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CHITOSAN NANOPARTICLE MODIFICATIONS FOR IMPROVED GENE DELIVERY IN AN ORAL DNA VACCINE APPLICATION

An Undergraduate Honors Thesis Submitted in Partial fulfillments of University Honors Program Requirements
University of Nebraska-Lincoln

by

Austin J. Helmink, BS
Biological Systems Engineering
College of Engineering

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Faculty Mentor: Dr. Angela Pannier, Department of Biological Systems Engineering

Table of Contents

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Figures and Tables

Abbreviations

^{1}H NMR	hydrogen-1 (proton) nuclear magnetic resonance
ACK	ammonium-chloride-potassium
APC	antigen presenting cell
BCA	bicinchonic acid assay
BMDD	bone marrow-derived dendritic cell
BMDM	bone marrow derived macrophage
CS	chitosan oligosaccharide lactate
CS NP	chitosan nanoparticle
CS-Zn-NIM	chitosan-zein nano-in-micro-particle
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EDC	N-(3-dimethylaminopropyl)-N'ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
HEK	human embryonic kidney
Man	mannose
MR	mannose receptor
NHS	N-hydroxysulfosuccinimide
PBS	phosphate buffered saline
mPEG	methoxy poly(ethylene glycol)-carboxyl aid
nPEG	poly(ethylene glycol) 2-aminoethyl ether acetic acid
pEGFP-LUC	enhanced green fluorescent protein-luciferase plasmid
Phe	phenylalanine
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
TCM	T-cell media
TE	tris-EDTA
TPP	sodium tripolyphosphate

Abstract

Vaccines represent one of the most significant medical innovations of the 20th century, resulting in the eradication or near eradication of a handful of deadly diseases. However, many infectious diseases remain resistant to effective vaccination, largely due to a lack full immune activation by traditional protein-based vaccines. A promising alternative vaccination strategy is the emerging development of DNA vaccines, which rely upon the delivery of exogenous genetic material to host cells encoding for a viral or bacterial antigen in order to induce a robust immune response by closely mimicking live infection. The delivery of genetic material requires a carrier in order to avoid degradation by host defenses in vivo. Non-viral gene delivery vectors avoid the immunogenicity and toxicity concerns of viral vectors, but suffer from low efficacy. Thus, techniques to improve the efficiency of gene delivery must be developed. Additionally, while many administration routes are viable, the oral route is preferable due to its ease of administration, high patient compliance, and ability to mimic live infection, which commonly occurs via the oral route. With these considerations in mind, we have previously formulated chitosan-zein nano-in-microparticles (CS-Zn-NIMs) as a dual-material oral gene delivery system consisting of chitosan /pDNA nanoparticle (CS/DNA NP) cores encapsulated within Zn microparticles. While we demonstrated moderate levels of transgene expression in vitro and successful immune responses in vivo, transfection efficiency remains relatively low. Thus, in this study we examine the potential of modifying the CS backbone with hydrophilic and hydrophobic molecules, which have been demonstrated to improve transfection efficiency via increased mucopenetration and cellular uptake, as well as with a molecule to specifically target antigen presenting cells (APCs), immune cells that a play a vital role in initiating robust immune responses. We describe the formulation and in vitro characterization of CS/DNA NPs using CS polymers modified with mannose (Man), poly(ethylene glycol) (PEG), and PEG-Man as well as the attempted formulation of CS-phenylalanine (CS-Phe)/DNA NPs. Modified CS/DNA NPs prepared by ionic gelation with sodium tripolyphosphate (TPP) demonstrated similar sizes to unmodified CS/DNA NPs and high DNA encapsulation efficiency. Furthermore, Man, PEG, and PEG-Man modified CS/DNA NPs exhibited improved transfection levels in vitro as compared to unmodified NPs. This work demonstrates the potential of CS-Man, CS-PEG, and CS-PEG-Man/DNA NPs for use in an oral gene delivery system.

1. Background

1.1. Vaccines

The concept of immunization via vaccination represents one the most successful and wide-reaching clinical interventions of the 20th century [1], if not of the entirety of human history [2]. The advent of vaccines has led to the complete eradication of smallpox, the near complete eradication of poliomyelitis, and a reduction of over 95% for incidences of diphtheria, tetanus, pertussis, measles, mumps and rubella [3]. The introduction of vaccination for infectious diseases, as well as the advent of antibiotics, has led to a dramatic increase in life expectancy and decrease in child mortality over the past century [3, 4]. However, despite these dramatic successes, many infectious diseases, including tuberculosis, hepatitis C, HIV, and influenza, continue to resist effective treatment via vaccination [5]. In fact, only 27 diseases are recognized by the CDC as preventable by vaccination [6]. This lack of efficacy can be attributed to a number of factors including an increased complexity in disease pathogenesis, extensive variability among disease strains, and the evolution of immunoevasive mechanisms to thwart the human immune system [5]. Thus, new approaches need to be considered in order to perpetuate the improvement of global health with vaccine strategies.

In general, vaccines work by taking advantage of the body's intrinsic immune system memory [7]. When infected with a pathogen, an organism's immune system will recognize the foreign substance and mount an attack against the pathogen. The immune system includes both innate defenses—skin, acidic environment of the stomach, and non-specific immune cells—as well as adaptive responses. The adaptive immune response can be classified into two categories: humoral immunity and cell-mediated immunity. Humoral immunity involves the production of antibodies by B-lymphocytes due to pathogen presence, and cell-mediated immunity involves the recognition and killing of viruses or virus-infected cells via cytotoxic T cells or Natural Killer cells. Upon primary exposure to a pathogen the immune system will mount a relatively weak response that takes on the order of weeks to be fully realized; however, following initial exposure, memory B and T lymphocyte cells will be maintained that are specific to that pathogen; thus, upon secondary exposure, a quicker, more robust immune response will be

initiated. Vaccines act upon this mechanism by inducing a primary response without actually causing disease [7].

Traditional vaccines are largely protein based and involve the direct, usually parenternal via intravenous injection, administration of dead or live-attenuated pathogens, recombinant proteins, or virus-like particles [6]. These protein-based vaccines have been effective in a number of pathologies, but often fail in others due to their inability to induce a robust immune response that includes both humoral and cell-mediated responses [6]. Traditional vaccines are primarily anti-body mediated (humoral) and fail to produce robust cell-mediated responses via cytotoxic T lymphocytes (CTLs). Traditional protein-based vaccines involve delivery of exogenous proteins that are internalized and processed by cells via the endolysosomal pathway, which results in degradation into peptides that are associated with MHC class II molecules and the induction of antibody-mediated (humoral) responses [17]. Alternatively, CTL responses result when proteins synthesized *in situ*, following pathogen infection, are process via the proteosomal pathway, which results in peptides that bind nascent MHC class I molecules for export to the cell surface for CTL induction [17]. The induction of a CTL response is necessary to provide immunity in many diseases due to their ability to directly kill pathogen-infected cells (as opposed to directly killing pathogens) and, most importantly, their ability to target antigens inaccessible to antibodies—antigens that, notably, are often epitopes of proteins that are highly conserved between pathogen strains. [17]. Furthermore, one of the most effective mechanisms of pathogen immunological escape is via mutation of the antibody ligand, which is the reason some vaccines require continual modification to target specific strains [17]. Thus, generation of a robust T-cell mediated cellular immune response in addition to the antibody mediated response would vastly improve the effectiveness of vaccination strategies. A further disadvantage of protein based-vaccines is their often time-consuming and expensive production and modification requirements as well as the necessity for cold chain storage due to formulation instability [8, 9]. Therefore, due to the drawbacks associated with protein-based vaccines, other strategies are needed to continue improving vaccine development and target a wider range of pathologies.

