Published in final edited form as:

Mol Pharm. 2012 April 2; 9(4): 946–956. doi:10.1021/mp200553x.

Dendritic Cell Targeted Chitosan Nanoparticles for Nasal DNA Immunization against SARS CoV Nucleocapsid Protein

Dharmendra Raghuwanshi, Vivek Mishra, Dipankar Das, Kamaljit Kaur*, and Mavanur R. Suresh

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2N8

Abstract

This work investigates the formulation and *in vivo* efficacy of dendritic cell (DC) targeted plasmid DNA loaded biotinylated chitosan nanoparticles for nasal immunization against nucleocapsid (N) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) as antigen. The induction of antigen-specific mucosal and systemic immune response at the site of virus entry is a major challenge for vaccine design. Here, we designed a strategy for non-invasive receptor mediated gene delivery to nasal resident DCs. The pDNA loaded biotinylated chitosan nanoparticles were prepared using a complex coacervation process and characterized for size, shape, surface charge, plasmid loading and protection against nuclease digestion. The pDNA loaded biotinylated chitosan nanoparticles were targeted with bifunctional fusion protein (bfFp) vector for achieving DC selective targeting. The bfFp is a recombinant fusion protein consisting of truncated core-streptavidin fused with anti-DEC-205 single chain antibody (scFv). The core-streptavidin arm of fusion protein binds with biotinylated nanoparticles, while anti-DEC-205 scFv imparts targeting specificity to DC DEC-205 receptor. We demonstrate that intranasal administration of bfFp targeted formulations along with anti-CD40 DC maturation stimuli enhanced magnitude of mucosal IgA as well as systemic IgG against N protein. The strategy led to the detection of augmented levels of N protein specific systemic IgG and nasal IgA antibodies. However, following intranasal delivery of naked pDNA no mucosal and systemic immune responses were detected. A parallel comparison of targeted formulations using intramuscular and intranasal route showed that the intramuscular route is superior for induction of systemic IgG responses compared with the intranasal route. Our results suggest that targeted pDNA delivery through non-invasive intranasal route can be a strategy for designing low-dose vaccines.

Keywords

Dendritic cells; DEC-205 receptor; Chitosan nanoparticles; Bifunctional fusion protein; Low-dose vaccines

1. Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that play a key role in the immune response by antigen uptake, processing and presentation. Among all the APCs, the DCs are considered as the most efficient cells for induction and regulation of immune responses.¹ Various strategies employed to harness DC selective targeting of vaccine

*Tel. 780-492-8917; Fax. 780-492-1217; kkaur@ualberta.ca.

Supporting Information Available: Figures S1 and S2 related to bfFp and SARS N protein purification. This material is available free of charge via the Internet at <http://pubs.acs.org>.

antigens have shown enormous potential at preclinical stage for designing low-dose vaccines.² DCs express a large number of endocytic receptors, such as C-type lectin receptors (CLRs) through which they recognize and uptake pathogens.

Different strategies employed for targeted delivery of antigen to DCs utilize the help of antibodies or natural ligands against DC restricted CRLs. The antigen targeting to CLRs have resulted in augmented immune response, dose-sparing of antigen and skewing of immune responses.²⁻⁴ Among CLRs, DC C-type lectin receptor DEC-205 (CD 205) receptor is best characterized. The DEC-205 is a member of type I C-type lectin receptor family, which is primarily expressed by immature DC in skin, lymph nodes and spleen.^{5, 6} The intracellular routing of DEC-205 targeted antigens enhances loading and presentation of antigenic peptides on MHC class I and class II molecules, leading to priming of antigen specific CD8⁺ and CD4⁺ T cells, respectively.^{7, 8} The *in vivo* targeting of antigens to murine DEC-205 receptor along with maturation stimuli has been shown to augment the efficiency of antigen presentation to both CD4⁺ and CD8⁺ T-cells.⁹

To achieve DC selective targeting of biotinylated protein antigen, we have previously developed a quadroma (hybrid-hybridoma) based full length bispecific monoclonal antibody (bsmAb). The full length hybrid-hybridoma based bsmAb can bind with biotinylated antigen through one arm and target DEC-205 through the other arm.¹⁰ Targeting of biotinylated ovalbumin (OVA) using bsmAb reduced the dose of antigen by ~500-folds compared with non targeted antigen. However, quadromas produce bsmAb along with parental and unwanted heavy and light chain combinations resulting in lower yield. Additionally, the antibody-based biotin binding is several orders weaker than the streptavidin binding.

Consequently, to overcome inherent limitations associated with bsmAb, we designed a recombinant bifunctional fusion protein (bfFp) vector for DC targeting.¹¹ A single chain variable fragment (scFv) that recognize mouse DC DEC 205 was fused with a truncated core-streptavidin domain and expressed in *Escherichia coli* using the T7 expression system. The truncated core-streptavidin arm can bind with any biotinylated antigen and anti-DEC-205 scFv impart targeting specificity to DC DEC-205 receptor. Using bfFp we have demonstrated *in vivo* targeting of four different classes of biotinylated antigens, namely, proteins, peptide, gangliosides and plasmid DNA, as low-dose vaccines.¹¹ *In vivo* studies in mice with biotinylated OVA have shown that in the presence of bfFp and anti-CD40 mAb, both humoral and cell-mediated responses can be augmented. In this targeting formulation, low concentration of antigen (200 ng) in saline was adequate to achieve a strong immune response in mice. In the multiple antigens targeting strategy, we also achieved enhanced humoral and cell-mediated responses for biotinylated OVA, SARS Spike, Ebola glycoprotein (GP1), MUC-1 peptide, and anthrax protective antigen.

Herein, we selected nucleocapsid (N) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) as vaccine antigen. The SARS-CoV contains four major structure proteins; membrane (M), spike (S), envelop (E), and nucleocapsid (N).^{12, 13} Studies have shown that N protein is highly conserved compared to other proteins such as S, E and M; therefore could serve as a stable vaccine candidate.¹⁴ Furthermore, N protein is abundantly shed during SARS infection and N protein specific antibodies and memory T cells can be detected in SARS-recovered patients.^{15, 16} A number of studies have used recombinant N protein^{17, 18} or DNA encoding N protein¹⁹⁻²¹ as vaccine antigen to elicit humoral and cellular immune responses in animal models. In this context, we selected plasmid DNA encoding N protein (pVAXN) as vaccine antigen and chitosan as DNA delivery vehicle.

