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Chitosan-Based Particulate Systems for Non-Invasive Vaccine Delivery

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Abstract The use of particulate systems is considered very promising for the delivery of antigenic molecules via parenteral and non-parenteral routes. They provide improved protection and facilitated transport of the antigen as well as more effective antigen recognition by the immune cells, which results in enhanced immune responses. The natural cationic polysaccharide chitosan has been investigated extensively both as an adjuvant and delivery system for vaccines. It has been shown to enhance both humoral and cellular responses. From the formulation point of view, chitosan-based particulate systems offer advantages over the other polymers used by avoiding the harsh conditions of heat and/or organic solvents for encapsulation of the antigen. Furthermore, versatility in the physicochemical properties of chitosan provides an exceptional opportunity to engineer antigen-specific adjuvant/delivery systems. In this review, the importance of chitosan in particulate systems for vaccine delivery will be emphasized according to administration routes, particularly focusing on non-invasive (needle-free) routes including oral, mucosal and pulmonary mucosae as well as skin.

Keywords Adjuvant · Chitosan · Needle-free · Particulate systems · Vaccine

Contents

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1 Introduction

In the area of vaccine development, the crucial concern is to balance safety with efficacy. Most of the vaccines involve killed or attenuated microorganisms (bacteria, viruses, fungi, etc.) or chemically detoxified toxins (toxoids) from bacteria. Killed bacterial or viral vaccines often have residual toxicity following inactivation and might contain toxic components, such as lipopolysaccharides [1]. Therefore, despite their efficacy, these type of vaccines exert serious safety issues. Well-defined, immunogenic fragments of pathogens, known as subunit antigens (such as purified protective proteins or carbohydrates), provide a much cleaner, safer and more immunologically defined alternative to live or killed whole cell vaccines. However, these subunit vaccines are weakly immunogenic on their own, hence incorporation of an adjuvant is required to enhance their ability to evoke effective immune responses [2–5]. Adjuvants (the word coming from the latin word *adjuvare*, which means to help or to enhance) are defined as molecules, compounds or macromolecular complexes that boost the potency and longevity of a specific immune response to antigens, causing only minimal toxicity or long-lasting immune effects on their own [6]. Adjuvants act by a diverse series of pathways, which may involve changing the properties of the antigen to provide a slow-release antigen depot that targets innate immune pathways to selectively activate specific pathways of immunity [7–11]. The mechanism of action of an adjuvant is mainly either as an immunostimulant or as a delivery system [12]. In spite of the intense studies on adjuvant strategies, adjuvant toxicity still remains the major limitation to be surpassed.

Most vaccines today are given by parenteral injection, which stimulates the immune system to produce antibodies in the serum but fails to generate a mucosal antibody response [13]. In general, topical application of vaccine is required to induce a protective immune response. The use of vaccines that induce protective mucosal and dermal immunity thus becomes attractive when one considers that most infectious agents come into contact with the host at these surfaces. Furthermore, immunization at one mucosal site can induce specific responses at distant sites. The search for needle-free (non-invasive) delivery of vaccine not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. Hence in recent years, needle-free administration, which is possible via oral, nasal, pulmonary and dermal routes, has become an alternative to parenteral route [14, 15]. However, poor immunogenicity and impaired antigen delivery with these systems still remains to be improved. For this purpose, particulate systems have been investigated as the appropriate vaccine formulations to target topical inductive sites and appropriately stimulate the innate system in order to generate effective adaptive immunity as well

as to prevent the antigen from physical elimination and enzymatic degradation. From the formulation point of view, needle-free vaccination also offers several benefits over parenteral routes, including ease and speed of administration, possibility of self-administration, reduced side effects, reduced risk of infection and disease transmission, improved safety and compliance, and reduced costs.

When all the limitations mentioned above are taken into consideration, chitosan seems to be a very promising material for vaccine delivery, both as an adjuvant and as a delivery system due to its numerous favorable properties such as bioadhesivity, biocompatibility, immunostimulating activity, and mucosal penetration enhancing effect [12, 13]. Furthermore, its positive charge and ability to form particulate systems without requiring harsh conditions of heat and/or organic solvents for encapsulation of antigen offer advantages over other polymers used for this purpose.

In this paper after explaining why particulate systems are preferred for vaccine delivery, a brief introduction to chitosan will be given, and the importance of chitosan in particulate systems for vaccine delivery will be emphasized according to administration routes, particularly focused on needle-free routes including, oral, mucosal and pulmonary mucosa as well as skin. For comparison, examples on parenteral route will also be mentioned.

2 Particulate Systems for Vaccine Delivery

Particulate carrier systems such as immune-stimulating complexes (ISCOMs) [16–18], liposomes [3, 19], and polymeric micro/nanoparticles [20–27] have been proven to be highly efficacious for antigen delivery. These particles, due to their similar size to the pathogens that invade the host, can be efficiently internalized by antigen-presenting cells (APCs). The uptake of microparticles (<10 μm) by phagocytic cells has been well recognized, and uptake into APCs is likely to be important in the ability of particles to perform as vaccine adjuvants. Microparticles are phagocytosed by a variety of cells including macrophages and dendritic cells (DCs) [28–30]. Once taken up by these cells, antigen is released and subsequently selected for presentation via major histocompatibility complex (MHC) II. Phagocytosis of microparticles and presentation of incorporated antigens have been reported to be strongly influenced by the chemical and physical nature of the particulate system. Cationic microparticles have been shown to be particularly effective for uptake into macrophages and DC. This is attributed to the enhancement of binding to the negatively charged cell surface by the positively charged particle, which subsequently sets off internalization into the cell [31]. Particulate systems have been shown to create a depot effect that helps to increase the persistence of antigens for a longer time, which is important for the induction of efficient protective T-cell responses [32].