1.2. Gene delivery and DNA vaccines

One promising alternative to traditional methods is the use of DNA vaccines. DNA vaccines rely upon the broader technology known as gene delivery or gene therapy. Gene delivery involves the delivery of exogenous genetic material to target cells for therapeutic gain [10]. In the case of DNA vaccines, the delivered gene encodes for a viral or bacterial antigen capable of being presented to antigen presenting cells (APCs) and inducing an immune response. Though the potential to modulate specific gene expression in target cells and tissues represents a promising route towards curing a wide variety of maladies (e.g. AIDS, cancer, genetic disorders, etc), significant challenges exist in achieving this goal [11]. Firstly, genetic material must be delivered to cells without being degraded by nucleases. Though initial efforts at delivering DNA to cells involved the injection of naked plasmid [12], numerous carrier vectors have been investigated to protect DNA and improve gene delivery efficiency, i.e. transfection efficiency. Generally, these vectors are grouped into two main categories: viral and non-viral delivery systems. The use of viral vectors is advantageous due to their high efficiency but remains unfavorable due to toxicity, immunogenicity, and oncogenic concerns as well as their limited DNA capacity and difficult manufacturing processes [10,13,14]. Consequently, non-viral delivery vectors are being investigated to address the perceived drawbacks of viral vectors. Non-viral vectors generally rely upon DNA complexation with cationic lipids or polymers in order to protect genetic material from degradation *in vivo* via nucleases [11]. Additionally, DNA complexes must remain small enough to allow for cellular uptake and subsequently promote endosomal escape and DNA dissociation. While non-viral delivery systems address many of the toxicity and immunogenicity concerns associated with viral vectors, non-viral vectors suffer from poor transfection efficiency. Thus, there remains a need to improve the efficiency non-viral vector transgene expression whilst remaining non-toxic.

Furthermore, many routes are being investigated for delivery of DNA vectors including parenteral routes (subcutaneous, intramuscular, intradermal, intravenous) or mucosal (oral, intranasal, and vaginal) [8]. Of particular interest is the oral route, which is preferable due to its ease of administration and dosing as well as high patient compliance [15, 16]. From a functionality standpoint, oral gene delivery provides significant advantages due to the large surface area it presents for cell uptake [18] as well as the potential for both local and systemic

delivery of genetic material [10]. The perceived advantages of oral gene delivery have resulted in it being an area of significant research in recent years [19-24].

Oral gene delivery for DNA vaccines provides the above advantages, in addition to providing the ability to target the intestinal mucosa to generate mucosal immunity [25]. Generation of mucosal immunity can greatly improve defense against pathogens that tend to cause disease at or enter the body through the mucosal lining [26]. However, successful oral delivery of genetic material faces a significant challenge in overcoming the harsh conditions of the gastrointestinal tract—low pH, nucleases, and gastric enzymes—as well as infiltrating the intestinal epithelium. One method being investigated to circumvent these barriers is the use of dual material systems that can protect DNA cargo through the varying physiological conditions of the GI tract [27-30] including the work in our lab on chitosan-zein nano-in-microparticles (CS-Zn-NIMs) [10]. This dual-approach composed of two highly biocompatible and biodegradable natural polymers has shown great potential as an oral DNA vaccine delivery system. We have demonstrated the ability of zein to protect chitosan/pDNA nanoparticles (CS/DNA NPs) during simulated gastric fluid (SGF) treatment followed by rapid release of the nanoparticles following treatment with simulated intestinal fluid (SIF) *in vitro* [10]. Additionally, we have demonstrated the ability of CS-Zn-NIMs to provide *in vivo* transgene expression and activation of mucosal immune response via anti-GFP IgA antibodies [10]. Although these results are promising, *in vitro* transgene expression and *in vivo* immune-activation remain relatively low. Thus methods to improve the transfection efficiency of the carrier system must be examined in order to generate more robust immune responses.

1.3. Chitosan modifications for improved transfection efficiency

The nature of the CS backbone allows for easy modification via its amine groups [31], which has led to numerous groups investigating the potential of chitosan modification for improving transfection efficiency [32, 59, 60, 61]. These include: hydrophilic modifications to improve systemic circulation time [33-38] and mucopenetrative capabilities [85-92]; hydrophobic modifications to improve cell adsorption and uptake as well as aiding in DNA release from complexes [39-45]; pH-sensitive modifications to improve endosome release via the proton-sponge effect [46-52]; temperature-sensitive modifications, which can aid in drug or

DNA dissolution due to heating/cooling above/below critical solution temperatures [53-58]; and specific-ligand conjugations such as galactose [62-70],transferrin [71-73], folate [74-76], and mannose [77-83] to improve immobilization on cell surfaces via cell receptor binding, often to specific target cells. For the purpose of improving gene delivery in a chitosan-based oral DNA vaccine application, the addition of several modifications should lead to improved transgene expression and immune system activation.

Firstly, a hydrophilic modification to improve mucopenetration and systemic circulation time is of vital importance to improve the immune response of oral DNA vaccines. Once released from zein microparticles within the small intestine, the CS/DNA NPs must be able to travel through the mucus layer lining the lumen before penetrating the epithelial lining. The mucus layer consists primarily of mucin fibers enmeshed in a dense network [94, 95]. These linear, glycosylated fibers contain hydrophobic serine residues along the backbone with widely branching polysaccharides in a bottle-brush configuration, which allows the mucus to trap molecules and prevent particle movement [96]. Ionic particles are particularly susceptible to mucus entrapment due to electrostatic interactions. Thus, improving movement through the mucus layer requires reducing these interactions. Viruses have been shown to quickly move through the mucus layer, which is attributed to their small size and nearly neutral surface charge [88]. In an attempt to mimic viral diffusion, hydrophilic polyethylene glycol (PEG) chains have been conjugated to nanoparticles to shield the positive surface charge and have demonstrated improved mucus diffusion [91, 97, 98, 99]. Additionally, once within the systemic circulation, the chitosan nanoparticles must avoid clearance in order to provide optimal immunostimulatory effects. The nature of cationic polymer/DNA complexes such as CS/DNA results in relatively short plasma circulation times due to hepatic clearance and accumulation in organs as a result of particle aggregation [32]. Aggregation occurs following interaction with blood components, such as albumin, that bind the cationic particles and consequently reduce the particle zeta potential resulting in less charge repulsion between particles. Thus it was hypothesized that the addition of a hydrophilic conjugate to the nanoparticle surface would reduce serum interactions and particle aggregation, which would then lead to improved plasma circulation time. PEGylation of polymeric gene delivery systems has been shown to prolong circulation times by providing a steric barrier to interaction with serum proteins and phagocytes [100]. Furthermore, PEGylated

CS/DNA NPs have even been shown to provide improved transfection efficiency *in vitro*, likely due to reduced aggregation in the transfection medium, which includes serum components [101].

