of 1/25 (w/v) chitosan/NaOH 50 % were treated at 95 °C for 48 h [30, 31, 33]. The solution was strained and rinsed with deionized water, neutralized with acetic acid, rinsed, and dried at 60 °C for 16 h. This hydrolyzed chitosan powder was used in all subsequent stages. A solution by 7.5 % (w/v) hydrolyzed chitosan/AcOH 70 % was created and then it had been shaking overnight at room temperature with gentle rotation. One milligram of lyophilized subunit antigen, rN-terminal region of IpaD, was reconstituted in 1 mL sterile distilled water to produce a stock recombinant antigens concentration of 1 mg/mL. An aqueous solution from the chitosan (untreated and hydrolyzed)/ AcOH solution and the diluted recombinant antigen were used in all encapsulation processes, a 1:1 relation by volume solution to create an end recombinant antigens concentration of 0.5 mg/mL.

4. Encapsulation through electrospinning process

The stock solutions for electrospinning were prepared and inserted into syringes with 0.7 mm inner capillary diameter and setup published previously was used for electrospinning [33]. The parameter utilized during electrospinning was as follows: the flow rate was used 8×10^{-2} mg/h (1.6 mm^3/h), the distance between the tip of the syringe and the collector was 16 cm and the voltage used was 17 kV. The collecting surface used was heavy-duty aluminum foil. The nanofibers electrospun mats produced through electrospinning of chitosan/AcOH solution without antigen and with diluted recombinant N-IpaD arrange were labelled with the numbers 1 and 2, respectively. All electrospinning experiments were carried out under ambient conditions (25 °C, 50 % humidity in average). After the electrospinning process complied, the solid electrospun membrane containing nanoencapsulated *Shigella* antigen, deposited onto the collecting surface was placed into PBS in a reagent reservoir. The membrane was allowed to dissolve directly off of the aluminum foil for a period of ten minutes to ensure of complete dissolution. The resulting solution had recombinant antigens concentration of 0.5 μg/μL and filtered through 0.22 μm filter.

5. Encapsulation efficiency and loading of N-IpaD antigen in chitosan nanofibers

The encapsulation efficiency and loading of the N-IpaD loaded in chitosan nanofibers membranes were determined by separating the nanofibers from the preparation media, by centrifugation at 16000 g for 40 min. The amount of free protein in the supernatant was measured by micro-BCA protein assay method [34]. The supernatant of blank chitosan nanofibers was used as blank. The encapsulation efficiency (EE) was calculated from standard curve and percent of EE was calculated from *Equation 1*:

$$EE\ \% = (\text{N-IpaD entrapment}/\text{N-IpaD total}) \times 100 \qquad \textbf{Eq. 1}$$

6. Short-term *in vitro* release of N-IpaD antigens from chitosan nanofibers

Aliquots of 1 mL N-IpaD in chitosan nanofibers membranes suspension prepared with Tween were centrifuged at 10000× g on a 10 μL glycerol bed for 15 min. The supernatant was decanted and the pellet was re-suspended in 1 mL phosphate buffered saline in microfuge tubes (PBS, 0.1 M, and pH 7.4). The tubes were incubated at 37 °C, under agitation (50 rpm), for 4 h. Each 30 min until 4 h, a tube was taken and centrifuged (at 18000× g for 10 min). The released protein in the supernatant was determined by the micro BCA protein assay (Pierce, USA). To eliminate background interference, a blank sample consisting of only chitosan nanofibers resuspended in PBS was used. The experiments were performed in triplicate.

7. Animals and immunization studies

Male Hartley Guinea pigs (200-250 g) were obtained from the Pasteur Institute of Iran. The animals were housed at controlled temperature with free access to rodent chow and water. All study animals were approved by the Institutional Animal Care and Use Committee at the University of Imam Hussein University, Tehran, Iran. Guinea pigs were divided into five groups and each group comprised of twelve to sixteen Guinea pigs. Before each immunization, Guinea pigs were anesthetized intramuscularly with a mixture of ketamine (30 mg/mL), xylazine (6 mg/mL) and atropine (0.1 mg/mL) in physiological saline (1 mL per Guinea pig), and then Guinea pigs were immunized by intranasal administration of electrospun membrane, containing nanoencapsulated antigen, and others formulations at three successive doses with 2 week intervals (days 0, 14, 28), into the nasal cavity. The intranasal administrated formulations were given by instillation in a total volume of 100 μL (50 μL into each nostril) containing 50 μg of N-IpaD subunit antigen, with a micropipette tip, which is an appropriate volume to prevent variable bioavailability due to the deposition of the

Table I - Immunization study design (A-E)*.