Various biodegradable polymers, including polyanhydrides, polyorthoesters, hyaluronic acid, alginate, chitosan, and starch as well as poloxamers (Pluronics), which self-assemble into particulates, and polyphosphazenes have been investigated

as vaccine delivery systems. Other adjuvants can also be incorporated into these systems to improve the immune response. With most of the polymers mentioned above, use of organic solvents is inevitable for particle preparation. Additionally, during the manufacturing process of the particles, the antigen may also be exposed to high shear stress and elevated temperatures. Hence, although these systems have advantage over other dosage forms in enhancing the immune response, there are limitations such as inefficient incorporation, limited antigen release, stability and integrity of the antigen during the formulation process as well as after administration or storage. The degradation and denaturation of proteins during encapsulation has been avoided by adsorbing the antigen onto the surface of the microparticles instead of encapsulating it [33]. Furthermore, there are potential limitations associated with regulatory and industrial issues related to the components of these systems, and also higher cost compared to other formulations [34].

It is well documented that the polymers used and formulation properties affect the success of the particulate systems for vaccine delivery [35]. Recently, Mohanan et al. [36] investigated the influence of the administration route of particulate systems on the type and strength of immune response elicited following immunization of mice by different routes, such as the subcutaneous, intradermal, intramuscular, and intralymphatic routes. Three delivery systems were used: ovalbumin-loaded liposomes, *N*-trimethyl chitosan (TMC) nanoparticles, and poly (lactide-*co*-glycolide) (PLGA) microparticles, all with and without specifically selected immune-response modifier (trehalose 6,6′-dibehenate, lipopolysaccharide, CpG). The route of administration was found to cause only minor differences in inducing an antibody response of the IgG1 subclass associated with Th2-type immune responses, and any such differences were leveled out after boosting. However, the administration route strongly affected both the kinetics and magnitude of the IgG2a response associated with Th1-type immune responses. A single intralymphatic administration of each of the delivery systems was found to induce a robust IgG2a response, whereas subcutaneous administration failed to elicit a substantial IgG2a response even after boosting, except with the adjuvanted nanoparticles. The intradermal and intramuscular routes generated intermediate IgG2a titers. It was suggested that all immunization routes mediated efficient drainage of all three delivery systems from peripheral non-lymphoid tissues to lymphoid organs, where strong immune responses were mounted against the antigen. No direct comparison was made between the three different formulations, stating that these systems differ in their chemistry, size, particle architecture, degradation rate, and erosion and antigen release kinetics.

3 Chitosan

Chitosan is a cationic polymer derived from chitin obtained from crustacean and insect skeletons. Structurally, it is a linear polysaccharide consisting of β (1-4)-linked D-glucosamine with randomly located *N*-acetylglucosamine groups

depending upon the degree of deacetylation of the polymer. The degree of acetylation represents the proportion of *N*-acetyl-D-glucosamine units with respect to the total number of units and can be employed to differentiate between chitin and chitosan. Chitin with a DD of 65–70% or above is generally known as chitosan. The DD is an important property of chitosan and defines its physicochemical and biological properties, hence determining its applications. Despite being the most abundant polymer in nature after cellulose, the utilization of chitin has been restricted by its intractability and insolubility. Chitosan has been favored more in a wide range of application owing to its better solubility and also to its free amine groups, which are an active site in many chemical reactions. Chitosan is readily soluble in dilute acidic solutions below pH 6.0. With increasing pH, the amino groups become deprotonated and the polymer loses its charge and becomes insoluble. The solubility of chitosan is dependent on the DD and the method of deacetylation used since the pK_a value is highly dependent on the degree of N-acetylation [37].

The positive surface charge of chitosan allows it to interact with macromolecules like exogenous nucleic acids, negatively charged mucosal surfaces, or even the plasma membrane [38, 39]. Chitosan is degraded by enzymes such as chitosanase and lysozyme. The rate of degradation of chitosan inversely depends on the degree of acetylation and crystallinity of the polymer. The highly deacetylated form exhibits the lowest degradation rates and may last several months in vivo. It is still not clearly known what the mechanism of degradation is when chitosan is injected intravenously. It has been reported that distribution, degradation, and elimination processes are strongly dependent on molecular weight and DD [40].

It is possible to prepare different forms of formulations such as aqueous dispersions, gels, sponges and micro/nanoparticles using chitosan and its derivatives. Furthermore, versatility in the physicochemical properties of chitosan allows the formulator an excellent opportunity to engineer antigen-specific adjuvant/delivery systems.

3.1 Mode of Action

Chitosan has been shown to induce both cellular and humoral responses when administered via parenteral, mucosal, or transcutaneous routes [41–43]. Various studies have demonstrated the activation of the DCs, macrophages, and lymphocytes by chitosan [44]. Maeda and Kimura [45] have reported that the presence of chitosan caused enhancement of the natural killer (NK) cell activity in intestinal intraepithelial lymphocytes and splenic lymphocytes. The NK activity of intraepithelial lymphocytes (IELs) or splenic lymphocytes treated with low molecular weight chitosan (LMWC; 21 and 46 kDa) was found to be stronger than that of lymphocytes treated with high molecular weight chitosan (HMWC; 130 and 650 kDa). In addition, IELs or splenic lymphocytes treated with LMWC were found to enhance the cytotoxic activity against sarcoma 180 cells. Hence, variations in

molecular weight or DD of chitosan can lead to different degrees of activation in cells from the immune system.

The uptake and distribution of chitosan, the phenotype of recruited APCs, the induction of cytokines tumor necrosis factor- α (TNF-α), interleukin (IL)-12, IL-4, IL-10, and transforming growth factor (TGF-β), and the activation of T lymphocytes were investigated in rats after oral feeding [46]. The orally administered chitosan in the absence of an antigen was found to enhance a naturally Th2/Th3-biased microenvironment at the mucosal level by stimulating the production of regulatory cytokines. McNeela et al. [47] showed in human as well that chitosan selectively enhanced the induction of Th2 cells following intranasal immunization with the genetically detoxified diphtheria toxin.