Secondly, due to the low transfection efficiency of CS/DNA NPs, the addition of a hydrophobic residue to the CS backbone should provide a general increase in transgene expression, which will aide in inducing more robust immune responses. Hydrophobic modifications are expected to increase transfection via increased cell surface adsorption and cellular uptake due to a greater ability to pass through the cell membrane [39]. Moreover, hydrophobic additions may assist in the decomplexation of DNA from chitosan carriers by reducing the strength of binding between chitosan and DNA thus leading to an increase in DNA available for gene expression. Many groups have demonstrated the ability of hydrophobically-modified CS to improve transfection efficiency [39-45, 103-106]. Specifically, the hydrophobic amino acid phenylalanine (Phe) has been used to form hydrophobically modified CS/DNA NPs with improved transfection capabilities due increased absorptive-mediated endocytosis and DNA dissociation [103-106].

Lastly, in order for successful DNA vaccine development, antigen-encoding DNA must be delivered to professional APCs, such as dendritic cells and macrophages, which are highly efficient at internalizing and presenting antigens to T-lympohocytes and inducing lympohocyte differentiation and proliferation [106-108]. Thus efficiently targeting APCs with CS/DNA NPs would be of enormous benefit in DNA vaccine applications. It has been noted that professional APCs overexpress the mannose receptor (MR) on their surface, leading several groups to attempt the conjugation of mannose to CS, which has shown significant ability to improve APC targeting and MR-mediated endocytosis [77-83, 106].

In this study, we report the formulation, characterization, and optimization for *in vitro* transfection of CS/DNA NPs modified with hydrophilic PEG, hydrophobic Phe, and APC-targeting mannose (Man) to improve transfection efficiency and cell-targeting for oral DNA vaccine applications.

We hypothesize that the addition of these ligands will improve *in vitro* transfection efficiency due to the conjugation of hydrophobic Phe, which will increase cellular uptake and complex dissociation leading to improved transgene expression. Futhermore, we theorize that the addition of mannose will demonstrate improved transfection in macrophage cells due to their overexpression of the mannose receptor. Finally, we posit that the addition of hydrophilic PEG

chains will enhance *in vitro* transfection efficiency due to reduced aggregation and will show promise for later incorporation into CS-Zn-NIMs to improve diffusion through the intestinal mucus barrier and prolong systemic circulation.

We provide evidence that the formulation of CS/DNA NPs using CS polymers modified with Man, PEG, and PEG-Man results in NPs of similar size to those formulated with unmodified CS as well as high DNA encapsulation efficiency. Furthermore, we provide evidence that CS-PEG-Man/DNA NPs can significantly improve transgene expression in vitro, which demonstrates their potential to be used within CS-Zn-NIMs to increase transgene expression and immune system activation in vivo.

2. Materials and Methods

2.1. Materials, cell lines, and cell culture

Chitosan oligosaccharide lactate (Avg MW 5000 g/mol), sodium tripolyphosphate (TPP), N-hydroxysulfosuccinimide sodium salt (NHS), N-(3-dimethylaminopropyl)-N'ethylcarbodiimide (EDC), D-(+)-mannose, α-D-mannopyranosylphenyl isothiocyanate (MPITC), poly(ethylene glycol) 2-aminoethyl ether acetic acid (nPEG) (Avg MW 2100 g/mol), and N-acetyl-L-phenylalanine, were purchased from Sigma-Aldrich (St. Louis, MO). Methoxy poly(ethylene glycol)-carboxyl aid (mPEG-AA, Avg MW 2000 g/mol) was purchased from Creative PEGWorks (Chapel Hill, NC). Human embryonic epithelial kidney cells, HEK 293T (ATCC,Manassas, VA), were cultured at 37^{o}C and 5% CO_2 in T-75 flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA) containing 4.5 g/L glucose and 2 mML-glutamine (Gibco), and supplemented with 10% fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin (Gibco) and 100 mM sodium pyruvate at a final concentration of 1%. Recombinant green fluorescent protein (GFP) from A. Victoria was purchased from Abcam (Cambridge, MA).

2.2. Isolation and culture of bone marrow-derived cells (BMDCs)

Additionally, primary murine bone marrow derived macrophage (BMDM) and dendritic (BMDD) cells were isolated and cultured from male BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME). Experimental animal procedures using mice were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln. Briefly, following administration of an anesthetic, mice were eutahnzed via cervical dislocation. Mice were then sprayed with 70% ethanol and the hind leg bones of the mice were dissected out with scissors and placed in a FBS wash buffer. In a fume hood, excess muscle was removed and bones were sterilized with 70% ethanol followed by a second FBS wash. Next, the ends of the bones were cut off and a 25-gauge needle was used to flush 5 mL of PBS through the medullary cavity into a petri dish to collect bone marrow. The PBS solution containing cells was then filtered through a 70 μm filter into a 50 mL conical using a 22-gauge needle followed b centrifugation at 1400 rpm and 4^{o}C for 7 min. 1 mL of 1x ACK (ammonium-chloride potassium) RBC (red blood cell) lysis buffer was added to the supernatant and 45 seconds later 35 mL 1% FBS wash buffer was added and the solution agitated. The sample was again centrifuged at 1400 rpm and 4^{o}C for 7 min and the pellet resuspended in TCM (T-cell media). Cells were then counted using a 1:10 dilution of trypan blue stain and plated into 6 well plates with 4 mL of TCM and 1 mL of cell suspension containing 5 ng/mL GM-CSF (granulocute macrophage-colony stimulating factor) at a cell density of $2x10^6$ cells/well. Cells were cultured at 37^{o}C and fed with a fresh 2.5 mL TCM and 5 ng/mL GM-CSF on days 3, 5, and 7, when transfection experiments took place.

2.3. Plasmid preparation

All transfection experiments were performed using the pEGFP-LUC (Clontoech, Mountain View, CA) plasmid encoding for both firefly luciferase protein and green fluorescent protein under the direction of the CMV promoter. The plasmid was purified from *E. coli* using a Qiagen Giga kit (Valencia, CA) and stored in Tris-EDTA (TE) buffer solution (10 mM Tris, 1 mM EDTA, pH 7.4) at -20^{o}C until use.

2.4. Synthesis and characterization of polymers

2.4.1 CS-PEG Synthesis

CS-PEG was synthesized by a carbodiimide-mediated coupling reaction between the carboxyl group of mPEG and the amine group of CS (Figure 2C). Briefly, 33.67 mg of mPEG and 30.81 mg of EDC (10-fold molar excess) were dissolved in 3 mL distilled water and stirred for 15 minutes. Then 87.39 mg of NHS (2.5 mol/mol EDC) was then added to the mixture and stirred for 45 minutes. The EDC/NHS activated mPEG was then added dropwise to 60 mg of CS (20 mol/mol mPEG) dissolved in 2 mL distilled water and reacted at 25^{o}C overnight under constant stirring. The solution was then dialyzed for 24 h (MWCO 5 kDA) and lyophilized to obtain CS-PEG.