Intranasal immunization
A: PBS (negative control)
B: N-IpaD solution
C: N-IpaD/chitosan solution
D: Electrospun N-IpaD chitosan nanofibers (N-IpaD/NFs)
Intramuscularly immunization (i.m.)
E: Alum-adsorbed N-IpaD (positive control)

*All formulations were prepared and/or resuspended in PBS.

formulation in the lung and lower respiratory tract [35]. For positive control group, lightly anesthetized Guinea pigs were intramuscularly (i.m.) immunized with 100 mL freshly prepared alum-adsorbed N-IpaD subunit antigen, with final concentration of 50 µg, in physiological saline, by injection into their left or right hind leg using the same schedule as was used with nasal administration (*Table I*).

8. Sample collection

Blood and the broncho-alveolar lavages (BAL) were collected before and after immunization (days 0, 14, 28) and after challenge (CLG) for analysis of humoral and mucosal immune responses. The blood samples were allowed to clot overnight and then centrifuged at 8000 g for 5 min at room temperature. After blood samples were taken, BALs were collected by gently washing the lungs with a volume of 6-8 mL of PBS (10 mL/kg of animal weight) supplemented with protease inhibitor cocktail (1 mM) (Sigma Aldrich) by using a catheter, inserted into the trachea. The BALs were centrifuged (10 min at 3000×g) to remove debris and the supernatants were stored at -20 °C till the day of analysis.

9. Determination of immune responses

Immune responses to *Shigella* antigens, N-IpaD, in various formulations were analyzed by Enzyme-Linked Immuno sorbent Assay (ELISA) in order to determine the levels of N-IpaD specific serum immunoglobulin G (IgG), immunoglobulin A (IgA) and secretory immunoglobulin A (sIgA) antibody as previously described [36, 37].

10. Serum (IgG and IgA) and secretory (S-IgA) antibodies response elicited against N-terminal region of IpaD following immunisation of Guinea pigs

ELISA plates were coated overnight at ambient temperature with rN-IpaD, 0.5 Lf in 100 µL/well, in coating buffer (0.05M carbonate/bicarbonate, pH 9.6). Plates were washed 6 times with PBS/Tween (PBS containing 0.05 % Tween, pH 7.6) and blocked by incubation with 2.5 % (w/v) bovine serum albumin (BSA) in 200 µL/well PBS/Tween for 1 h at 37°C. Subsequently, the plates were washed with PBS containing 0.05 % PBS/Tween. Primary antibodies collected from the sera and BAL lavages of immunized Guinea pigs were used for the analysis of immune response. Appropriate dilutions of sera and non-diluted BAL of each individual Guinea pig were applied to the plates, serially diluted two-fold in PBS/Tween and incubated for 2 h at 37 °C. After washing with PBST, plates were incubated with Goat anti-Guinea Pig IgG (diluted 1:5000 in PBS/Tween, 100 µL/well) HRP conjugated and Sheep anti-Guinea Pig IgA HRP conjugated (diluted 1:1000 in PBS/Tween, 100 µL/well) for 1 h at 37 °C. Afterwards, the plates were washed 6 times with PBS/Tween and once with PBS. Specific antibodies were detected by staining with O-phenyldiamine dihydro-chloride, as described previously [36]. After 10 min, the reaction was stopped by adding 100 µL/well of 1 NH_2SO_4. Antibody titers are expressed as the reciprocal of the calculated sample dilution corresponding with an A492 of 0.2 above the background.

11. Assessment of vaccine protective efficacy (conjunctival challenge test)

Three weeks after final immunization (day 49), groups of vaccinated and sham immunized Guinea pigs were challenged intraocularly by the Sereny keratoconjunctivitis test by administering with 6×10^8 cfu virulent *S. flexneri* 2a in 25 µL of normal saline into the conjunctival sac of one eye and scored daily for disease, as described previously [38]. Guinea pigs were examined for 7 days by an observer who was unaware of the immunization status of the animals, and the degree of inflammatory response, if any, was graded according to the following scale: 0, no inflammation or mild irritation; 1, mild keratoconjunctivitis or clearing; 2, keratoconjunctivitis without purulence; and 3, severe keratoconjunctivitis with purulence. Protection percent was calculated by the following formula: [% disease in controls - % disease in vaccines)/(% disease in controls)] ×100.