Recently, the effects of chitosan oligosaccharides (COS) with different polymeri zation degree (3–7 and 7–16) on the activation of spleen CD11c-positive ($CD11c^+$) dendritic cells (SDCs) and the role of Toll-like receptor 4 (TLR4) in this process was investigated by Dang et al. [48]. It was shown that SDCs could be induced to a mature state, secrete TNF-α, and promote the proliferation of $CD4^+$ T cells by B-COS. TLR4 may play a critical role in this process. The biological activity of COS was suggested to be dependent on the molecular size or polymeration degree of COS. But, the mechanisms of B-COS recognition and the B-COS-related signaling pathway have not yet been described.

The immunostimulating activity of chitosan and its derivatives have also been investigated in combination with other adjuvants. Combinations of chitosan and its trimethyl derivative with LTK63 mutant, which is a mucosal adjuvant, were investigated in an effort to enhance the immunogenicity and protective efficacy of the CRM-MenC conjugate vaccine given intranasally to mice [49, 50]. At very low doses of the LTK63 adjuvant, high bactericidal antibody titers were induced only in the presence of TMC. The quality of this protective immune response was reported to be modulated depending on the appropriate dosing of the mucosal adjuvants.

In recent years, new information about the functions of immunomodulatory cytokines and the discovery of TLRs have provided promising new alternatives for adjuvants [51]. Based on the delivery potential of chitosan and its compatibility with IL-12, Heffernan et al. [52] combined chitosan and IL-12 as an adjuvant system for subcutaneous administration of a model antigen, ovalbumin (OVA). The chitosan/IL-12/OVA vaccine was found to elicit greater antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, as determined by $CD4^+$ splenocyte proliferation, Th1 cytokine release, $CD8^+$ T-cell interferon-γ release, and MHC class I peptide pentamer staining. The IgG2a and IgG2b antibody responses to OVA were also enhanced with a combination of chitosan and IL-12. Various TLR ligands including CpG-containing oligonucleotides, which have shown adjuvant activity when administered mucosally, were combined with chitosan. Recombinant hepatitis B surface antigen (HBsAg)-loaded nanoparticles were associated with Class B CpG ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′) [74] . The generation of Th1-biased antigen-specific systemic antibodies was observed only when HBsAg-loaded nanoparticles were applied together with Class B CpG ODN.

Sometimes, conflicting results have been reported by different groups on stimulatory activity of chitosans; however, this is probably due to the differences in experimental conditions or parameters evaluated. Besides its immunostimulating activity, the bioadhesive and penetration enhancing properties of chitosan also contribute to the immune system stimulation by enhancing paracellular absorption at the mucosal site and allowing increased uptake of antigens [26, 53].

In the following sections, the application of chitosan-based particulate systems for vaccine delivery will be reviewed according to the administration routes (mucosal, dermal and parenteral) after giving a brief introduction to each route.

4 Mucosal Delivery

Mucosal immunization has been focused on oral, nasal, and aerosol vaccines. The use of vaccines that induce protective mucosal immunity becomes very attractive when one considers that most infectious agents come into contact with the host at mucosal surfaces. The mucosal immune system, which protects the host from pathogenic microorganisms at mucosal surfaces, is an integrated network that permits communication between the organized lymphoid tissues (inductive sites) and the diffuse mucosal tissues (effector sites) [54, 55]. Through innate and adaptive immunity, the mucosal immune system maintains immunological homeostasis along the vast expanse of the epithelial surface area, including oral and nasal cavities, respiratory, intestinal and genito-urinary tracts. The initiation of mucosal immune responses occurs with the transport of antigen through specialized epithelial cells (M cells) that are present in the organized mucosa-associated lymphoid tissues (MALT) (including Peyer's patches, mesenteric lymph nodes, solitary follicles in the intestine, appendix, tonsils and adenoids). After antigens are taken up via M cells, they are entrapped by APCs (DCs, B lymphocytes and macrophages) and presented to $CD4^+$ and $CD8^+$ T cells [56]. Upon sensitization by the antigens, B cells proliferate and switch to IgA-committed cells. These B cells eventually leave the MALT and migrate through the systemic circulation to various mucosal sites, including the initial induction site for terminal differentiation to sIgA-producing plasma cells. Recent evidence shows that TLRs recognize specific patterns of microbial components, especially those from pathogens, and regulate the activation of both innate and adaptive immunity. TLRs are membrane bound pattern recognition receptors (PRRs) responsible for detecting most antigen-mediated infections [57]. There are at least 13 different forms of TLR, each with its own characteristic ligand.

The oral route would be considered to be the most suitable route for mucosal immunization with regard to the ease and acceptability of administration. However, the acidic pH as well as the presence of the digestive enzymes limits the antigen's access to the mucosal epithelium, which makes oral immunization complicated. Hence, over the past two decades, new strategies for design of delivery systems and adjuvants have been investigated with the aim of enhancing immune responses