2.4.2 CS-Man Synthesis

CS-Man was synthesized by a carbodiimide-mediated coupling reaction between the carboxyl group of MPITC and the primary amines on the CS backbone (Figure 2A). Briefly, 60 mg CS was dissolved in 2 mL distilled water and 10 mg MPITC was dissolved in 1800 μL dimethyl sulfoxide (DMSO). MPITC solution was added to the CS solution and reacted overnight at 25^{o}C followed by dialysis for 24 hours (MWCO 2 kDa) and lyophilization to obtain CS-Man.

2.4.3 CS-PEG-Man Synthesis

CS-PEG-Man was synthesized in a two-step process (Figure 2D). First, MPITC was conjugated to nPEG via carbodiimide-mediated coupling reaction between the carboxyl group of MPITC and the amino terminus of nPEG. Briefly, 33.67 mg nPEG were dissolved in 2 mL distilled water and 10 mg MPITC was dissolved in 1800 μL DMSO (2:1 molar ratio) and reacted overnight at 25^{o}C followed by dialysis for 24 hours (MWCO 2 kDa) and lyophilization to obtain PEG-Man. The PEG-Man was subsequently conjugated in a similar manner to the CS backbone. Briefly, the PEG-Man was dissolved distilled water and 60 mg CS was dissolved in 2 mL distilled water. The PEG-Man

solution was added to the CS solution and reacted overnight at 25^{o}C followed by dialysis for 25 hours (MWCO 2 kDa) and lyophilization to obtain CS-PEG-Man.

2.4.4 CS-Phe Synthesis

CS-Phe was synthesized by carbodiimide mediated coupling reaction between the carboxyl group of N-acetyl-L-phenylalanine and the primary amines on the CS backbone (Figure 2B) according to a previously established reaction scheme [106]. Chitosan was dissolved in distilled water at a concentration of 12 mg/mL. N-acetyl-L-phenylalanine (0.3 mol/mol of sugar unit of chitosan) and EDC (3 mol/mol of N-acetyl-L-phenylalanine) were dissolved in ethanol and continued to stir for 1 h followed by addition of N-hydroxysuccinimide (3 mol/mol of N-acetyl-L-phenylalanine). The mixture of phenylalanine solution containing EDC and NHS was added slowly to the chitosan solution. The reaction was continued at room temperature for 24 h with constant stirring. After that, the reaction mixture was refluxed with 1 N hydrochloric acid for 30 min to removes the N-acetyl protecting groups of L-phenylalanine [106]. The refluxed solution was neutralized with 1 N sodium hydroxide followed by dialysis against distilled water for 48 h using dialysis membrane (MWCO: 1 kDa) and then lyophilized to obtain CS-Phe.

2.5. Formation of CS/DNA NPs

CS/DNA NPs encapsulating pEGFP-LUC were prepared using an ionic gelation method [109]. Briefly, 5 mg/mL solutions of all chitosan oligosaccharide lactate derivatives were made by dissolution of powder form in ultrapure water. The resulting solutions were filtered with a 0.22 μm syringe filter (EMD Millipore, Billerica, MA) to remove any impurities. Similarly, a 0.5 mg/mL TPP solution was prepared in ultrapure water. To prepare nanoparticles, the stock 5.0 mg/mL CS solutions were diluted in ultrapure water to various concentrations (0.5-2.0 mg/mL). Plasmid DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) was added to the TPP solution, vortexed for 5 s, and added to the CS solution. The resulting NP suspension was immediately

vortexed for 10 s and then allowed to incubate at room temperature for 20 min. The amount of TPP solution used in the particle formation was varied to achieve different CS:TPP ratios (w/w). For all characterization and transfection studies, the CS:DNA ratio was held constant at 10:1, unless otherwise noted.

2.6. Characterization of CS/DNA NPs for size, charge, and DNA encapsulation efficiency

The size of CS/DNA NPs was evaluated by dynamic light scattering and the zeta potential by Laser Doppler micro-electrophoresis using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). Size measurements were taken at 25^{o}C at a scattering angle of 90^{o}, and size was reported as the Z-average diameter. Zeta potential measurements were also taken at 25^{o}C using folded capillary cells with the measurement mode set to automatic. DNA encapsulation efficiency of the CS NPs was determined by measuring the amount of free DNA in the aqueous particle suspension after particle formation. Freshly prepared NPs were centrifuged at 10,000 x g for 20 min and the amount of DNA in the supernatant was measured using the Hoechst assay. Briefly, supernatant samples were diluted in 1 x TNE buffer (10 mM Tris; 0.2 M NaCl; 1 mM EDTA; pH 7.4) to a final volume of 1 mL and mixed with 1 volume of a 200 ng/mL solution of Hoechst 33258 dye. After a 5 min incubation period at room temperature, fluorescence was measured using a modulus luminometer/fluorometer (Turner Biosystems, Sunnyvale, CA). The encapsulation efficiency of the CS NPs was determined by calculating the difference between the amount of measured free DNA and the initial amount of DNA used in the particle preparation according to Eq. (1):

$$EE\% = \frac{Initial\ amount\ of\ DNA\ used\ in\ particle\ preparation - DNA\ in\ supernatant}{Initial\ amount\ of\ DNA\ used\ in\ particle\ preparation} \quad (1)$$

2.7. In vitro transfection efficiency of CS/DNA NPs

Transfection studies with CS/DNA NPs encapsulating pEGFP-LUC were performed using the human embryonic kidney cell line (HEK293T). Cells were seeded in 48 well plates at a seeding density of 36,000 cells/well (unless otherwise noted) in a total volume of 300 μL of

complete Dulbecco's Modified Eagle's Medium (DMEM) 18 h prior to delivery of CS/DNA NPs. The day of transfection, CS/DNA NPs were prepared as previously described and diluted in an appropriate volume of serum free Opti-MEM to deliver a total volume of 75 μL/well equating to 1 μg of DNA per well. The NPs were allowed to incubate with the cells for 24 h at which point transfection was assessed qualitatively using fluorescent microscopy. Quantitative evaluation of transfection efficiency was carried out using the luciferase assay system (Promega, Madison, WI) to measure luciferase activity. Briefly, cells were lysed by adding 200 μL of 1X reporter lysis buffer (Promega) and the cell lysates were measured for luciferase activity using a luminometer with an integration time of 10 s by adding 20 μL of sample to 100 μL luciferase assay reagent followed by brief vortexing and insertion into luminometer. Measured relative light units (RLU) were normalized to total protein content as measured by the BCA protein assay (Pierce).

2.8. Statistical analyses

All experiments were performed in triplicate on duplicate days (n=6) unless otherwise noted. Comparative analyses were completed using one-way ANOVA followed by Tukey's post-test for comparing multiple treatment conditions, both at a 95% confidence level using Prism software (GraphPad Prism 5, LaJolla, CA). All values are reported as mean ± standard error of the mean.