Chitosan is a natural polysaccharide consisting of repeated D-glucosamine and N-acetyl-D-glucosamine units, linked via β (1, 4) glycosidic bond. The chitosan and its derivatives are

ideal nucleic acid delivery vehicles due to their excellent biocompatible, biodegradable and non-toxic nature.²² The presence of high cationic charge on chitosan provides strong binding affinity with nucleic acids resulting in excellent gene delivery vehicle.²³

The aim of the present work was to develop and characterize dendritic cell targeted chitosan nanoparticles as vaccine delivery systems via nasal route. Murine respiratory DCs subsets, such as airway DCs and alveolar DCs, express the DEC-205 receptor.²⁴ These respiratory DCs could serve as primary target for uptake of targeted nanoparticles. Our strategy combines the pDNA carrying capacity of chitosan and selective targeting specificity of bFp to respiratory DCs. Ultrapure water soluble biotinylated chitosan hydrochloride was used to formulate pVAXN loaded nanoparticles. These pVAXN loaded biotinylated chitosan nanoparticles were surface functionalized with bifunctional fusion protein to achieve nasal DC targeting (Figure 1). Different NP formulations (Table 1) were given intranasal or intramuscular to the mice to elicit the immune response and the results of the immune response are presented here.

2. Materials and Methods

2.1. Materials

Ultrapure chitosan hydrochloride salt (Protasan UP CL 113) was purchased from FMC Biopolymers AS (Novamatrix, Norway). pVAX1 vector and Lipofectamine 2000 were from Invitrogen (USA). Biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sulpho-NHS-LC-Biotin) and isopropyl- β -D-thiogalactopyranoside (IPTG), chitosanase (from *Streptomyces griseus*) and lysozyme were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ni-NTA agarose resin was from Qiagen (Mississauga, Canada). Rat anti-mouse CD40 mAb was purified from 1C10 hybridoma, a kind gift from Dr. M. Gold (University of British Columbia, Canada). The TMB (3, 3', 5, 5' - tetramethylbenzidine) peroxidase substrate was from Kirkegaard and Perry Laboratory Inc. (Gaithersburg, MD). HRP based mouse antibody isotyping kit was obtained from Southern Biotech (Birmingham, AL USA). African green monkey SV40 transformed kidney (COS-1) cells were from American type culture collection (ATCC). Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin-L-glutamine (PSG) and fetal bovine serum (FBS) were procured from Gibco (Burlington, Canada).

2.2. Plasmid DNA (pVAXN) construction and detection of SARS-N protein

SARS-CoV nucleocapsid (N) DNA sequence was polymerase chain reaction (PCR) amplified using pFastBacNP as DNA template with gene specific primers. The 5' primer contains a BamHI restriction site, Kozak translation initiation sequence and initiation codon (ATG) and 3' primer contains the stop codon and EcoRI restriction site. The PCR fragment was electrophoresed in 1% low melting point agarose gel and gel-purified using Qiagen gel extraction kit. The purified fragment was then double digested with BamHI and EcoRI and further gel purified. The purified fragment was ligated to BamHI and EcoRI digested pVAX1 mammalian expression vector (Invitrogen, USA). The ligation mixture was transformed into TOP10 *E. coli* cells by heat shock method and plated onto LB agar plate containing 50 μ g/ml of Kanamycin. Recombinant positive clones were screened by plasmid DNA isolation using Qiagen plasmid DNA isolation kit and restriction digestion fragment mapping. The resultant plasmid is designated as pVAXN and used in the present study. pVAXN used for experiments was purified using the EndoFree Plasmid Mega Kit (Qiagen, Mississauga, Canada).

To confirm the expression of encoded N-protein, COS-1 cells were grown in DMEM medium supplemented with 10% FBS and 1% PSG. The cells were seeded at density of

1X10⁶ per well in 6-well plate and grown overnight to 85 to 95 % confluence. Next day cells were transfected with pVAXN using lipofectamine 2000 reagent as per manufacturer's instructions and allowed to grow for 48 hr in humidified 5% CO₂ atmosphere at 37°C. Cells were trypsinized and pelleted and pellet was washed twice with PBS and lysed with RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA). Cell lysate was cleared by centrifugation (10 min at 10,000 rpm) and cleared lysate was analyzed using 10% reducing SDS PAGE. Subsequently the proteins were electrophoretically transferred on to nitrocellulose membrane. The membrane was blocked with 5% skim milk and probed with anti-SARS-N protein monoclonal antibody (19C7) previously developed in our lab.²⁵ Finally, unbound 19C7 antibody was washed and the membrane incubated with goat anti-mouse IgG HRPO (1:5000). Blot was developed with ECL plus Western blotting reagent (Amersham Pharmacia Biotech, BaiedUrfe, Canada).

2.3. Preparation of biotinylated chitosan and estimation of chitosan modification using fluorescamine

Chitosan hydrochloride was biotinylated using biotinamidohehexanoic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-NHS-LC-biotin). Briefly, chitosan solution (20 mg/mL) was prepared in PBS (pH 7.4) and biotinylation was initiated by adding 10 mg of sulfo-NHS-LC-biotin. The reaction was carried out for 2 h at room temperature and biotinylated chitosan was dialyzed against PBS overnight at 4°C and lyophilized for 48 hr.

The extent of amine group modification on glucosamine monomer of chitosan was determined to verify the attachment of biotin. The percentage of biotin substitution at primary amine group on glucosamine monomer was determined by fluorescamine assay.²⁶ Aqueous solutions of plain and biotinylated chitosan were made at various concentrations (0.01%, 0.03%, 0.04% and 0.05% w/v). A 50 µl/well of respective chitosan solutions were added in triplicate to 96-well black fluorescent microplate and to this 100 µl of fluorescamine (0.2% w/v in anhydrous DMSO) was added. After incubation for 30 min fluorescence intensity was recorded using a Synergy fluorescence plate reader (BioTek Instruments, Winooski, VT) with a 360 nm, 40 nm bandwidth excitation filter and a 460 nm, 40 nm bandwidth emission filter. The sensitivity was set at 40%.

2.4. Formulation of pVAXN loaded biotinylated chitosan nanoparticles

Plasmid DNA (pVAXN) loaded biotinylated chitosan nanoparticles (NPs) were prepared using modified complex coacervation method as described previously.²³ The conditions for formulation of complexes between chitosan and pVAXN were optimized. Briefly, biotinylated chitosan was dissolved in sodium acetate buffer (5 mM, pH 5.5) to obtain a final concentration of 0.1% w/v and passed through 0.22 µm syringe filter. To obtain different weight ratios of chitosan to DNA (1 to 6), pVAXN solutions were prepared at different concentrations (1, 0.5, 0.333, 0.25, 0.2 and 0.166 mg/mL) in sodium sulphate (25 mM). The chitosan and DNA solutions were separately pre-heated to 50–55 °C on the water bath. Biotinylated chitosan (100 µL, 1 mg/mL in 5 mM sodium acetate buffer, pH 5.5) solution was added to pVAXN solution (100 µL) and the mixture was immediately vortexed for 15 seconds. The particles were left at room temperature for 30 minutes for stabilization. After stabilization, NPs were centrifuged at 14,000 rpm for 20 minutes and supernatants were analyzed for the presence of free DNA at absorbance 260 nm/280 nm using NanoDrop ND-1000 (Nanodrop Technologies Inc. Wilmington, Delaware) for analysis of encapsulation efficiency. The DNA encapsulation efficiency was calculated using the formula: Encapsulation efficiency (EE) = $\frac{A-B}{A} \times 100$, where A is the total DNA amount and B is the free DNA in the supernatant.