based on entrapping or adsorbing the selected potent antigens into a variety of micro- and nanoparticulate systems in the absence or presence of another adjuvant [58, 59]. Such particles can protect the antigens from degradation in the stomach and intestine, and deliver them efficiently to the gut-associated lymphoid tissue (GALT) located in the lower portion of the small intestine. When such microparticles reach the Peyer's patches in the GALT, they can be taken up by M cells. However, the ability of microparticles to perform as adjuvants or delivery systems following oral mucosal administration has not been very encouraging in either animals or human [60]. Due to these limiting issues in oral vaccination, nasal mucosa, which does not have the issues of instability in the gastrointestinal region and permits lower doses to be used, has been investigated as an alternative route for mucosal immunization [61–63]. The formulation of antigens in particulate delivery systems for intranasal administration protects the antigen from mucosal enzymes and facilitates the preferential uptake of the antigen by specialized M cells of the nasal-associated lymphoid tissue (NALT) [34]. Nasal immunization with particulate systems has provided more encouraging data than oral immunization thus far in both animal and human studies. Targeting of antigens to alveolar macrophages has also been explored using the pulmonary route [64]. Lungs are highly vascularized, have a large absorptive surface area because of the alveoli structure, and contain bronchoalveolar lymphoid tissue. Furthermore, local APCs are ideally located for antigen sampling and subsequent presentation to T cells. Pulmonary vaccination has also the advantage of inducing both systemic and local immunity (IgA and IgG) in the respiratory tract. However, for pulmonary protein delivery, poor deposition of protein formulations at the alveoli (the absorption site) is still a major limitation [65].

4.1 Oral Delivery

Due to the limitations with the gastrointestinal tract, there are few studies available on chitosan-based delivery systems for oral vaccine delivery (Table 1). Van der Lubben et al. [66] were among the first to demonstrate that chitosan microparticles with a particle size smaller than 10 μm, incorporated with the model protein OVA as well as diphtheria toxoid (DT), were taken up by the Peyer's patch after intragastric administration to mice. A dose-dependent immune reaction was observed for mice vaccinated with different doses of DT associated to chitosan microparticles [67]. It was also observed that the immune response started only 1 week after the boosting, indicating the formation of memory cells after priming.

Chitosan formulations administered mucosally have been shown to enhance tolerance induction in murine models of allergy. Besides protecting against antigenic entry to the systemic immune system, mucosal immune system also provides to be unresponsive to food antigens. Chitosan/DNA nanoparticles carrying the gene for a principal peanut allergen, Arah2 were prepared for oral immunization and their efficacy in modulating antigen-induced hypersensitivity in a murine model of peanut allergy was demonstrated [68]. Oral administration using chitosan/DNA

Table 1 Chitosan-based particulate systems for oral, dermal and parenteral delivery of vaccines

Antigen	Dosage form	Immunization route	Chitosan type	Adjuvants	Animal model	Immune response	References
Ovalbumin Diphteria toxoid	Microparticles 4.3 ± 0.7 μm	Oral	Chitosan (DD 93%) (Primex, Avaldsnes, Norway)	–	Female BALB/c mice	Taken up by the epithelium of the murine Peyer's patches; protective systemic and local immune response against diphteria toxoid	[66, 67]
DNA	Nanoparticles 150–300 nm	Oral	Chitosan (MW 390 KDa)	–	AKR/J mice	Modified immune system and protection against food allergen-induced hypersensitivity	[68]
Der p1. DNA	Nanoparticles 506.9 ± 9.7 nm	Oral	Chitosan C390 (MW 390 kDa), (DA 83.5%) (Vanson, Redmond, WA)	–	6- to 8-week-old female BALB/cJ mice	Increased immune responses	[69]
Ovalbumin	Microparticles 1–3 μm and 300–800 nm	Sublingual	Chitosan (310–375 kDa and 190–310 kDa), (references 419419 and 448877, Sigma)	–	BALB/c mice	Enhanced tolerance induction only with highly positively charged microparticles	[70]

(*continued*)

Table 1 (continued)

Antigen	Dosage form	Immunization route	Chitosan type	Adjuvants	Animal model	Immune response	References
Plasmid DNA	Chitosan oligomer/CMC nanoparticles 93–270 nm Chitosan/CMC nanoparticles 180–650 nm	Intradermal	Chitosan (MW 102 kDa; DD 80%) (Natural Biopolymer, Raymond, WA)	–	BALB/C mice	Quantifiable levels of luciferase expression in skin after 24 h; signicant antigen-specific IgG titer to expressed β-galactosidase 28 days after the first application	[42]
Diptheria toxoid Ovalbumin	Nanoparticles 100 nm Solution	Transcutaneous	TMC (quaternization: 15% and 30%)	– Before and after microneedle array used	Female BALB/c mice	Strong IgG and neutralising antibody titres No effect observed with microneedle application or type of microneedle array	[71, 72]
DNA-based respiratory syncytial virus (RSV)	Nanoparticles 80–150 nm	Parenteral (intravenous)	Chitosan	–		Higher level of RSV protein expression in mouse tissues	[73]
Hepatitis B surface antigen (HBsAg)	Alginate-coated chitosan nanoparticles 643 ± 171.7 nm	Parenteral (subcutaneous)	Chitosan (DD 95%) (Primex BioChemicals AS, Avaldsnes, Norway)	Class B, CpG ODN (1826) (5′-TCC ATG ACG TTC CTG ACG TT-3′)	7- week-old female BALB/cAnNHsd mice	High anti-HBsAg IgG titer, majority of antibodies being of Th2 type No significant differences in antigen-specific	[74]

						splenocyte proliferation and in secretion of IFN-γ and IL-4 Increased IgG2a/IgG1 ratio in presence of the adjuvant Increased IFN-γ production	
Ag85B–MPT64–Mtb8.4 (AMM) from *Mycobacterium tuberculosis* genes	Microparticles 5.78±0.65 μm	Parenteral (subcutaneous)	Chitosan (DD 85%) (Sigma–Aldrich, USA)	Incomplete Freund's adjuvant	6-week-old female C57BL/6 mice	Higher levels of IFN-γ Higher levels of Ag85B-specific IgG(H+L), IgG1 and IgG2a	[75]

DD degree of deacetylation, *TMC* trimethylated chitosan, *CMC* carboxymethylcellulose, *IFN-γ* interferon-γ, *IL* interleukin

nanoparticles for raising specific antibodies to native Der p1, which is regarded as the most important *Dermatophagoides pteronyssimus* allergen and is a major triggering factor for mite allergy worldwide, was investigated in mice [69]. The nanoparticle formulations were shown to prime Th1-skewed immune responses against both domains of Der p1. It was suggested that by resorting to an oral route of DNA delivery, it was possible to induce Der p1-specific immune responses that were undetectable using a parental route of immunization.