12. Statistical analysis

Statistical analyses of individual experiments are described in the text or in the figure legends where appropriate. For analysis of immunogenicity in Guinea pigs, the geometric mean titers (GMTs) for each group of Guinea pigs were calculated, using Prism 4 (Graphpad Software, Inc) with 95 % confidence intervals. Different groups were compared with one-way analysis of variance (ANOVA) using Bonferroni's multiple comparison test with significance level of $P < 0.05$. A statistical comparison of the Guinea pig protection data was determined by using Fisher's exact test. P values of <0.05 were considered significant.

I. RESULTS

1. Electrospinning

The morphology and diameter of the electrospun nanofibers of chitosan were analyzed before and after the antigens encapsulation process. The powders of chitosan stock and different antigens loaded chitosan were mounted on circular aluminium stubs with double-sided sticky tape, coated for 250 s with 15 nm gold. Afterward, examined and photographed by a LEO Electron Microscopy Model 1455 variable pressure-scanning electron microscope (VP-SEM) at an accelerating voltage of 10 kV. In *Figure 1*, the morphology of samples a and b can be observed.

Nanofibers without antigen content show defects free morphology and its diameter distribution gives an average value of 87 ± 2.06 nm, which one is within the nanometer range. Once the antigens were encapsulated inside the nanofibers through electrospinning, the fiber diameter was increased to 103 ± 1.53 nm. However, this increase was not appreciative, conserving the characteristic large surface area of the nanofibers. In *Figure 1*, the appearance of entangled fibers along with bead defects can also be observed.

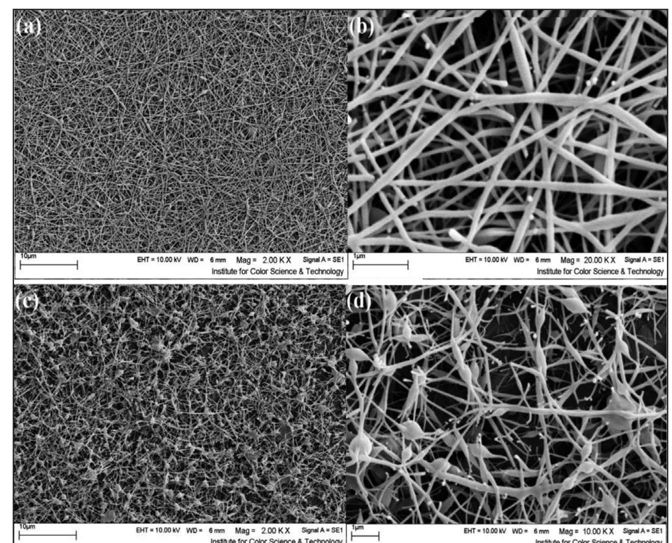

Figure 1 - SEM images of chitosan nanofibers in the samples were the antigens solution was utilized as external element in the electrospinning: (a) and (b) chitosan nanofibers without antigen, (c) and (d) N-IpaD/chitosan nanofibers.

2. Presence of the antigen in the electrospun nanofibers

In produced samples using the antigens as internal element, the encapsulation of the antigen inside the chitosan nanofibers were confirmed through infrared spectroscopy analysis (FTIR) (samples a and b). The IR spectra of both the chitosan alone and the antigen loaded chitosan nanofibers are shown in *Figure 2*. The FTIR spectrum of chitosan showed a strong amino characteristic peaks at 3435 cm^{-1}, also the chitosan fibbers exhibit others bands at: 2876 cm^{-1} corresponding to stretching vibration of C-H bond; at 1658 cm^{-1}, a characteristic amide I band attributed to C=O vibration of the acetylated units (-$CONH_2$ groups). The peak at 1379 cm^{-1} was the joint contribution of the vibration of -OH and -CH. The band at 1167 cm^{-1} corresponds to the symmetrical stretching of C-O-C; bands at 1075 cm^{-1} were associated to the C-O stretching vibration [39].