3. Results

3.1. Modified CS polymer syntheses and characterizations

The objective of this study was to improve the gene delivery ability of CS/DNA NPs for use in an oral gene delivery application. Specifically, we aimed to improve the transfection efficiency and cell-targeting ability of CS/DNA NPs for use in the dual-material CS-ZN-NIMs previously developed by our lab for oral DNA vaccine applications [10]. To this end we investigated three modifications to the chitosan backbone—poly (ethylene glycol) (PEG), phenylalanine (Phe) and mannose (Man)—which we hypothesized would improve transfection

efficiency via improved mucopenetration and systemic circulation time, cell encapsulation and DNA dissociation, and APC targeting, respectively. However, it should be noted that we are unable to test for improved mucopenetration and circulation time.

The CS-Phe was synthesized in a three-step reaction by EDC/NHS mediated coupling of N-acetyl-L-phenylalanine to the primary amine groups of CS (Fig. 1 B) [106]. The acetylated form of phenylalanine was used to protect the amine groups of phenylalanine from coupling with each other during the reaction. Following lyophilization CS-Phe was unable to be resuspended in aqueous solution and thus further optimization of synthesis procedures is necessary before use within particles. Consequently, CS-Phe was not used in the remainder of the study. The CS-PEG and CS-Man were similarly synthesized via EDC/NHS-mediated coupling to primary amines of CS (Fig. 1 A, C). The synthesis of CS-PEG-Man necessitated first coupling PEG and Man via EDC/NHS coupling followed by conjugation to the CS backbone in order to establish PEG chains attached to CS at the C-terminus and mannose at the N-terminus (Fig. 1 D).

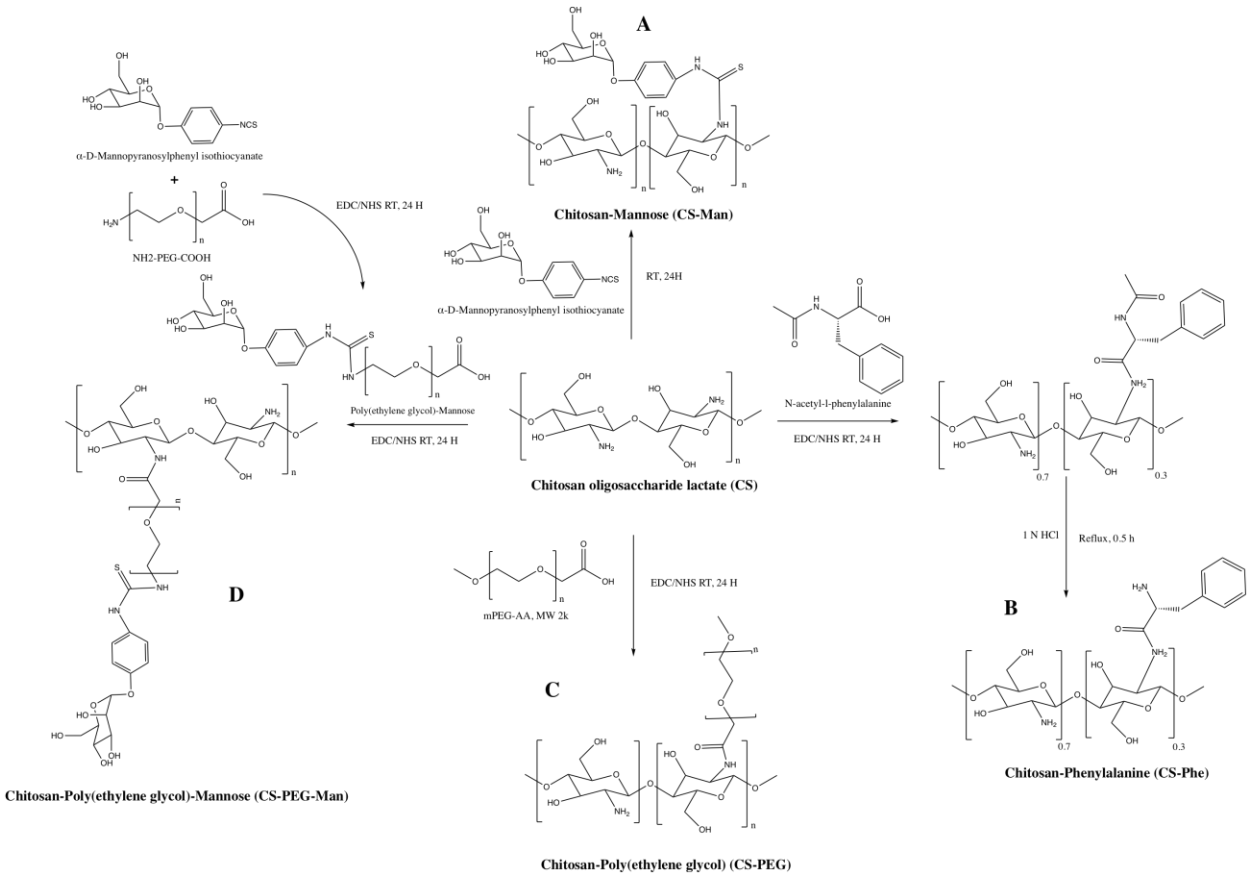

Figure 1. Synthetic schemes of chitosan modifications: (A) Chitosan-Mannose (CS-Man), (B) Chitosan-Phenylalanine (CS-Phe), (C) Chitosan-Poly(ethylene glycol) (CS-PEG), and (D) Chitosan-Poly(ethylene glycol)-Mannose (CS-PEG-Man). All reactions involved EDC/NHS-mediated carbodiimide coupling reactions to the primary amines of chitosan.

3.2. CS NP characterization: size, zeta potential, and DNA encapsulation efficiency

The size and surface charge of the CS/DNA NPs are highly important properties affecting all steps in the gene delivery process, including cellular uptake, endosomal escape, complex dissociation, and nuclear import. Thus, modulation of these two factors is vital to successful gene transfection, and their characterization was essential. Thus, CS/DNA NPs were formed with all four modified CS polymers, as well as unmodified CS, at varying CS:TPP ratios (6 to 12) with an initial CS concentration of 0.5 mg/mL and CS to DNA ratio of 10 to 1. The resulting NPs were characterized for size, zeta potential, and DNA encapsulation efficiency (Fig. 2). For all NPs, size was dependent on the CS:TPP ratio, with increasing CS:TPP generally positively correlated with increasing size. Unmodified CS NPs showed increasing size from 309.7 nm (CS:TPP 6:1) to 366.1 nm (CS:TPP 12:1) (Fig. 2A, Table 1). CS-Man NPs showed a similar