Chitosan has also been investigated for sublingual immunotherapy (SLIT), which is a noninvasive and efficacious treatment of type I respiratory allergies. Two types of chitosan microparticles with distinct size and charge characteristics were investigated for their ability to facilitate allergen uptake by various murine APC populations as well as to enhance allergen-specific tolerance when administered sublingually to OVA-sensitized mice with asthma [70]. Animals were treated sublingually with soluble or chitosan-formulated OVA twice a week for 2 months. Airway hyperresponsiveness (AHR), lung inflammation, and T-cell responses in cervical and mediastinal lymph nodes (LNs) were assessed. Only highly positively charged (which had the highest mucoadhesivity as well) and microparticulate form of chitosan was found to enhance OVA uptake, processing, and presentation by murine bone marrow DCs and oral APCs. Targeting OVA to DCs with this formulation increased specific T-cell proliferation and interferon γ (IFN-γ)/IL-10 secretion in vitro, as well as T-cell priming in cervical LNs in vivo. Sublingual administration of such chitosan-formulated OVA particles was reported to enhance tolerance induction in mice with established asthma, with a dramatic reduction of both AHR, lung inflammation, eosinophil numbers in bronchoalveolar lavages, as well as antigen-specific Th2 responses in mediastinal LNs. Due to both its mucoadhesive property and availability to form particles with different properties (size, charge), chitosan was suggested to be very promising for sublingual immunization. These results indicated that chitosan can mediate an adjuvant effect by itself that is dependent on the type and properties of the chitosan used as well as the route of administration.

4.2 Nasal Delivery

There are numerous studies demonstrating that chitosan and its derivatives enhance the immune response upon nasal immunization [12, 34]. Recent studies on chitosan-based particulate systems for nasal immunization are summarized in Table 2. The immune response has been shown to be affected by the particle size, DD, and molecular weight of the chitosan used. We have prepared nanoparticulate systems using differently charged chitosan derivatives, TMC (polycationic) and mono-*N*-carboxymethyl chitosan (MCC, polyampholytic), for nasal immunization [95]. This was the first time the negatively charged chitosan derivative, MCC, was investigated for mucosal immunization. Enhanced immune responses were obtained with intranasal (i.n.) application of tetanus toxoid (TT)-loaded nanoparticle formulations

Table 2 Chitosan-based particulate systems for nasal delivery of vaccines

Antigen	Dosage form	Chitosan type	Adjuvants	Animal model	Immune response	References
Bacillus anthracis PA and PA-conjugate	ChiSys Dry powder	Protasan UP G 213 (MW 200–600 kDa; DD 75–90%; Novamatrix, Norway)	MPL	Female New Zealand white rabbits	Induction of IgG levels	[76]
Recombinant Anthrax protective antigen (rPA)	ChiSys Dry powder	Protasan UP G 213 (MW 200–600 kDa; DD 75–90%; Novamatrix)	MPL	Female New Zealand white rabbits	Induction of IgG levels	[77]
BBD	Microspheres 0.29–5.3 μm	Chitosan (MW 10, 100 and 300 kDa; DD 90.8%; Jakwang, Korea)	–	BALB/c female mice	Induction of IgG titer in serum Induction of IgA in nasal wash	[78]
BBD	Microspheres 1.94–5.21 μm	Chitosan (MW 10 kDa; DD 80.4%; Jakwang)	Pluronic F127	Mice	Induction of IgG and IgA levels in serum, saliva and nasal wash Significant TNF-α values	[79]
BBD	Microspheres 5–6 μm	Water-soluble chitosan (MW 100 kDa; DD 95.4%; Sunchon National University) Mannosylated chitosan	–	Female mice	Induction of IgG and IgA levels in serum, saliva and nasal wash	[80]
BBD	Microspheres	Chitosan (MW 10 kDa; DD 80.4%; Jakwang)	–	Colostrum-deprived pigs	Induction of IgG and IgA levels in serum, IgA levels in nasal wash	[38]
FMDV DNA antigen	Nanoparticles 255 nm	Chitosan (MW 170 kDa; DD >85%; Dalian Xindie Chitin, China)	Provax IL-15	Female BALB/c mice, guinea pigs	Induction of IgG levels in serum, IgA levels in lung and vaginal wash Induction of cellular immune response (IL-4 and IFN-γ in $CD4^+$ cells and IFN-γ in $CD8^+$ T cells)	[81]
Plasmid DNA HbsAg	Glycol chitosan-coated liposomes 775 nm	Glycol chitosan-(MW 250 kDa; DD 88%; Sigma, USA)	–	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal, saliva, vaginal wash	[82]

(*continued*)

Table 2 (continued)