The IR spectrums of the antigen loaded chitosan nanofibers (*Figure 1*) displayed band at 1654 and 1541 cm^{-1} in N-IpaD/NFs attributed to -COO^- and -$NH3^+$ groups stretching vibration, respectively. The coacervation between the antigen and chitosan changed the FTIR in the carbonyl-amide region (*Figure 2*). The -$NH3^+$ groups, bands at 1658 cm^{-1}, and asymmetric and -COO^- stretching vibration at 1679 and 1679 cm^{-1} in antigen loaded chitosan nanofibers, were changed, indicating the electrostatic interaction among the carboxyl groups of different antigens (-COO^-) and amine groups of chitosan (-$NH3^+$). Moreover, the spectrum of antigen loaded chitosan nanofibers coacervate showed a narrow and changed band at around 3000 to 3600 cm^{-1}, indicating enhanced hydrogen bonding compared to that of chitosan. This implied that hydrogen bonding was also involved in the interaction between the antigens and chitosan, as well as, the anterior absorption bands are features of proteins, which is indicative of the presence of the antigen in the chitosan nanofibers.

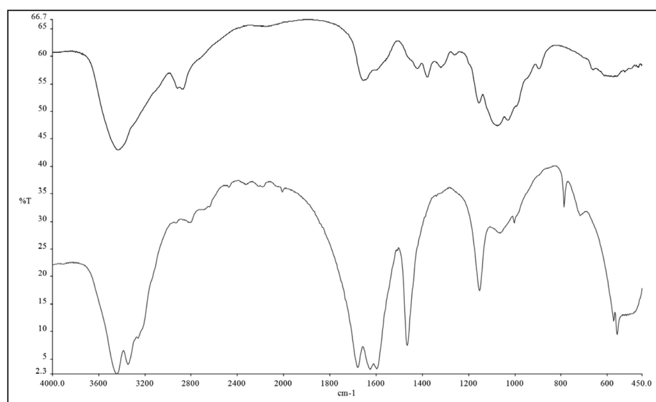

Figure 2 - FTIR spectra of chitosan nanofibers with different antigens as internal element: chitosan without antigen (up), N-IpaD/NFs (down).

3. Loading and release study of N-IpaD antigens from chitosan nanofibers

Encapsulation efficiency of N-IpaD was calculated as 64.7 ± 14.3 %. This high entrapment efficiency could be attributed to positive charge of chitosan and negative charge of N-IpaD. It seems that electrostatic interactions have a positive effect on N-IpaD loading.

As it is seen in *Figure 3*, *in vitro* release studies showed that around 20 % of the loaded protein was immediately released into PBS. Nearly all of electrospun antigen has been released within 2.5 h. After 2.5 h, the graph reached to plateau.

This high observed stability might be ascribed to protein molecules that were tightly bound to chitosan nanofibers as well as high capacity of chitosan nanofibers for antigen trapping. It is important that the protein is not released to a large extent before the protein loaded-chitosan nanofibers pass the nasal epithelial barrier during their residence in the nasal cavity.

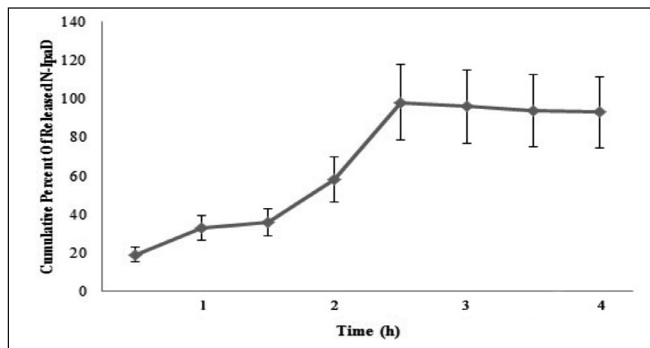

Figure 3 - Release profile of N-IpaD from chitosan nanofibers. The N-IpaD-loaded nanofibers were suspended in 1 mL of distilled water and incubated in 37 ºC under continuous shaking. The amount of released N-IpaD in supernatant was determined by micro-BCA protein assay method each 30 min until 4 h.