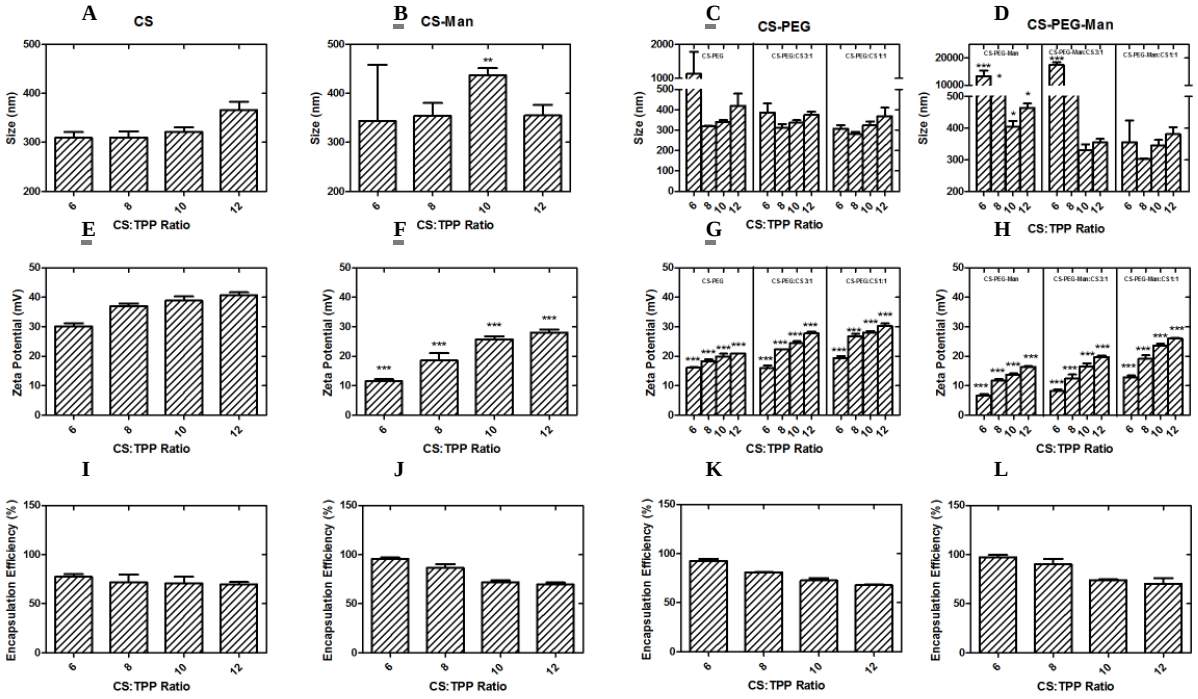

Figure 2. Characterization of CS/DNA NPs formed via ionic gelation with unmodified and modified CS. CS/DNA NPs formed using CS, CS-Man, CS-PEG, and a 1:1 mixture of CS and CS-PEG-Man at varying CS:TPP ratios were characterized for size (A-D), zeta potential (E-H), and DNA encapsulation efficiency (I-L). The concentration of CS in solution and the CS:DNA ratio were held constant at 0.5 mg/mL and 10:1, respectively. All data represented as mean ± SEM (n=3). Asterisks (*) denote significance as compared to CS conditions (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

but more variable trend, ranging from 343.9 nm (CS:TPP 6:1) to 354.8 nm (CS:TPP 12:1) with a CS:TPP ratio of 10:1 resulting in significantly larger particles (436.5 nm) (Fig. 2 B, Table 1). CS-PEG demonstrated a trend with a high particle size at a CS:TPP ratio of 6:1 (1130.4 nm), which then decreased at CS:TPP ratio of 8:1 (318.0 nm) before rising again to 419.9 nm at a CS:TPP ratio of 12:1 (Fig. 2 C, Table 1). CS-PEG was also mixed with unmodified CS at CS-PEG:CS ratios of 3:1 and 1:1, with similar trends resulting but with a much lower particle size at a CS:TPP ratio of 6:1. Finally, CS-PEG-Man was similarly characterized with varying ratios of modified to unmodified CS with similar trends resulting wherein low CS:TPP ratios result in large particle diameters, which then decrease as the CS:TPP ratio increases (Fig. 2 D). Mixing CS with CS-PEG-Man at a ratio of 1:1 allowed nearly complete recovery of particle size to sub-500 nm levels, ranging from 355.3 to 380.4 nm as the CS:TPP ratio was increased from 6 to 12 (Table 1). Particle formulations were next analyzed for surface charge as determined by zeta potential. All particles showed a consistent trend of a positive zeta potential that increases with an increasing CS:TPP ratio (Fig. 2 E-H). Zeta potential ranged from 30.2 to 40.8 mV, 11.5 to 28.0 mV, 16.2 to 20.9 mV, and 12.8 to 26.0 mV for CS, CS-Man, CS-PEG, and CS-PEG-Man:CS 1:1, respectively (Table 1). Finally, DNA encapsulation efficiency was determined via a Hoescht assay. For all formulations, as the CS:TPP ratio increased, encapsulation efficiency decreased (Fig. 2 I-L) from 77.4 to 69.7 % for CS, 95.7 to 69.6% for CS-Man, 92.5 to 68.0% for CS-PEG, and 97.1 to 70.3% for CS-PEG-Man 1:1 (Table 1).

Table 1. CS NP size, zeta potential, and DNA encapsulation efficiency with differently modified CS backbones.

NP Type	Average particle size (nm)				Average zeta potential (mV)				Average encapsulation efficiency (%)			
CS:TPP Ratio	6	8	10	12	6	8	10	12	6	8	10	12
CS	309.7 ± 11.1	310.2 ± 12.3	321.4 ± 9.3	366.1 ± 16.8	30.2 ± 1.0	37.0 ± 0.9	38.9 ± 1.4	40.8 ± 0.9	77.4 ± 2.8	71.6 ± 8.3	70.9 ± 6.4	69.7 ± 2.3
CS-Man	343.9 ± 114.31	353.8 ± 26.6	436.5 ± 14.6	354.8 ± 21.8	11.5 ± 0.7	18.6 ± 2.4	25.7 ± 1.1	28.0 ± 1.1	95.7 ± 1.0	86..3 ± 4.1	71.8 ± 2.0	69.6 ± 2.1
CS-PEG	1130.4 ± 649.6	318.0 ± 5.2	340.6 ± 8.4	419.9 ± 60.2	16.2 ± 0.2	18.4 ± 0.6	19.9 ± 1.0	20.9 ± 0.1	92.5 ± 2.2	81.0 ± 0.2	72.7 ± 2.3	68.0 ± 0.8
CS-PEG-Man:CS 1:1	355.3 ± 68.4	302.3 ± 2.0	345.9 ± 16.9	380.4 ±22.5	12.8 ± 0.7	19.1 ± 1.3	23.7 ± 0.5	26.0 ± 0.2	97.1 ± 2.5	90.2 ± 5.4	74.1 ± 0.6	70.3 ± 5.9

3.3. CS NP transfection efficiency

CS/DNA NP formulations were next evaluated for their ability to mediate transgene expression in vitro using HEK293T cells. Transfection efficiency was analyzed 24 hours post-transfection using a luciferase assay normalized to protein content as determined by BCA protein assay. Transgene expression was dependent on the CS:TPP ratio and similar trends were observed for all conditions as the CS:TPP ratio was increased with transfection efficiency

initially increasing before reaching a peak and decreasing (Fig. 3 A-D). Naked CS/DNA NPs demonstrated the lowest transgene expression with CS-Man and CS-PEG showing moderate increases over CS. CS-PEG-Man mixed with CS at a ratio of 1:1 demonstrated the highest levels of transgene expression, and a comparison of the optimal CS:TPP ratios for each NP formulation (6, 6, 10, and 10 for CS, CS-Man, CS-PEG, and CS-PEG-Man:CS 1:1, respectively) demonstrated that CS-PEG-Man mediated significant increases in transfection over CS and CS-Man.