2.5. Physicochemical characterization of biotinylated chitosan NPs

2.5.1. Electrophoretic gel mobility assay—The complex formation between chitosan and DNA was analyzed by electrophoretic gel mobility assay. Samples of NPs (prepared at 1 to 6 weight ratios of biotinylated chitosan to pVAXN DNA) were mixed with the loading dye and loaded to a 1% agarose gel containing ethidium bromide. The gel was immersed in 1× tris-acetate/EDTA buffer and was exposed to 100 V for 45 min. The DNA bands were visualized using the Alpha Imager (Alpha Innotech; San Leandro, CA). Unless otherwise mentioned, the NPs prepared at biotinylated chitosan to pVAXN weight ratio of 4 were used for all studies.

2.5.2. Size and zeta potential—The particle size and zeta potential of pVAXN loaded biotinylated chitosan nanoparticles was measured using Zetasizer 3000 (Malvern, UK). The measurement of mean size diameter of the nanoparticles was based on the dynamic light scattering technique. For zeta potential measurements, nanoparticles were suspended in deionised water and the measurements were performed in the automatic mode.

2.5.3. DNase I protection assay and plasmid integrity—To see whether nanoencapsulated pVAXN is protected against nuclease digestion, nanoparticles encapsulated pVAXN and naked pVAXN was subjected to DNase I digestion. Briefly, naked pVAXN or nanoencapsulated pVAXN (4 µg) in 40 µl of deionised water was incubated with 10 U DNase I (Invitrogen) for 30 and 60 min at 37 °C. The DNase activity was stopped by adding 0.5 M EDTA solution to a final concentration of 50 mM. Alternatively, following incubation with DNase I, the integrity of plasmid DNA within particles was analyzed by lysis of particles with chitosanase and lysozyme.^{23,26} Briefly, after treatment with DNase I for 60 min, the NPs were centrifuged and washed three times to remove DNase I and were resuspended in NaAc-HOAc buffer (50 mM). For digestion, the NPs were incubated with chitosanase (50 µL, 1 U/ml) and lysozyme (20 µL, 500 U/ml) for 4 hr at 37 °C. The integrity of DNA samples was analyzed by 1% agarose gel using Alpha Imager (Alpha Innotech; San Leandro, CA).

2.6. Expression and purification of bifunctional fusion protein (bfFp) and SARS-CoV N protein

The expression and purification of bifunctional fusion protein (bfFp), specifically anti-DEC-205 scFv fused with truncated core streptavidin, has been described in our previous communication with slight modifications.¹¹ Briefly, pWET7 vector was transformed into BL21-CodonPlus® (DE3)-RIPL *E. coli* cells (Stratagene, Cedar Creek, TX) using heat shock method. The *E. coli* transformant was cultured in 2xYT medium containing 100 µg/ml of ampicillin and 50 µg/ml of chloramphenicol and induced with 0.5 mM IPTG. Following induction, the culture was grown at 26 °C for 5 hr and harvested by centrifugation. Periplasmic and cytoplasmic soluble bfFp was extracted with Bugbuster master mix reagent (Novagen Inc.) according to manufacturer's instructions and lysate was cleared by centrifugation and loaded onto Ni-NTA agarose. Purification of periplasmic soluble protein was performed using immobilized metal affinity chromatography (IMAC). The IMAC fractions were analyzed for purity by 10% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue (Figure S1, supporting Information).

Expression and purification of full length SARS-CoV N protein was carried out according to our published method.²⁷ Briefly, full length N protein gene was codon optimized for high level bacterial expression and chemically synthesized from Geneart Inc. The N protein was expressed as insoluble inclusion bodies in Top 10 *E. coli* strain and purified under denaturing conditions using IMAC column and refolded. The purified and refolded fractions of N protein were analyzed on 10% SDS page under reducing conditions (Figure S2). The

recombinant full-length N protein was used as antigen to analyze magnitude of antibody responses and as recall antigen for stimulation of splenocytes.

2.7. Immunization studies

Ten to twelve weeks old female BALB/c mice used for this study were procured from Charles River Laboratories Inc. (Canada). Animals were housed at Health Sciences Laboratory Animals Services (HSLAS) at the University of Alberta, Edmonton, Canada. Animal treatment, care and euthanasia were carried out according to the Canadian Council of Animal Care guidelines. A total of five mice per group (n=5) were used for evaluating immune response against vaccine constructs. The mice were immunized either through intranasal or intramuscular route on day 1 and day 21 in the various combinations (Table 1). A 5 µg/mouse dose of pVAXN was administered as soluble, NPs or bFp targeted NPs in the presence or absence of DC maturation stimuli (anti-CD 40 mAb). For intranasal delivery, mice were held in vertical position perpendicular to the bench. While holding tightly in vertical position the vaccine formulation (40 µl, 20 µl in each nostril) was delivered drop wise through pipette tip right into the nostrils. The applied volume was naturally inhaled and adequate care was taken to assure proper delivery of the drops in nostrils, while avoiding any incident of swallowing. Following administration of formulations the mice were held in vertical position for some time to ensure proper delivery. Intramuscular administration was done by injecting the vaccine formulation with 26 1/2 gauge needle in the quadriceps muscle in a total volume of 50 µl.

2.7.1. Humoral immune responses—Serum IgG titers were evaluated using recombinant N protein. Tail vein bleeds were performed on day 0, 14, 28 and 46 and terminal bleed (day 56) was collected by cardiac puncture following CO₂ asphyxiation and cervical dislocation. Nasal washings were performed immediately after cardiac puncture. Briefly, trachea of each mouse was opened and a fine micropipette tip was inserted. The nasal fluids were collected by flushing PBS (200 µl) containing BSA (0.1%; w/v) through the nasal cavity and washes were collected from the nostrils. The nasal washes were kept at −20 °C till the day of analysis.

SARS N protein specific IgG and IgA titers were evaluated using enzyme-linked immunosorbent assay (ELISA). The Nunc MaxiSorp flat-bottom 96-well ELISA microplates were coated with N protein solution (100 µl/well, 10 µg/ml) overnight at 4°C. The following day, plates were washed three times with PBST (0.1 % Tween 20 in PBS pH 7.4) and blocked with 1% BSA solution (200 µl) for 1 hr at 37°C. The plates were again washed with PBST (3X) and 100 µl of diluted serum (1:100) from each mouse was added in duplicate and incubated overnight at 4°C. The plates were washed with PBST and 100 µl of goat-anti-mouse IgG HRP (1:5000) was added followed by incubation for 1 hr at 37°C and the plates were washed with PBST. Finally TMB substrate was added and after 15 minutes optical density was measured at 650 nm using ELISA Vmax kinetic microplate reader (Molecular Devices Corp, CA). The nasal IgA levels and the serum isotypes (IgG1, IgG 2a and IgG 2b), against N-protein were determined using respective isotypes from Southern Biotechnology (Birmingham, AL). Same procedure as described above was followed except that at the end IgG1, IgG2a or IgG2b HRPO (1:400 dilution) or IgA HRPO (1: 250 dilution) were added to the plates for analyzing N protein specific IgA content in the nasal washes. The ABTS substrate was prepared as per manufacturer's instructions and added to plates and optical density was recorded at 405 nm.