4. N-IpaD-specific serum IgG and IgA antibody

IgG and IgA titers at day 0 were not detectable but the IgG titers following the first booster were substantially increased (*Figure 4*). On day 14, antigen, rN-IpaD, in chitosan nanofibers membranes (D group) and with chitosan solutions (C group) exerted a higher induction of the immune responses in comparison with subunit antigen in saline solution (B group), but less than antigen injected in alum i.m. (E group). Comparing the results of group D with C, showed that the IgG levels obtained from Guinea pigs which have received N-IpaD in chitosan nanofibers membranes, were slightly higher than those obtained from Guinea pigs which have received N-IpaD in chitosan solution; but the difference observed between these two groups was not significant ($p < 0.089$). Significant different IgG levels were observed when group E with group C ($p < 0.001$), and D ($p < 0.001$) was compared. On day 28 and after challenge (GLC), compare to negative control (A), the IgG antibody responses to subunit antigens in all formulations were greatly increased.

A high statistical significant different IgG levels were found between group D, which have received N-IpaD in chitosan nanofibers membranes, with groups which have received antigens in saline or chitosan solutions (B and C), but not with group E which have received antigen injected i.m. These results show that antigen in chitosan nanofibers membranes could elicit higher IgG responses than antigens in chitosan or saline solutions. Most significantly sera collected from the N-IpaD/NFs immunized Guinea pigs showed about 5.19 ($p < 0.001$) and 8.59 ($p < 0.001$) times greater IgG responses than those collected sera from N-IpaD in chitosan and PBS solutions, on GLC, respectively (*Figure 4A*). IgG responses obtained with Guinea pigs in E group which was vaccinated i.m. by the antigen in alum were also significantly higher than those of the Guinea pigs immunized i.n. with antigen in different formulations (*Figure 4A*).

In general, the results of IgA responses of the various groups of Guinea pigs were similar to the results of the IgG responses. IgA levels obtained from Guinea pigs having received subunit antigen, N-IpaD, in alum injected i.m. were again significantly higher than those obtained from Guinea pigs which were vaccinated intranasal with subunit antigens in chitosan nanofibers membranes and in chitosan or saline solutions (*Figure 4B*). The IgA response in group D, which have received antigen in chitosan nanofibers membranes, was highly more significant than other intranasal vaccinated groups ($p < 0.001$). However, significant different IgA levels were observed on day 28 and GLC in comparison of group E with others groups ($p < 0.001$).

5. N-IpaD-Secretory IgA (S-IgA) antibody

Since mucosal immunization has great potential of inducing local immune responses, particularly S-IgA, the IgA levels were measured in the BAL lavages of the Guinea pigs on days 0, 14 and 28. *Figure 5* illustrates

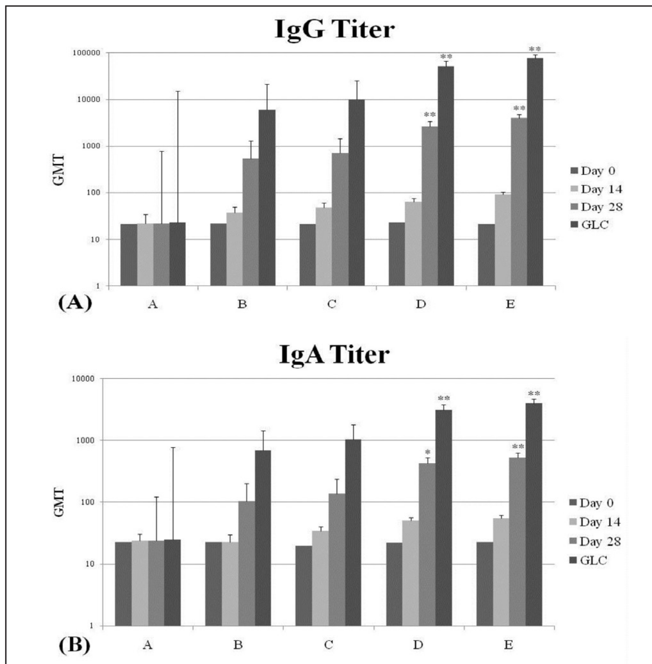

Figure 4 - Serum IgG- and IgA-specific antibody titers against N-IpaD were measured in Guinea pigs immunized i.n. or i.m. Formulations: PBS (A), N-IpaD solution (B), N-IpaD/chitosan solution (C), N-IpaD/NFs (D), Alum-N-IpaD (E). The immune responses were determined for days 0, 14, and 28. The geometric mean titer (GMT) for each group of Guinea pigs was calculated and is shown on the y-axis. Serum immune responses were also measured 2 weeks after challenge of immunized Guinea pigs with wild-type *S. flexneri* 2a (CLG). 95 % confidence intervals are indicated by error bars. Significant differences between control groups and vaccinated groups were expressed as *p < 0.05, **p < 0.001, respectively.