Antigen	Dosage form	Chitosan type	Adjuvants	Animal model	Immune response	References
					Induction of cellular immune response (IL-2 and IFN-γ)	
Plasmid DNA HbsAg	Nanoparticles 300–400 nm	Chitosan (MW 400 kDa; DD 85%; Fluka, Switzerland)	–	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal, saliva, vaginal wash Induction of cellular immune response (IL-2 and IFN-γ)	[83]
Recombinant HbsAg	Alginate-coated chitosan nanoparticles 300–600 nm	Chitosan (DD 95%; Primex BioChemicals)	CpG ODN	Female BALB/cAnNHsd mice	Induction of IgA levels in feces, nasal and vaginal wash Induction of cellular immune response (IFN-γ) Induction of Th2-type immune response (IgG1/IgG2a > 1)	[74, 84]
Plasmid DNA Hepatitis B core antigen	Nanoparticles 340–380 nm	Chitosan (MW 173 kDa; Shanghai KABO Trading Co., China)	–	Male New Zealand white rabbits	Induction of serum IgG levels	[85]
Inactivated influenza virus	Nanoparticles	Chitosan (DD 17%; MW 23 kDa, 43 kDa; Primex) TMC		Female C57-BL/6 mice (Charles River)	Induction of stronger total IgG, IgG1 and IgG2a/c responses	[86]
Inactivated influenza virus	Dry powder <100 μm	Chitosan (MW 161 kDa; DD 92%; Vanson HaloSource, USA)	–	Female brown Norway rats	Induction of IgG levels in serum and IgA levels in nasal wash	[87]
Subunit influenza antigen	Nanoparticles 850 nm	Chitosan (MW 177 kDa; DD 93%; Primex); TMC	–	Female C57BL/6 (B6) mice	Induction of Th2 type immune response (IgG1/IgG2a > 1) Induction of IgA immune response in nasal wash	[88]
CRM197	Microparticles or powder 4.5 μm (chitosan) 2.5 μm (TMC)	Chitosan (DD 94.5%; Primex) TMC	LTK63	Female BALB/c mice	Induction of IgG levels in serum and IgA levels in nasal and vaginal wash	[50]

MCP-CRM197	Powder	Chitosan glutamate Protasan UP G 213 (Novamatrix)	–	Female and male healthy volunteers	Induction of IgG2, IgG1, IgG4 Induction of IgA response in nasal wash	[89]
Tetanus toxoid	Nanoparticles Chitosan 300–400 nm MCC 40–90 nm TMC 300–400 nm	Chitosan glutamate Protasan UP G 113 (MW 150 kDa; DD >75–90%) (Novamatrix, Norway) MCC, TMC	–	BALB/c mice	Induction of Th2-type immune response (IgG2a/IgG1 < 1)	[95]
Tetanus toxoid	Nanoparticles 280 nm	MCC-TMC	–	BALB/c mice	MCC-TMC nanoparticles induced significantly higher immune responses than TMC, MCC, or chitosan nanoparticles	[90]
Bovine herpes virus	Microparticles 35–50 μm	Protasan UP Cl213 (Novamatrix, Norway) Chitopharm S, Chitopharm M, Chitopharm L (Cognis, Germany)		In vitro cell culture	Infectivity decreased with increasing molecular weight of chitosan	[91]
Ovalumin	Nanoparticles 380 nm	TMC	CpG DNA (ODN 2006)	Female BALB/c mice	Replacing TPP by CpG as acrosslinking agent modulated the immune response towards a Th1 response after nasal vaccination	[92]
Ovalbumin	Nanoparticles 450 nm (TMC-PLGA) 280 (TMC)	TMC TMC-coated PLGA	–	Female BALB/c (nu/nu) mice	TMC nanoparticles superior over PLGA and PLGA/TMC nanoparticles	[93]
Recombinant HbsAg	Nanoparticles 160–200 nm	Protasan UP Cl 113 (MW 125 kDa; DD 14%; Novamatrix)	–	Female BALB/c mice	Induction of anti-HBsAg IgG levels Prolonging antibody response	[94]

BBD Bordetella bronchiseptica dermonecrotoxin, *CpG* trehalose 6,6′-dibehenate, lipopolysaccharide, *CRM* cross-reacting material, *DD* deacetylation degree, *FMDV* foot and mouth disease virus, *HbsAg* hepatitis B surface antigen, *IFN-γ* interferon, *IL* interleukin, *MCC* mono-*N*-carboxymethyl chitosan, *MCP* meningococcal C-polysaccharide, *MPL* monophosphoryl lipid A, *MW* molecular weight, *ODN* oligodeoxynucleotide, *PA* protective antigen, *PLGA* poly (lactic-*co*-glycolic acid), *TMC N*-trimethyl chitosan, *TPP* tripolyphosphate

in BALB/c mice. Chitosan and TMC nanoparticles (300–400 nm), which have positively charged surfaces, induced higher serum IgG titres compared to those prepared with MCC, which are negatively charged and smaller in size (40–90 nm). The aqueous dispersions of chitosan, TMC, and MCC incorporating TT were also investigated in vivo. Significantly higher immune responses were obtained with aqueous dispersions following intranasal application compared to that of TT solution in phosphate-buffered saline, whereas with subcutaneous administration, no enhancement in immune response was observed. This is attributed to the bioadhesive property inherent to chitosan that prolong the contact time of the formulation on the nasal mucosa, thereby leading to higher antibody titres. Both dispersion and nanoparticle systems prepared by chitosan derivatives were found to enhance mucosal immune responses. Certainly, encapsulation of TT into the nanoparticles would bring advantages over a simple aqueous dispersion, especially in protecting the antigen from the potential undesired environment at the nasal surface and preventing the loss of the antigen before reaching the target M cells within the delivery system. Moreover, the retention time of the nanoparticles, which was longer than that of the solutions, would allow efficient uptake of antigen through the mucosa thus improving the immune response. Our results showed that the nature of the surface charge and particle size exert an important role in obtaining an enhanced immune response using carriers prepared with chitosan. We have also developed nanoparticles by means of TMC and MCC complexation without using any crosslinker, and also expected to enhance the immunostimulant effect by using two chitosan derivatives in the same formulation [90]. TT was incorporated into the nanoparticles (280 nm) and the immune responses were investigated after nasal immunization in mice model. The TMC/MCC complex nanoparticles were found to induce significantly higher immune responses than the nanoparticles prepared with TMC, MCC, or chitosan alone. These results indicated that the developed TMC/MCC nanoparticles with very mild processing and high loading efficiency, maintaining the protein integrity, are very promising both as an adjuvant and as a delivery system for antigens.