2.7.2. Cytokine assay—Spleens from each group of mice were pooled and cell suspension of splenocytes was prepared by disrupting the spleen between frosted slides. The splenocytes were passed through cell strainer (70 µm) to obtain single cell suspension. The

red blood cells were lysed with ACK lysis buffer and cells were washed twice with serum free media. Finally, the splenocytes were resuspended in complete DMEM medium and cell density was adjusted at $1 \times 10^7/\text{ml}$. The splenocytes were seeded at the density of $1 \times 10^6/\text{well}$ and stimulated with N protein, concanavalin A (Con A) as positive control and media as sham control. The culture supernatants were harvested after 72 hr incubation at 37°C in the humidified atmosphere at 5% CO_2 level. The cytokine contents of supernatants were analyzed by cytokine ELISA kit using 96-well Corning microplate as per manufacturer's instructions.

2.7.3. Statistical analysis—Data are presented as mean $\pm$ standard deviation (SD) throughout the manuscript. Statistical significance of difference was tested using one way ANOVA test with Turkey's multiple comparison test. The significance level (α) was set at 0.05. Statistical difference was denoted as $*p < 0.05$, ns = no significant difference ($p > 0.05$). The data analysis was performed using Graphpad Prism, version 5.00 (Graphpad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Construction and expression of pVAXN DNA vaccine

The DNA vaccine vector used here was constructed by inserting full length SARS-CoV N protein gene sequence between BamHI and EcoRI restriction sites in pVAX1 mammalian expression vector to obtain pVAXN. Recombinant positive clones were isolated with kanamycin selection pressure and screened by plasmid DNA isolation using plasmid DNA isolation kit (Qiagen), restriction digestion fragment mapping and DNA sequencing. The integrity of the pVAXN construct was evaluated using 1% agarose gel analysis (Figure S3).

To confirm the expression of encoded antigen, pVAXN DNA encoding for N protein was transiently transfected to COS-1 cells using lipofectamine 2000. The transient expression of N protein was detected by Western blot using anti-N-protein monoclonal antibody (19C7) previously developed in our lab.²⁵ The result of Western blot confirms expression of N protein specific band on the COS-1 cell lysates. Mock transfected cells showed no cross-reactivity with 19C7 antibody (Lane 2 in Figure 2).

3.2. Biotinylation of chitosan and formulation of pVAXN loaded nanoparticles

The water soluble chitosan hydrochloride salt was biotinylated with long chain water soluble sulfo-N-hydroxysuccinimide ester. This derivative of biotin incorporates an extended spacer arm provided by hexanoic acid. The spacer arm improves the interaction between avidin and biotinylated macromolecules by overcoming steric hindrance at the biotin binding sites of avidin (Figure 3a).

The extent of biotinylation (i.e substitution at the primary amine group) at the glucosamine amine group of chitosan was determined using fluorescamine assay.²⁶ The fluorescamine reagent reacts rapidly with primary amines and can be used to estimate the magnitude of substitution at primary amine groups. When excited at $\sim 365 \text{ nm}$ wavelength, the fluorescence of the primary amine-dye complex has an emission wavelength of approximately 470 nm . The decrease in the fluorescence intensity of biotinylated chitosan can be correlated to obtain percentage substitution of biotin on amine groups of chitosan backbone. The results show the overall extent of glucosamine modification was found to be between 6 to 14 percent compared with plain chitosan hydrochloride (Figure 3b). Chitosan with a concentration of 0.03% (w/v) showed highest decrease in the fluorescence intensity ($13.2 \pm 1.9\%$) and therefore was most labeled with biotin. This sample was used for subsequent experiments.

Nanoparticles were formulated using modified complex coacervation between negatively charged pVAXN DNA and positively charged biotinylated chitosan. Agarose gel analysis was performed to determine optimal weight ratio of biotinylated chitosan to plasmid DNA to achieve maximum encapsulation efficiency (Figure 4a). Results of agarose gel show that the weight ratio of 4:1 (biotinylated chitosan to plasmid DNA) yielded maximum efficiency of complexation with almost negligible free plasmid DNA. The loading efficiency at 4:1 weight ratio of biotinylated chitosan to plasmid DNA was very efficient and consistently encapsulation efficiency of pVAXN was found to be in the range of $97.6 \pm 2.1\%$ as determined by the NanoDrop experiment.

Next, the pVAXN loaded biotinylated chitosan nanoparticles were characterized for size, surface charge and loading efficiency. The average hydrodynamic diameter of nanoparticles determined by zetasizer was found to be 210 ± 60 nm with zeta potential $+10 \pm 1.7$ mV.

Further, the results of nuclease protection assay suggest that nanoencapsulated pVAXN could be protected at high concentration of DNase I digestion (Lanes 2 and 4, Figure 4b). While it turned out that naked pVAXN was completely degraded under DNase I digestion (lane 1 and 3 Figure 4b). DNase I treated NPs were digested with chitosanase and lysozyme. This resulted in the release of intact DNA and showed no signs of degradation (Lane 5, Figure 4b) suggesting that formulation process did not affect the integrity of the nanoencapsulated DNA. The nuclease protection to DNA by chitosan nanoparticles would be meaningful for the maintenance of integration and function of DNA vaccine.

3.3. Expression and medium-scale purification of bFfp and N protein

The dendritic cell targeting vector (bFfp) was expressed and purified as periplasmic soluble protein as per our previous published methods with modifications.^{11, 28} The pWET7 construct encoding for anti-DEC-205 scFv-core-streptavidin fusion protein (bFfp) was subjected to medium scale expression. The expression of bFfp was verified using anti-His6 mAb Western blot. The bacterial cell pellet was lysed with Bug Buster Master mix reagent for efficient extraction of periplasmic and cytoplasmic soluble protein. bFfp was purified from lysate using IMAC column with a gradient of imidazole concentration in the native protein elution buffer (Figure S1). The final yield of bFfp was found to be approximately 1.0 mg/L of induced bacterial culture.