the specific anti-N-IpaD S-IgA titers in the BAL lavages of the Guinea pigs immunized i.n. with different formulations. High and significant S-IgA titers were detected in all Guinea pigs after i.n. immunizations with the chitosan nanofibrous membrane containing N-IpaD. On days 14 and 28, difference between group D with others groups was highly considerable (p < 0.001). In contrast, Guinea pigs immunized i.n. with free antigen or antigen mixed with chitosan gave low IgA titers. These results indicate that antigen in chitosan nanofibers membranes can elicit very high IgA responses than antigen in chitosan or saline solutions.

No detectable S-IgA response was obtained after i.m. immunization with N-IpaD in alum (data not shown).

6. Protection against conjunctival challenge

In an efficacy study, Guinea pigs were ocularly challenged about 3 weeks after final immunization with virulent *S. flexneri* 2a. The

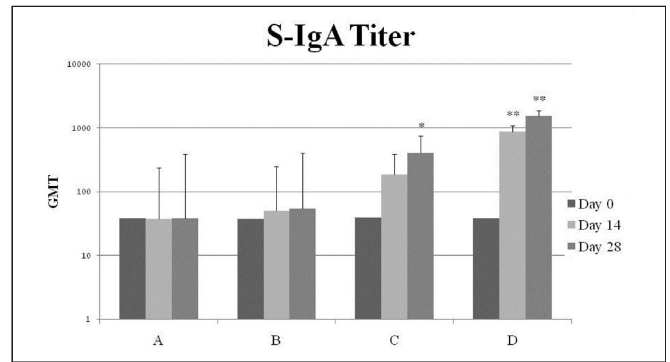

Figure 5 - N-IpaD-Secretory IgA (S-IgA) antibody titers in BAL of guinea pigs immunized with PBS (A), N-IpaD solution (B), N-IpaD/chitosan solution (C), N-IpaD/NFs (D). The GMT (y-axis) was determined for day 0, 14, and 28 for each group of Guinea pigs. The error bars indicate 95 % confidence intervals. Significant differences between control groups and vaccinated groups were expressed as *p < 0.05, ** p < 0.001, respectively.

Guinea pig eyes were daily monitored for 7 days after challenge for the development of a reaction, and scored according to a pre-established grading method (ranging from 0 to 3), described in Section 2 (*Table II*). Full blown keratoconjunctivitis was observed in the control groups of animals immunized with empty PBS or within 1-2 days after challenge with *S. flexneri* 2a (*Table II*). Moreover, the animals immunized with N-IpaD in saline solution (B group) and also, immunized i.m. with the antigen in alum (E group), showed poorly protection following challenge (*Table II*). The Guinea pigs in group C which have received the antigens in chitosan solution were slightly protective against virulent *S. flexneri* 2a after challenge. In contrast, in animals intranasal immunized with N-IpaD-containing chitosan nanofibers membranes (D group), low levels of disease were seen during 7 days. The protective efficacy for the group D, vaccinated with IpaD/NFs, which was calculated to be 93.75 % (*Table II*).

III. DISCUSSION

Development of successful *Shigella* vaccines has been elusive. Over a span of multiple decades various approaches, such as killed whole cell, live attenuated and subunit vaccine strategies have been investigated [12, 13]. In this study, we investigated *Shigella* subunit antigen delivery as a novel nasal *Shigella* vaccine through a novel intranasal delivery system.

Locally produced secretory IgA is considered to be one of the most important protective humoral immune factors. This antibody constitutes over 80 % of all antibodies produced in mucosa associated tissues and it has been proposed to play the first line of defense against pathogens in the mucosal surfaces [14-17]. Oral immunization may achieve intestinal immunity but is often difficult to consistently accomplish due

Table II - Protection following challenge with wild-type *S. flexneri* 2a.