Recently, we have prepared chitosan microparticles for mucosal delivery of bovine herpes virus (BHV-1), which is a major pathogen of cattle causing serious infections including infectious bovine rhinotracheitis (IBR)/infectious pustular vulvovaginitis (IPV) and is still a big threat in Turkey [91]. The integrity and antigenicity of the virus incorporated into microparticles (35–50 μm) prepared with various types of chitosan with different molecular weight and solubility were evaluated through direct immunofluorescent staining in which the virus infected cells were fluorescently labeled with BHV-1-specific FITC antibody conjugate. The results showed that the solubility of chitosan did not have any significant effect on integrity and potency of the antigen, whereas the infectivity decreased with increasing molecular weight of chitosan. Cytopathic effect (CPE) plaques after inoculation with BHV-1 loaded microparticles were observed in 16 h with water soluble chitosan particles whereas with base chitosan particles the post-infection was observed in longer period of time (LMWC 23 h; medium and HMWC 42 h].

Vila et al. [96] have also investigated the immune responses of nanoparticles prepared using different molecular weight chitosans. The mode of action of chitosan nanoparticles loaded with TT was reported not to be affected significantly by the chitosan molecular weight. Greater response was observed with LMWC particles at early times (12 weeks), and for HMWC at late times (24 weeks). This was attributed to the different release rate of TT from LMWC and HMWC formulations.

The potential of LMWC (5 and 8 kDa) for DNA vaccine delivery via nasal mucosa was evaluated in vitro and in vivo [97]. The in vitro transfection efficiency of chitosan/DNA polyplexes were investigated, and the LMWC was found to have lower binding affinity to DNA and mediated higher transfection efficiency. The capabilities of the polyplexes based on LMWC to elicit serum IgG antibodies and to attenuate the development of atherosclerosis after intranasal vaccination were compared with the polyplexes based on HMWC (32, 173, and 425 kDa) in a rabbit model. Intranasal vaccination with LMWC/DNA polyplexes could elicit significant systemic immune responses, modulate the plasma lipoprotein profile, and attenuate the progression of atherosclerosis. Those aspects were comparable to those obtained by HMWC/DNA polyplexes. The LMWC/DNA polyplexes were shown to remain stable on the nasal mucosa, and were internalized by nasal epithelial cells, which was similar to the case of HMWC/DNA polyplexes.

Microspheres using different molecular weight chitosans were prepared for nasal delivery of *Bordetella bronchiseptica* dermonecrotoxin (BBD), a major virulence factor of a causative agent of atrophic rhinitis in pigs [78]. The highest BBD release was obtained with lower molecular weight of chitosan and at higher pH. TNF-α and nitric oxide from RAW264.7 cells exposed to BBD-loaded chitosan microspheres (BBD-CMs) were gradually secreted with time, showing immunostimulating activity in vitro. Furthermore, the effect of Pluronic F127-associated microparticles on immunostimulating activity was investigated in mice [79]. Protective immunity was measured by survival rate after challenge with *B. bronchiseptica* via the nasal cavity. The survival rate of the group treated with BBD-CMs/F127 was reported to be higher than those of the other groups. Pluronic F127-associated chitosan microparticles were suggested as an efficient adjuvant for nasal delivery of BBD.

For influenza vaccines, nasal administration has been regarded as a good alternative to parenteral injection because of the enhancement of the mucosal immune response and the ease of vaccine administration. Chitosan and its derivatives have also been investigated for nasal delivery of influenza virus. In a study performed in humans, standard inactivated trivalent influenza vaccine with chitosan was administered nasally and intramuscularly [98]. Although the induction of mucosal antibodies was not investigated, the vaccine was reported to be safe and well tolerated by the subjects. Overall, the geometric mean serum HI antibody titres induced by the vaccine were reported to meet the regulatory requirements, and the seroconversion rate and protective antibody levels achieved were not statistically different from those for saline vaccines given intramuscularly.

Intranasal vaccination studies with whole inactivated influenza virus (WIV) adjuvanted with *N,N,N*-trimethylchitosan (TMC-WIV) have shown promising

results [86] in mice. No significant differences in the nasal residence time between WIV and TMC-WIV were observed. However, a remarkable difference in the location and distribution of WIV in absence and presence of the TMC was observed, which was correlated with the observed differences in immunogenicity of these two formulations. TMC-WIV was reported to allow a much closer interaction of WIV with the epithelial surfaces, potentially leading to enhanced uptake and induction of immune responses. Both WIV and TMC-WIV formulations were shown to induce minimal local toxicity.

In a study performed in humans, *Neisseria meningitidis* serogroup C polysaccharide (MCP)-CRM197 conjugate vaccine mixed with chitosan was administered in two nasal insufflations 28 days apart and the effects compared to those of intramuscular injection with alum. Nasal immunization was reported to be well tolerated, with fewer symptoms reported than after intramuscular injection. Increases in CRM197-specific IgG and diphtheria toxin-neutralizing activity were observed after nasal or intramuscular immunization, with balanced IgG1/IgG2 and higher IgG4. Significant MCP-specific secretory IgA was detected in nasal wash only after nasal immunization and predominantly on the immunized side [89].

Recently, in another study performed in humans, intranasal vaccine against norovirus infection (known as the "stomach flu") incorporated in a dry powder formulation based on virus-like particle (VLP) antigens including Monophosphoryl Lipid A (GlaxoSmithKline) and chitosan was reported to show immunogenicity in clinical Phase I studies [99].