The prokaryotic full-length SARS-CoV N protein was used as antigen to analyze the antibody titers and as recall antigen for stimulating splenocytes. The N-protein with C-terminal His₆ tag was expressed and purified from inclusion bodies under denaturing conditions (Figure S2) according to our previous protocols.²⁷

3.4. Systemic N-protein specific IgG antibody response

The pVAXN loaded biotinylated chitosan nanoparticles were appended with bFfp for DEC 205 restricted DC targeting, and were evaluated for their *in vivo* potential to elicit immune responses following nasal administration. As the positive controls, these formulations were also administered through intramuscular route (Table 1). In addition, the bFfp targeted biotinylated nanoparticles were compared for immune responses in the presence or absence of DC maturation stimuli (anti-mouse CD40 mAb). The agonistic anti-CD40 mAb binds to CD40 on DCs, resulting in activation of cells replacing the CD40-CD40L signalling via CD4⁺ T cells.²⁹ Anti-CD40 mAb has been used as adjuvant in combination with DEC-205 targeted protein based vaccines,^{30, 31} and as an effective mucosal adjuvant upon intranasal administration in combination with influenza peptide encapsulated liposomal vaccine.³²

To examine the systemic immune responses elicited using targeted vaccines through intranasal (IN) or intramuscular (IM) routes, the presence of N protein specific IgG

antibodies was analyzed by indirect ELISA on serum samples collected during different time-points (Day 0, 14, 28, 46 and 56). The serum IgG profile (Figure 5a) shows that the nasal administration of bfFp targeted nanoparticles in combination with anti-CD40 mAb elicits higher levels of systemic IgG compared to naked DNA vaccine formulation administered through IN or IM route. Additionally, the bfFp targeted nanoparticles in the absence of any maturation stimuli (anti-CD40 mAb) elicited significantly lower levels ($*p<0.05$) of IgG titer compared to bfFp targeted nanoparticles administered along with anti-CD 40 mAb. This trend was observed, when formulations were administered either by IN or IM route. As expected, the intramuscular group immunized with targeted nanoparticles along with maturation stimuli induced the highest level of antibody titers. Serum titers of targeted formulations (NP+bfFp) were significantly ($*p<0.05$) higher than naked DNA formulations following intranasal as well as intramuscular administration (Figure 5a). In addition, in a separate set of experiment we found that immune response of NPs with anti-CD 40 mAb was significantly lower than that observed from the targeted NPs with anti-CD 40 mAb (unpublished results).

Interestingly we did not observe N protein specific IgG titers following nasal delivery of naked pVAXN or nanoparticle encapsulated pVAXN. The possible reason could be the low-dose of naked or nanoencapsulated DNA, as only 5 μ g/mice DNA (pVAXN) was administered. The above results show that the use of DC targeting ligand such as bfFp and the presence of maturation stimuli lead to enhanced immunogenicity of DNA vaccines. Such pDNA vaccines could provide augmented serum IgG titer at low doses.

The serum IgG isotyping profile specific for N protein following pDNA vaccine, irrespective of route of administration, indicates that targeted formulations augmented the IgG1, IgG2a and IgG2b levels. The intramuscular route, however, was found to be superior compared with the nasal route. The ratio of IgG1 to IgG2a and IgG2b indicates a Th1 biased immune response (Fig. 5b).

3.5. Mucosal N-protein specific antibody response

The mucosal surfaces are rich in antigen presenting cells such as DCs. The uptake of antigen by resident DC in nasal-associated lymphoid tissue (NALT) is necessary in the induction of mucosal immune responses. Production of SARS N protein specific mucosal IgA antibodies could be vital for protection against SARS CoV infection. Previous studies have shown that the mucosal IgA plays an important role in protection against SARS-CoV virus in animal models.³³ We found significantly higher mucosal IgA levels in nasal washings following nasal administration of DC targeted nanoparticles in combination with anti-CD40 mAb compared with intranasal delivery of pVAXN, NP and bfFp targeted NPs ($*p<0.05$) (Fig. 6). Detectable mucosal IgA response was also observed following intramuscular administration of bfFp targeted nanoparticles alone or in combination with anti-CD40 mAb as maturation, and there was no significant difference between the response from either formulation (ns, $p>0.05$). These findings can be explained based on the previous observations that systemic administration of DEC-205 targeted protein vaccines could provide protection against mucosal pathogen.⁹ However, merely detectable IgA response was observed following intramuscular administration of pVAXN formulations.

The results clearly indicate that chitosan nanoparticles administered by nasal route efficiently elicit mucosal immune response (IgA level). Thus, nasal administered DC targeted nanoparticles can provide additional advantage of mucosal immunity, which could lead to neutralizing the pathogen following exposure. These observations are interesting since SARS virus primarily infects the upper respiratory tract and induction of mucosal immunity (mucosal IgA) could limit viral attachment and pathogenesis.³⁴

3.6. Interferon-gamma cytokine profile

Interferon-gamma (IFN- γ) levels were measured to determine whether intranasal and intramuscular administration of targeted and non-targeted vaccines elicits cellular immunity. The recombinant N protein was used as a recall antigen, concanavalin A (con A) served as a positive control, while media was treated as a sham control. As shown in Figure 7, splenocytes from a group of mice treated with targeted formulations (NP+bfFp) secreted significantly higher levels of IFN- γ on stimulation with N protein by either intranasal or intramuscular route. The vaccination of mice with targeted chitosan nanoparticles and anti-CD40 mAb maturation stimuli resulted in the highest levels of IFN- γ secretion, followed by targeted chitosan nanoparticles without maturation stimuli (* $p < 0.05$). Non targeted or naked pDNA vaccine formulations were found to less effective to provoke significant IFN- γ levels. No detectable levels of IFN- γ were observed for splenocytes cultures treated with media alone, whereas, high levels of IFN- γ (1795–2526 pg/ml) were present following stimulation with Con A.

4. Discussion

SARS is an emerging infectious disease caused by a novel coronavirus named as SARS-CoV.¹² At the start of twenty first century SARS outbreak has seriously threatened healthcare agencies around the world, over 8,000 peoples were infected, resulting in 774 deaths. Till date, there is no therapeutic treatment/vaccine available for containing future threats of SARS endemic or pandemic.³⁴ Since SARS is transmitted through respiratory route, the respiratory tract serves as most common route for virus entry and as a first-line of defense. It becomes mandatory to design a vaccine delivery system which can provide antigen specific mucosal immunity. The respiratory tract harbours local DCs subsets, which routinely sample and process innocuous agents. Hence, respiratory tract resident DCs could serve as ideal target for uptake of targeted nanoparticles administered through non-invasive means.