Immunizing strain	No. of eyes inoculated	No. of eyes with the indicated rating[a]				Disease/ total[b]	Disease (%)	Protection[c] (%)	p-value[d]
		0	1	2	3				
Intranasal immunization (i.n.)									
A	12	0	0	3	9	12/12	100	-	1
B	16	0	1	9	6	14/16	93.75	6.25	0.189
C	16	1	3	7	5	12/16	75	25	0.013
D	16	13	2	1	0	1/16	6.25	93.75	< 0.001
Intramuscular immunization (i.m.)									
D	16	1	2	8	5	13/16	81.25	18.75	0.189

[a]Inflammation rating: 0, no inflammation or mild irritation; 1, mild keratoconjunctivitis; 2, keratoconjunctivitis without purulence; 3, severe keratoconjunctivitis with purulence. [b]Guinea pigs were considered diseased if they had a rating of 2 or 3. [c] % protection was calculated by the following formula: [(% disease in controls - % disease in vaccinees)/% disease in controls] × 100. [d]p-value was determined using Fisher's exact test.

to delivery restrictions, particularly for inactivated or subunit vaccines. Moreover, it is established that nasally administered vaccines can provide effective immuno-stimulation, both in terms of humoral and cell-mediated responses, especially if the antigen is adjuvant by means of an immunostimulator or a delivery system [15-17]. However, the nasal mucosal immunity has several limitations, such as limited diffusion [21], rapid mucociliary clearance [22] and enzymatic degradation [27]. Chitosan is a safe and effective mucosal adjuvant or absorption enhancer for overcoming the limitation of mucosal immunization and induction of effective mucosal immune responses, and also it is a spinnable polymer with necessary parameters to make it suitable to produce proper nanofibers and is widely used in medical sciences such as drug delivery, tissue engineering and wound healing [23].

Chitosan, in nano and/or microparticle formation, has been widely used for protein and antigen delivery [24]. In current study; the novel method for nasal vaccine delivery utilizes the high surface area to volume ratio offered by antigen-containing nanofibers membranes, which were prepared by the electrospinning technique. We produced chitosan nanofibers membranes containing *Shigella* subunit antigen, as a model protein antigen, via chitosan alone and not co-administration with any second polymer, and then were delivered to NAT (nasal associated tissue) by intranasal administration for the first time. Recombinant *Shigella* antigen, N-IpaD, was successfully encapsulated in chitosan nanofibers, using electrospinning as an effective technique to encapsulation process. The electrospun nanofibers morphology was analyzed by SEM and gives us an acceptable morphology without sudden diameter changes or bead defects. Besides, the morphology of samples b and c gives entanglement and agglomerates morphology in the nanofibers surface, suggesting that antigens were encapsulated well within the fibers (*Figure 1*). Also, the presence of the antigens in the electrospun nanofibers was confirmed by infrared spectroscopy (FTIR). The changes in chitosan FTIR before and after the antigens encapsulation process indicate that presence of the antigens in the chitosan nanofibers (*Figure 2*).

In this study encapsulation efficiency of N-IpaD antigen was calculated as 64.7 ± 14.3 %, which is an acceptable loading. One of the important features of particulate delivery systems is the rate and profile of the release. It is important that the protein is not released to a large extent before the protein loaded-nanoparticles pass the nasal epithelial barrier during their residence in the nasal cavity. The normal mucociliary clearance rate in healthy human's nose is about 20 min [22]. At the present study, to give particulate nature to N-IpaD solution, it was encapsulated with chitosan nanofibers membranes. It has been shown in several studies that particulate antigens could better interact with APCs and also could be better uptaken by microfold cell of mucosa associated lymphoid tissues (MALT) [24, 24], therefore it is preferred that most of antigen keep its particulate nature and the least release rate is the most optimum condition. In the release profile of N-IpaD from of chitosan nanofibers membranes (*Figure 3*), nearly all of encapsulated N-IpaD has been released within 2.5 h.

To study the immune responses to *Shigella* antigens, N- terminal region of IpaD, in PBS, chitosan nanofibers membranes, chitosan solutions and in alum, the Guinea pigs were vaccinated by various formulations on days 0, 14 and 28, respectively. Sera and mucosal samples were collected on days 0, 14, 28 and after challenge (CLG). The results have obviously showed that nasal administration of this antigen in chitosan nanofibers membranes is able to enhance the IgG antibody, and also result in high IgA antibody response.