Transfection experiments were also conducted with both RAW 264.7 macrophages and the primary BMDCs obtained from mice; however, no transfection was observed in either one with any of the CS/DNA NP formulations.

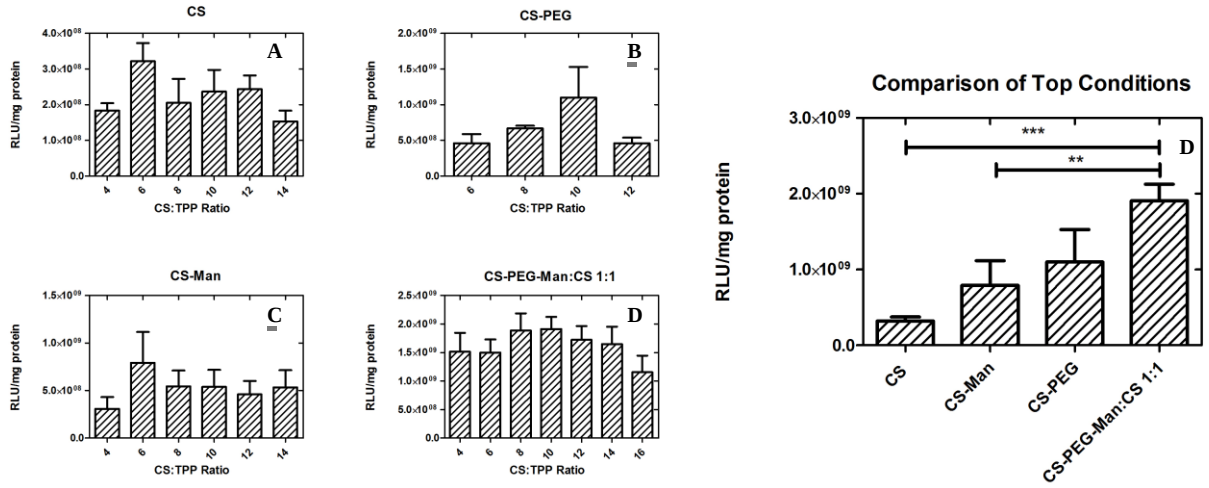

Figure 3. Transgene expression levels of CS/DNA NPs. Particles were formed with (A) CS, (B) CS-PEG, (C) CS-Man, and (D) CS-PEG-Man:CS 1:1. The CS:TPP ratio was varied from 4 to 14 for CS and CS-Man, 6 to 12 for CS-PEG, and 4 to 16 for CS-PEG-Man:CS 1:1. All data are represented as mean ± SEM (n≥6 except for n=3 for CS-PEG). Asterisks (*) denote significance as compared to CS conditions (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

4. Discussion

Despite the dramatic success of vaccination strategies in treating a handful of potentially deadly infectious diseases, many pathogens remain resistant to effective immunization via vaccines. One of the major drawbacks of traditional vaccine technologies is their failure to induce a robust response in both aspects of the adaptive immune system, namely the antibody-mediated (humoral) and cell-mediated systems. While traditional vaccines typically are only

capable of inducing humoral immunity, live pathogens will induce both. Thus, a need remains to more closely mimic live infection and induce both of these systems. In order to induce a cell-mediated immune response, antigens must be presented on the surface of infected cells where cytotoxic T cells can recognize and destroy infected cells. DNA vaccines involving the delivery of exogenous DNA encoding for a pathogenic antigen are being investigated towards this end in order to more closely mimic live infection. While many delivery routes are being investigated, the oral route is preferable due to the ease of administration and high patient compliance, as well as the potential to induce mucosal immunity ability and to mimic live infection, since the pathogens often enter by way of the oral cavity.

Consequently, the oral delivery of exogenous genetic material has the potential to be an effective DNA vaccination and gene therapy strategy, but significant barriers remain towards fully realizing this potential. Both the harsh conditions of the gastrointestinal tract and the low efficacy of non-viral delivery strategies remain significant challenges towards this end. Previously, we have developed a dual-material nano-in-microparticulate system for the oral delivery of genetic material using an inner chitosan/DNA core with an outer zein shell, known as CS-ZN nano-in-microparticles (CS-Zn-NIMs). In response to promising initial results, we wished to further improve the efficiency of gene delivery. Thus, in this study, we describe the modification of the CS backbone with two small molecules—mannose (Man) and phenylalanine (Phe)—and the polymer poly(ethylene glycol) (PEG)—in order to improve the *in vitro* transfection efficiency of CS NPs.

CS, which has been widely investigated as a DNA delivery vector [6,8,10,16,21,32,34], was chosen as the inner core polymer for DNA complexation due to its biodegradability and biocompatibility as gene delivery vector, as well as the presence of primary amine groups in its backbone, which have a pKa of approximately 6.5 allowing for protonation in physiological conditions and a net positive charge that lends itself towards easy electrostatic complexation with negatively charged DNA. In this study, we used oligosaccharide CS due to its improved water solubility to formulate CS/DNA NPs, which we further crosslinked with TPP.

Additionally, the primary amines present on the CS backbone allow for simple modification via carbodiimide coupling in order to modulate the NP properties. A wide variety of modifications to CS have been investigated [31,32,59-61]. To address the unique challenges presented by oral gene delivery for a DNA vaccination application, we investigated modifying

CS with PEG, Phe, and mannose. PEG was chosen to fulfill the need for a hydrophilic molecule to shield the positive charge of CS in order to reduce interactions with mucus and blood components and increase mucopenetration and systemic circulation time [32, 91,97-101]. Phe was chosen as we hypothesized that the addition of a hydrophobic ligand could improve both cellular encapsulation, by improving direct transport through the inherently hydrophilic interior of the cell membrane and complex dissociation by reducing the strength of interaction between CS and DNA [39-45, 103-106]. Third, mannose was investigated as potential modification due the reported overexpression of the mannose receptor (MR) on the cell surface of professional antigen-presenting cells (APCs), cells that specialize in present antigen components to T cells as part of the cell-mediate immune response. Addition of mannose to NPs has been shown to improve cellular-targeting of NPs to APCs, a highly desirable feature in a DNA vaccine [77-83, 106].

Synthesis of modified CS oligosaccharide polymers was carried out using EDC/NHS-mediated carbodiimide coupling reactions between the primary amines of CS and carboxyl groups on the ligands. CS-PEG was synthesized using methoxy PEG (mPEG), which possesses a carboxyl group on one end, for attachment to CS, and a methoxy group on the other, which is unreactive in this scheme to prevent self-reaction of PEG chains or attachment of both sides to the backbone. CS-Man was synthesized according to previous report [79,83] using the modified mannose reagent α-D-mannopyranosylphenyl isothiocyanate (MPITC), which allows formation of an isothiocyanate bridge via conjugation of the isothiocyanate carbon with the nucleophilic primary amines of CS. A PEG and Man dual-modified polymer was also formulated. In order to attach PEG to CS on one end while conjugating mannose to the other end, a PEG chain with both an amino terminus and a carboxyl terminus was utilized. In order to avoid self-reaction between the N- and C-terminuses of individual PEG chains, as well as to ensure selective attachment of mannose to PEG chains and not onto the CS backbone, PEG-Man polymer was first synthesized to attach mannose to the N-terminus, followed by attachment of the C-terminus to CS, both via EDC/NHS-mediated carbodiimide coupling. Finally, CS-Phe was formulated according to previous report [106] using N-acetyl-L-phenylalanine. The acetyl group acted as a protecting group for the primary amine on Phe in order to prevent self-reaction between the carboxyl and amino groups of Phe molecules. Following carbodiimide coupling, acetyl protecting groups were removed by refluxing with 1 N HCl for 30 min, followed by dialyzing and lyophilizing.