Previous studies have shown that N protein is highly conserved; therefore could serve as a stable vaccine candidate.¹⁴ However, studies performed using N protein as antigen are based on systemic immunization and no attempt has been made to naturally mimic the route of viral infection and to target mucosal DCs. The logistics behind targeting of pDNA vaccine to DCs stems from the fact that the traditional intramuscular route of pDNA immunization results in the expression of antigens on myocytes. However, the myocytes are not professional antigen presenting cells thus can only present antigens through MHC class I pathway. Furthermore, myocytes do not migrate to draining lymph nodes and express costimulatory molecules, both of these features are essentially required for priming and activation of T cells.³⁵

Therefore, the main objective of this work was to investigate mucosal DC targeting potential of the pDNA loaded chitosan nanoparticles. The mucosal delivery of DNA vaccines mimic the natural mode of virus infection and induce both humoral and cellular immune responses.³⁶ Naked DNA is ineffective in crossing mucosal barriers, and it is rapidly degraded by nucleases.³⁷ Contemporary efforts have been directed to improve the immunogenicity of DNA vaccines. A wide range of strategies have been adopted in the literature to increase immunogenicity profile of DNA vaccines, such as the use of heterologous prime-boost regimes, co-expression of cytokines, electroporation and the use of viral and non-viral gene delivery vehicles.³⁶

In context of design of effective mucosal pDNA delivery, the non viral vectors such as polymeric nano/micro particles and liposomes protected plasmid DNA have shown better immunogenicity profile. These systems can specifically deliver the pDNA to antigen

presenting cells such as dendritic cells and macrophages. Additionally, these delivery systems could be surface functionalized with targeted ligands to achieve DC-selective targeted delivery of plasmid DNA through different immunization routes.^{22, 38} Numerous research findings have explored the nano/micro particles formulated using chitosan and their derivatives as gene delivery vehicles.³⁹ Chitosan is believed to show better nasal delivery prospects through two mechanisms.⁴⁰ First, the cationic charge on chitosan confers binding to negative sialic residues in the mucus lining the nasal epithelial cells thereby slowing clearance. Secondly, chitosan transiently opens the tight junctions to allow an increased paracellular transport across nasal mucosa. Additionally, the attachment of receptor specific ligands on chitosan microspheres facilitates receptor mediated endocytosis resulting in cell-specific delivery vaccine formulations.⁴¹

We have synthesized biotinylated chitosan and used it for the formulation of pVAXN loaded nanoparticles. The biotin substitution on the chitosan backbone facilitates the attachment of bifunctional fusion protein (bfFp) through core-streptavidin arm and anti-DEC-205 scFv arm assists in guiding the nanoparticle toward DCs. The N protein gene was cloned in US FDA approved pVAX1 plasmid vector, containing a CMV promoter and BGH polyadenylation sequence for mammalian protein expression. Further, the nanoencapsulated form of pVAXN was found to resist nuclease digestion following incubation with DNase I, which is vital for *in vivo* performance of the nanoparticulate formulations. The intranasal application of bfFp targeted nanoparticulate vaccines induces systemic and mucosal immune responses against N protein antigen. In addition, mere administration of naked pDNA or nanoencapsulated pDNA was not sufficient enough in provoking detectable serum titers against N protein. We speculate this could be due to the low-dose of the pVAXN antigen (5 µg/mouse). Contemporary studies have shown immune response following the use of pDNA encapsulated chitosan particles where a high dose of encapsulated pDNA or frequent dosing was used.^{42, 43}

From our results, it is apparent that both systemic and mucosal N protein specific responses were induced in mice by using bfFp based targeting of nanoencapsulated pVAXN. The N protein specific serum IgG, IgG1, IgG2a and IgG2b antibodies were elicited to higher magnitude when the targeted formulations were co-administered with DC maturation/activation stimuli (anti mouse-CD40 mAb). The splenocytes were capable of producing IFN-γ, a Th1 cytokine upon *in vitro* stimulation with recombinant N protein. However, a significantly higher level of IFN-γ was detected for targeted formulations compared with non-targeted formulation. Co-delivery of DC maturation stimuli with targeted formulations resulted in augmented IFN-γ levels, irrespective of the route of immunization.

5. Conclusion

We report a promising strategy for enhancing immunogenicity of low-dose DNA vaccine through targeted delivery to nasal resident dendritic cells. The vaccine strategy demonstrated here could provide a better understanding of non invasive means of targeting vaccine antigens to the dendritic cells. The antigen formulations developed here warrant further evaluation in SARS-CoV virus challenge experiments. This study has important implications for designing vaccines against SARS or infections with similar mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funding from the National Institute of Health (NIH) and Alberta Ingenuity Centre for Carbohydrate Science (AICCS) to M.R.S. D.R. would like to thank Canadian Commonwealth Scholarship Program (CCSP) for the financial support. V.M. would like to thank DBT, Govt. of India for Overseas Associateship Award.

Abbreviations

APC	antigen presenting cells
αCD 40	rat anti-mouse CD 40 monoclonal antibody
bsmAb	bispecific or bifunctional monoclonal antibody
CLR	C-type lectin receptor
DC	dendritic cells
scFv	single chain antibody
bfFp	bifunctional fusion protein
SARS-CoV	severe acute respiratory syndrome-coronavirus
ATCC	American type culture collection
His	histidine
HRPO	horseradish peroxidase
IMAC	immobilized metal affinity chromatography
MMR	macrophage mannose receptor
MHC	major histocompatibility complex
ng	nanogram
N protein	nucleocapsid protein
NP	biotinylated chitosan nanoparticle containing pVAXN DNA
pVAXN	pVAX1 vector carrying gene of SARS CoV nucleocapsid protein
Sulpho-NHS-LC-biotin	Biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt
PBST	PBS with 0.1% Tween 20
PSG	penicillin, streptomycin and L-glutamine
RT	room temperature
V_L	variable light chain
V_H	variable heavy chain
TMB	3,3',5,5'-tetramethylbenzidine

References

1. Steinman RM. Dendritic Cells In Vivo: A Key Target for a New Vaccine Science. *Immunity*. 2008; 29(3):319–324. [PubMed: 18799140]
2. Tacken PJ, De Vries IJM, Torensma R, Figdor CG. Dendritic-cell immunotherapy: From ex vivo loading to in vivo targeting. *Nat Rev Immunol*. 2007; 7(10):790–802. [PubMed: 17853902]
3. Keler T, He L, Ramakrishna V, Champion B. Antibody-targeted vaccines. *Oncogene*. 2007; 26(25): 3758–3767. [PubMed: 17530028]