I.m. immunizations with subunit antigen in alum resulted in strong IgG immune responses with higher specific *Shigella* antibody levels than obtained with the i.n. administered vaccine associated with chitosan nanofibers membranes and vaccine solution both in chitosan and PBS solutions (*Figure 4A*). The effective immune-induction was substantiated by the increase of IgA immune responses in both serum and BALs after intranasal administration of antigen-containing chitosan nanofibers membranes, N-IpaD, vaccine (*Figures 4B and 5*). Our results have showed that N-IpaD specific IgA titer of BALs in the groups with administration of antigen-containing chitosan nanofibers membranes was significantly higher than those of other groups (*Figure 5*).

In this study, we used a small and unique protein which was an Ag and it has just one epitope, N terminal region of IpaD [10, 11], for intranasal delivery via chitosan nanofibers. Because this protein in chitosan nanofibers membranes were recognized by Guinea pigs immune systems and also the subsequently IgG, IgA and s-IgA antibodies which produced against it, matched with recombinant and native Ag particularly, in ELISA and challenge tests very well, respectively, indicated that the integrity of N-IpaD has been maintained during preparation, therefore Ag has been safe and maintained its folding.

These results have demonstrated that chitosan nanofibers membranes hold great promise as a delivery system for intranasal vaccination. Chitosan nanofibers membranes, containing nanoencapsulated *Shigella* subunit antigen, which are attached to mucosa and thereby, can prolong the residence of the antigen in nasal cavity. Therefore, more time is available for the antigen to be taken up in nasal tissues. The immune responses elicited by the antigen-containing chitosan nanofibers were probably based on improving cellular uptake of the nanoparticles by M-cells and APC in the nasal epithelium and NALT and more efficient delivery to mucosal lymph nodes, and/or more efficient stimulation of subunit antigens after uptaking and subsequent accessing of the vaccine to sub-mucosal lymphoid tissues.

The nasal vaccine significantly induced higher local IgA in nasal washings and local cell-mediated immunity but less high serum antibody titers than the injectable vaccine [16]. It has been shown that i.n. antigen exposure elicited IgA responses in salivary, respiratory, intestinal and vaginal secretions suggesting trafficking of NALT lymphocytes especially to distant mucosae and to peripheral lymph nodes [15]. It is clear from the results that the i.n. administered antigen-containing chitosan nanofibers were the formulations that induced highest S-IgA titers after three immunizations in all Guinea pigs (*Figure 5*), and because of the high S-IgA titer, animals immunized with chitosan nanofibers membranes, containing nanoencapsulated *Shigella* subunit antigen, N-IpaD, resulted in a high protective immunity in these groups (*Table II*). In conclusion, i.n. administered antigen-containing chitosan nanofibers membranes can induce an S-IgA response at the site of bacteria entry. In contrast, i.m. administered antigen formulations did not show S-IgA response, which is consistent with published observations that i.m. administrations of antigen formulations were not able to induce any S-IgA response, not even after several booster immunizations, which in the absence of S-IgA resulted in very low protective immunity in these groups [36].

In this study, we have showed that the intranasal vaccination by chitosan nanofibers membranes containing nanoencapsulated antigens is capable to induce a protective immune response in Guinea pigs. However, our finding has indicated that, N-terminal region of IpaD by chitosan nanofibers membranes system delivery has great potential as navel nasal vaccine candidate for development an adequate protective immunity against Shigellosis.

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In conclusion, biodegradable chitosan ultrafine chitosan nanofibers membranes, containing nanoencapsulated *Shigella* subunit antigen, N-IpaD, are prepared via electrospinning and are characterized by SEM and FTIR. The chitosan nanofibers membranes have an acceptable loading capacity for proteins, and a positive surface charge, suitable to attach to nasal mucosa. The integrity of the loaded antigen was preserved. I.n. administration of encapsulated *Shigella* subunit antigen in chitosan nanofibers enhanced noticeably the systemic and local immune responses, comparing to i.m. in alum or i.n. administration of soluble *Shigella* subunit vaccine.

In vivo studies confirmed that this system is capable of delivering

the antigen with chitosan nanofibers membranes to nasal mucosa. This makes the chitosan nanofibrous membrane a promising vehicle for intranasal delivery of *Shigella* antigens, and most probably other antigens.

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