Following synthesis, full characterization of CS/DNA NPs formulated with modified CS polymers was essential to understand and optimize particle formation characteristics that affect transfection efficiency. Previously we have demonstrated the importance of the CS:TPP ratio on particle size, zeta potential (surface charge), and DNA encapsulation efficiency (EE) [10], all parameters related to the efficiency of transfection [110,111]. In our initial studies, CS/DNA NPs were fully characterized for size, charge and EE at CS:TPP ratios from 4:1 to 14:1 at a CS:DNA ratio of 10:1 [10]. Generally, complex size and zeta potential increased with increasing CS:TPP ratios and a CS:TPP ratio of 6:1 was found to be optimal for transfection efficiency, likely due to the high EE at lower CS:TPP ratios combined with smaller size, as small complex size has been shown to be a critical factor in the efficiency of endocytosis [112,113]. Thus, here we formulated and similarly characterized modified CS/DNA NPs at varying CS:TPP ratios. In agreement with our previous work, increasing CS:TPP ratio was generally correlated with increasing CS/DNA NP size (Fig. 2 A-D) and increasing positive surface charge (Fig. 2 E-H), likely due to decreasing amounts of negatively-charged crosslinking TPP, with several notable exceptions. The formulation of CS/DNA NPs with CS-Man, CS-PEG, and CS-PEG-Man at lower CS:TPP ratios (6 for CS-Man and CS-PEG and 6, 8 for CS-PEG-Man) demonstrated large diameters, particularly for CS-PEG-Man, as measured by dynamic light scattering. However, visible accumulation within NP solutions signified the presence of large aggregations of particles rather than large NPs. In addition, we noted that the modifications of CS with Man, PEG, and Man-PEG all led to significantly reduced zeta potentials, with the CS-PEG-Man NPs having the lowest. The combination of these observations led us to hypothesize that the conjugation of Man and PEG to the amine groups on the CS backbone, which are normally positively charged, led to a decrease in overall positive charge and the formation of NPs with near-neutral zeta potentials, causing them to aggregate in solution. Additionally, the long PEG chains may be shielding much of the remaining positive charge on the backbone, as we had hypothesized would happen, which would explain why aggregation was much more significant with CS-Man-PEG than CS-Man even though their zeta potentials were nearly equivalent and why CS-PEG demonstrated more significant aggregation than CS-Man despite having a more positive zeta potential. In order to prevent the aggregation of NPs and allow for successful transgene expression, we mixed solutions of CS-PEG-Man and CS in ratios of 3:1 and 1:1 and demonstrated the ability to achieve NPs with sub-400 nm diameters. The trend of decreasing encapsulation efficiency with

increasing CS:TPP ratio is seemingly counterintuitive—larger particles should hold more DNA—but may be due to the increased size, which leads to DNA be less surrounded by positive charges and enables DNA to more easily escape.

In order to induce significant immune responses in vivo, CS/DNA NPs must be capable of successfully mediating transgene expression in vivo. In order to analyze the potential of the described modifications to improve transfection of CS/DNA NPs for later in vivo application, we measured transgene expression in vitro using HEK293T cells and a luciferase assay system. Again, similar trends were observed to those found in our previous work [10], with transgene expression levels, normalized to total protein content, initially increasing before decreasing as CS:TPP ratios increased (Figure 4). The higher transfection at lower CS:TPP ratios is likely the direct result of smaller sizes and higher encapsulation efficiency. All three modified CS polymers resulted in CS/DNA NPs with improved transfection levels over unmodified CS/DNA NPs, with CS-PEG-Man, with a nearly 10-fold increase in transgene expression seen when comparing the optimal CS:TPP ratios of each NP formulation, being statistically significant. The improvements in transfection were somewhat unexpected, as the hypothesized benefits of adding Man and PEG were expected to only be perceived in vivo, where NPs will need to penetrate the mucus layer and avoid aggregation with serum components, the expected benefit of adding PEG, as well as specifically interact with mannose receptor-containing APCs, the expected result of adding Man. However, we hypothesize that improvements seen with CS-PEG and CS-PEG-Man NPs were the result of decreased interaction with serum components present within the cellular media, which allowed for improved cellular uptake. Additionally, the CS-PEG and CS-PEG-Man NPs showed much higher encapsulation efficiencies at lower CS:TPP ratios, as compared to CS NPs, which likely also contributed to the improvements in transgene expression. The most modest improvement was found with CS-Man, and while the improvement is not statistically significant, it at the very least demonstrates that CS-Man NPs are comparable to CS NPs and hold the potential to improve transfection when utilized in vitro in the presence of APCs.

CS/DNA NPs were also used to attempt to transfect RAW 264.7 macrophages and primary BMDCs. No transfection was observed in either with any of the CS/DNA NP formulations. The lack of transgene expression could be due to a number of factors. For one, macrophage cell lines and primary macrophages are notoriously hard to transfect, due in part to their nature as phagocytes that are highly efficient at degrading exogenous genetic material

[114]. Furthermore, though a similar study reported significant levels of transgene expression in RAW 264.7 with CS/DNA NPs, they formulated NPs with a mean diameter of 130 nm [106], while our particles are over twice as large, and another study demonstrated more than a two-fold increase in cellular uptake of particles when size was reduced from 250 nm to 150 nm [115]. With respect to the primary BMDCs, the above reasons may also have contributed to their lack of transfection, in addition to the fact that primary cell lines are generally much more difficult to transfect [116].

5. Conclusions

In this study, we have described the synthesis of CS oligosaccharide modified with Man, PEG, PEG-Man, and Phe as well as the formulation and characterization of CS/DNA NPs formed with the modified CS polymers. We successfully fabricated CS/DNA NPs with tunable size, charge and transfection efficiency and showed that the modified NPs could result in improved transfection levels in vitro. This study demonstrates the potential of these NPs to improve transgene expression in vivo following encapsulation within zein microparticles for use in an oral DNA vaccination application.

6. Future Directions

Future studies will further examine the use of CS-Phe in forming CS/DNA NPs by modifying the synthesis scheme. Additionally, the potential of modified CS NPs to improve transgene expression in vitro following encapsulation in zein microparticles and incubation in SGF and SIF will be examined. The encapsulation efficiency and size of CS-Zn-NIMs will be determined and cumulative release profiles determined. CS-Zn-NIMs formed with modified CS polymers will then be utilized in vivo to determine their ability to induce immune responses and the characterization of the induction of the immune response generated will be fully characterized.

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