4. Proudfoot O, Apostolopoulos V, Pietersz GA. Receptor-mediated delivery of antigens to dendritic cells: Anticancer applications. *Mol Pharm.* 2007; 4(1):58–72. [PubMed: 17228857]
5. Inaba I, Swiggard WJ, Inaba M, Meltzer J, Mirza A, Sasagawa T, Nussenzweig MC, Steinman RM. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145 I. Expression on dendritic cells and other subsets of mouse leukocytes. *Cell Immunol.* 1995; 163(1): 148–156. [PubMed: 7758125]
6. Witmer-Pack MD, Swiggard WJ, Mirza A, Inaba K, Steinman RM. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145 II. Expression in situ in lymphoid and nonlymphoid tissues. *Cell Immunol.* 1995; 163(1):157–162. [PubMed: 7758126]
7. Jian W, Swiggard WJ, Heufler C, Peng M, Mirza A, Steinman RM, Nussenzweig MC. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature.* 1995; 375(6527):151–155. [PubMed: 7753172]
8. Mahnke K, Guo M, Lee S, Sepulveda H, Swain SL, Nussenzweig M, Steinman RM. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol.* 2000; 151(3): 673–683. [PubMed: 11062267]
9. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii SI, Soares H, Brimnes MK, Moltedo B, Moran TM, Steinman RM. In Vivo Targeting of Antigens to Maturing Dendritic Cells via the DEC-205 Receptor Improves T Cell Vaccination. *J Exp Med.* 2004; 199(6):815–824. [PubMed: 15024047]
10. Wang WW, Das D, Tang XL, Budzynski W, Suresh MR. Antigen targeting to dendritic cells with bispecific antibodies. *J Immunol Methods.* 2005; 306(1–2):80–92. [PubMed: 16202424]
11. Wang WW, Das D, Suresh MR. A versatile bifunctional dendritic cell targeting vaccine vector. *Mol Pharm.* 2009; 6(1):158–172. [PubMed: 19053535]
12. Drosten C, Günther S, Preiser W, Van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RAM, Berger A, Burgüiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Müller S, Rickerts V, Stürmer M, Vieth S, Klenk HD, Osterhaus ADME, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med.* 2003; 348(20):1967–1976. [PubMed: 12690091]
13. Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, Nicholls J, Yee WKS, Yan WW, Cheung MT, Cheng VCC, Chan KH, Tsang DNC, Yung RWH, Ng TK, Yuen KY. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet.* 2003; 361(9366):1319–1325. [PubMed: 12711465]
14. Surjit M, Lal SK. The SARS-CoV nucleocapsid protein: A protein with multifarious activities. *Infect Genet Evol.* 2008; 8(4):397–405. [PubMed: 17881296]
15. Peng H, Yang Lt, Wang Ly, Li J, Huang J, Lu Zq, Koup RA, Bailer RT, Wu Cy. Long-lived memory T lymphocyte responses against SARS coronavirus nucleocapsid protein in SARS-recovered patients. *Virology.* 2006; 351(2):466–475. [PubMed: 16690096]
16. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, Peñaranda S, Bankamp B, Maher K, Chen MH, Tong S, Tamin A, Lowe L, Frace M, DeRisi JL, Chen Q, Wang D, Erdman DD, Peret TCT, Burns C, Ksiazek TG, Rollin PE, Sanchez A, Liffick S, Holloway B, Limor J, McCaustland K, Olsen-Rasmussen M, Fouchier R, Günther S, Osterhaus ADHE, Drosten C, Pallansch MA, Anderson LJ, Bellini WJ. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science.* 2003; 300(5624):1394–1399. [PubMed: 12730500]
17. Gupta V, Tabiin TM, Sun K, Chandrasekaran A, Anwar A, Yang K, Chikhlikar P, Salmon J, Brusica V, Marques ETA, Kellathur SN, August TJ. SARS coronavirus nucleocapsid immunodominant T-cell epitope cluster is common to both exogenous recombinant and endogenous DNA-encoded immunogens. *Virology.* 2006; 347(1):127–139. [PubMed: 16387339]
18. Schulze K, Staib C, Schätzl HM, Ebensen T, Erfle V, Guzman CA. A prime-boost vaccination protocol optimizes immune responses against the nucleocapsid protein of the SARS coronavirus. *Vaccine.* 2008; 26(51):6678–6684. [PubMed: 18805454]
19. Kim TW, Lee JH, Hung CF, Peng S, Roden R, Wang MC, Viscidi R, Tsai YC, He L, Chen PJ, Boyd DAK, Wu TC. Generation and Characterization of DNA Vaccines Targeting the

- Nucleocapsid Protein of Severe Acute Respiratory Syndrome Coronavirus. *J Virol*. 2004; 78(9): 4638–4645. [PubMed: 15078946]
20. Zhao P, Cao J, Zhao LJ, Qin ZL, Ke JS, Pan W, Ren H, Yu JG, Qi ZT. Immune responses against SARS-coronavirus nucleocapsid protein induced by DNA vaccine. *Virology*. 2005; 331(1):128–135. [PubMed: 15582659]
 21. Zhu MS, Pan Y, Chen HQ, Shen Y, Wang XC, Sun YJ, Tao KH. Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine. *Immunol Lett*. 2004; 92(3):237–243. [PubMed: 15081618]
 22. Kang ML, Cho CS, Yoo HS. Application of chitosan microspheres for nasal delivery of vaccines. *Biotechnol Adv*. 2009; 27(6):857–865. [PubMed: 19583998]
 23. Mao HQ, Roy K, Troung-Le VL, Janes KA, Lin KY, Wang Y, August JT, Leong KW. Chitosan-DNA nanoparticles as gene carriers: Synthesis, characterization and transfection efficiency. *J Control Release*. 2001; 70(3):399–421. [PubMed: 11182210]
 24. Wikstrom ME, Stumbles PA. Mouse respiratory tract dendritic cell subsets and the immunological fate of inhaled antigens. *Immunol Cell Biol*. 2007; 85(3):182–188. [PubMed: 17262055]
 25. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zayas-Zamora G, King M, Suresh MR. A rapid point of care immunoswab assay for SARS-CoV detection. *J Virol Methods*. 2008; 152(1–2):77–84. [PubMed: 18620761]
 26. Corsi K, Chellat F, Yahia LH, Fernandes JC. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials*. 2003; 24(7):1255–1264. [PubMed: 12527266]
 27. Das D, Suresh MR. Copious production of SARS-CoV nucleocapsid protein employing codon optimized synthetic gene. *J Virol Methods*. 2006; 137(2):343–346. [PubMed: 16904198]
 28. Wang WWS, Das D, McQuarrie SA, Suresh MR. Design of a bifunctional fusion protein for ovarian cancer drug delivery: Single-chain anti-CA125 core-streptavidin fusion protein. *Eur J Pharm Biopharm*. 2007; 65(3):398–405. [PubMed: 17257818]
 29. Elgueta R, Benson MJ, De Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev*. 2009; 229(1):152–172. [PubMed: 19426221]
 30. Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8+ T Cell Tolerance. *J Exp Med*. 2002; 196(12):1627–1638. [PubMed: 12486105]
 31. Trumpfheller C, Finke JS, López CB, Moran TM, Moltedo B, Soares H, Huang Y, Schlesinger SJ, Park CG, Nussenzweig MC, Granelli-Piperno A, Steinman RM. Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med*. 2006; 203(3):607–617. [PubMed: 16505141]
 32. Ninomiya A, Ogasawara K, Kajino K, Takada A, Kida H. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. *Vaccine*. 2002; 20(25–26):3123–3129. [PubMed: 12163263]
 33. See RH, Zakhartchouk AN, Petric M, Lawrence DJ, Mok CPY, Hogan RJ, Rowe T, Zitzow LA, Karunakaran KP, Hitt MM, Graham FL, Prevec L, Mahony JB, Sharon C, Auperin TC, Rini JM, Tingle AJ, Scheifele DW, Skowronski DM, Patrick DM, Voss TG, Babiuk LA, Gaudie J, Roper RL, Brunham RC, Finlay BB. Comparative evaluation of two severe acute respiratory syndrome (SARS) vaccine candidates in mice challenged with SARS coronavirus. *J Gen Virol*. 2006; 87(3): 641–650. [PubMed: 16476986]
 34. Roper RL, Rehm KE. SARS vaccines: Where are we? *Expert Rev Vaccines*. 2009; 8(7):887–898. [PubMed: 19538115]
 35. Kutzler MA, Weiner DB. Developing DNA vaccines that call to dendritic cells. *J Clin Invest*. 2004; 114(9):1241–1244. [PubMed: 15520855]
 36. Kutzler MA, Weiner DB. DNA vaccines: Ready for prime time? *Nat Rev Genet*. 2008; 9(10):776–788. [PubMed: 18781156]

37. Wang D, Christopher ME, Nagata LP, Zabielski MA, Li H, Wong JP, Samuel J. Intranasal immunization with liposome-encapsulated plasmid DNA encoding influenza virus hemagglutinin elicits mucosal, cellular and humoral immune responses. *J Clin Virol.* 2004; 31(SUPPL 1):S99–S106. [PubMed: 15567101]
38. Garg NK, Mangal S, Khambete H, Sharma PK, Tyagi RK. Mucosal delivery of vaccines: Role of mucoadhesive/biodegradable polymers. *Recent Pat Drug Deliv Formul.* 2010; 4(2):114–128. [PubMed: 20380624]
39. Csaba N, Garcia-Fuentes M, Alonso MJ. Nanoparticles for nasal vaccination. *Adv Drug Deliv Rev.* 2009; 61(2):140–157. [PubMed: 19121350]
40. Yeh TH, Hsu LW, Tseng MT, Lee PL, Sonjae K, Ho YC, Sung HW. Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening. *Biomaterials.* 2011; 32(26): 6164–6173. [PubMed: 21641031]
41. Jiang HL, Kang ML, Quan JS, Kang SG, Akaike T, Yoo HS, Cho CS. The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. *Biomaterials.* 2008; 29(12):1931–1939. [PubMed: 18221992]
42. Khatri K, Goyal AK, Gupta PN, Mishra N, Vyas SP. Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. *Int J Pharm.* 2008; 354(1–2):235–241. [PubMed: 18182259]
43. Xu J, Dai W, Wang Z, Chen B, Li Z, Fan X. Intranasal Vaccination with Chitosan-DNA Nanoparticles Expressing Pneumococcal Surface Antigen A Protects Mice against Nasopharyngeal Colonization by *Streptococcus pneumoniae*. *Clin Vaccine Immunol.* 2011; 18(1): 75–81. [PubMed: 21047997]

Figure 1.

Schematic representation of dendritic cell targeting strategy using bifunctional fusion protein (bfFp). The bfFp is a recombinant fusion protein consisting of truncated core-streptavidin fused with anti-DEC-205 single chain antibody (scFv). The core-streptavidin arm of fusion protein binds with biotinylated nanoparticles while anti-DEC-205 scFv imparts targeting specificity to DC DEC-205 receptor.

Figure 2.

Analysis of recombinant N protein expression. The COS-1 cells were transiently transfected with pVAXN or pVAX-1 (control). N protein expression on cell lysate was analyzed by probing Western blot with mouse anti-N-specific monoclonal (19C7).

Figure 3.

(a) Schematic for the synthesis of biotinylated chitosan. (b) Estimation of chitosan modification using fluorescamine assay. The extent of biotinylation of the amine groups of chitosan was determined using fluorescamine, which reacts with primary amine groups. The decrease in fluorescence intensity is proportional to the percentage of biotinylated amine groups.

Figure 4.

Formulation of pVAXN loaded biotinylated chitosan nanoparticles. (a) Agarose gel analysis of pVAXN DNA loaded biotinylated chitosan nanoparticles prepared at different weight ratios of B-chitosan to pVAXN DNA (1 to 6). (b) Agarose gel electrophoresis of DNase I digested naked pVAXN and nanoencapsulated pVAXN. Lane 1: pVAXN (30 min); Lane 2: nanoencapsulated pVAXN (30 min); Lane 3: pVAXN (60 min); Lane 4: nanoencapsulated pVAXN (60 min); Lane 5: Lane 4 digested with chitosanase and lysozyme.

Figure 5.

(a) Analysis of time-dependent serum IgG antibody against SARS-CoV N protein following IN and IM immunization with different vaccine formulations. Balb/c mice (5 mice per group) were immunized with 5 μ g of pVAXN on day 1 and day 21. The mouse serum was collected at day 0, 14, 28, 42 and 56 post immunization and IgG against N protein was detected using ELISA. The data are presented as group mean $\pm$ SD at various time-points. ELISA was performed at 1:100 diluted sera. (b) Detection of N protein specific serum IgG isotypes in mice vaccinated with different formulations. IgG isotypes were detected in serum samples obtained on day 56. The ELISA was performed at 1:100 diluted sera. Data are presented as group mean $\pm$ SD. Statistical differences between groups are denoted as * p <0.05 and ns = no significant difference (p >0.05). The horizontal bars compare statistical difference in IgG1 between different groups.

Figure 6.

SARS-CoV N protein specific IgA levels in the nasal washes. The nasal washes were collected on day 56 following euthanasia and assayed for the presence of N protein specific IgA using ELISA. Each sample was analyzed in triplicate and data are representative of group mean $\pm$ SD of five mice. Statistical differences between groups are denoted as * $p < 0.05$ and ns = no significant difference ($p > 0.05$). The horizontal bars with * denote statistical difference in IgA between different groups compared with NP+bIFP+αCD40 group (IN).

Figure 7.

Analysis of IFN- γ levels in mice immunized with different vaccine formulations.

Splenocytes obtained from a group of immunized mice (5 mice per group) were pooled and cultured in the presence of recombinant N protein (4 μ g) in 96-well flat bottom plate with a final volume of 200 μ L. Culture supernatants harvested after 72 hr were analyzed for IFN- γ levels using ELISA kit. Data represents mean $\pm$ SD of triplicate cultures stimulated with N protein. Statistical differences between groups are denoted as * p <0.05. * denote statistical difference in IFN- γ between different groups. IFN- γ levels obtained following stimulation with Con A were 1795–2526 pg/ml in different groups, whereas, no detectable levels were observed for splenocytes culture stimulated with media alone.

Table 1

Different vaccine formulations used for intranasal and intramuscular immunization in mice (n= 5 per group)

Groups	Formulations *
<i>Intranasal (IN)</i>	
1.	pVAXN
2.	NP
3.	NP + bfFp
4.	NP + bfFp + α CD 40
<i>Intramuscular (IM)</i>	
1.	pVAXN
2.	NP + bfFp
3.	NP + bfFp + α CD 40

* 5 μ g/mouse of pVAXN was administered either naked or as nanoparticles. A mass dose of 25 μ g of nanoparticles was used per dose, while α CD 40 mAb's dose was 25 μ g/mouse. pVAXN, DNA vaccine vector; NP, nanoparticle with encapsulated pVAXN; bfFp, bifunctional fusion protein; α CD 40, monoclonal antibody against anti CD 40 protein