4.3 Pulmonary Delivery

Immunization by the pulmonary route in order to establish a mucosal as well as systemic immune response against airborne pathogens is an emerging field [100, 101]. The effect of chitosan particles as vaccine carriers in endotracheal immunization has been investigated by several groups (Table 1). An HLA-A2 transgenic mouse model was used to investigate the effects of pulmonary delivery of a DNA vaccine encoding HLA-A*0201-restricted T-cell epitopes of *Mycobacterium tuberculosis* formulated in chitosan nanoparticles [102]. It was shown that the chitosan/DNA formulation was able to induce the maturation of DCs while chitosan solution alone could not, indicating that the DNA released from the particles was able to stimulate DCs. Pulmonary administration of the DNA plasmid incorporated in chitosan nanoparticles was shown to induce increased levels of IFN-γ secretion compared to pulmonary delivery of plasmid in solution or the more frequently used intramuscular immunization route. These results indicated that pulmonary delivery of DNA vaccines against tuberculosis may provide an advantageous delivery route compared to intramuscular immunization, and that increased immunogenicity can be achieved by delivery of this DNA encapsulated in chitosan nanoparticles.

The potential of a chitosan derivative, TMC, for pulmonary delivery of DT was also investigated [88]. Pulmonary immunization with DT-TMC microparticles

containing 2 or 10 Lf units of DT resulted in a strong immunological response, as reflected by the induction of IgM, IgG, and IgG subclasses (IgG1 and IgG2) antibodies, as well as neutralizing antibody titers comparable to or significantly higher than those achieved after subcutaneous administration of alum-adsorbed DT (2 Lf). Moreover, the IgG2/IgG1 ratio after pulmonary immunization with DT-TMC microparticles was substantially higher than that of the subcutaneously administered alum-adsorbed DT. DT-TMC microparticles were also able to induce detectable pulmonary secretory IgA levels. Recently, Heuking et al. [103] has developed a new water-soluble chitosan derivative (6-*O*-carboxymethyl-*N*,*N*,*N*-trimethyl chitosan) that was functionalized with a TLR-2 agonist as a material for pulmonary vaccine delivery. Furthermore, TLR-2-decorated CM-TMC nanocarriers (400 nm) by complexation with pDNA were prepared and shown to protect pDNA against enzymatic degradation [104].

The kinetics and toxicity of the nanoparticles (350 nm) prepared using hydrophobically modified glycol chitosan (HGC) were evaluated after intratracheal instillation to mice [105]. The half-life of HGC nanoparticles in the lung was determined as 131.97 $\pm$ 50.51 h, showing rapid uptake into the systemic circulation and excretion via urine, which peaked at 6 h after instillation. HGC nanoparticles were found to be distributed to several extrapulmonary organs; however, the levels were extremely low and transient. Transient neutrophilic pulmonary inflammation was induced from 6 h to day 3 after instillation. Expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokine (MIP-1α) in lung showed an increase after instillation and recovered thereafter. The HGC nanoparticles were suggested as successful candidates for pulmonary delivery owing to their excellent biocompatibility, transiency, low pulmonary toxicity, and property of rapid elimination without accumulation.

5 Transdermal and Intradermal Delivery

The skin is accepted to be a good target organ to generate both cellular and humoral immune responses, hence it is used as an alternative route for vaccine delivery [106–109]. Studies in several animal species and clinical trials in humans have established the proof of principle [14]. The skin is rich in DCs found at high density in the dermis and Langerhans cells (LCs), mostly localized in the epidermis. Application of vaccine antigens and adjuvants to the skin, also known as transcutaneous immunization (TCI) involves the topical application of the formulation directly onto the skin surface [110]. The quality and duration of the humoral and cellular responses to transcutaneous vaccination depend on the appropriate targeting to the APCs, vaccine dose, route of administration, and use of adjuvant. Various methods of vaccination have been designed to target the antigen to LCs and DCs for the induction of immune responses. Among the different parameters that have to be considered in formulation of particulate sytems for transcutaneous vaccine delivery, the size of the particles and their physical and chemical properties that allow

their internalization by APCs are important. Limitations in particle-based TC vaccination are imposed by skin layers and hair follicles.

In recent years, utilization of chitosan and its derivatives for TC immunization has been investigated and compared to to intradermal delivery (Table 1). Bal et al. [71] first studied the immunogenicity of both OVA-loaded TMC nanoparticles (200 nm) and TMC/OVA solutions after intradermal (ID) injection. TMC/OVA nanoparticles were shown to be taken up in vitro by DCs and the nanoparticles and mixtures were able to induce upregulation of MHC-II, CD83, and CD86 whereas a solution of plain OVA did not induce DC maturation or T cell proliferation. In vivo studies were also performed in mice by injecting three times with TMC-based formulations containing either OVA or DT. All TMC-containing formulations were shown to increase the IgG titres compared to antigen alone, and induced Th2-based immune responses. Hence TMC was suggested as an immune potentiator for antigens delivered via the skin. The same formulations were applied onto the skin in a solution before or after microneedle treatment with two different 300-μm microneedle arrays and also injected intradermally [72]. As a positive control, alum-adjuvanted DT (DT-alum) was injected subcutaneously. Microneedle-based application of a TMC/DT mixture was also shown to increase the antibody titres when compared to application of the DT solution. However, topically applied DT-loaded TMC nanoparticles were not able to enhance the immune response. Modifying the method of microneedle application or the type of microneedle array used was not helpful in improving the immunogenicity of the TMC nanoparticles.

In another study, reporter antigen expression and immune responses of plasmid DNA-condensed chitosan nanoparticles and pDNA-coated on preformed cationic chitosan/carboxymethylcellulose (CMC) nanoparticles were investigated following topical application onto the skin of shaved mice [42]. Significant levels of luciferase expression was obtained in skin after 24 h, and significant antigen-specific IgG titers to expressed β-galactosidase were seen 28 days after the initial application to the skin. No general correlation between the levels of gene expression in skin at 24 h and IgG titer on day 28 was found for the formulations studied except for chitosan oligomer/CMC nanoparticles with both the highest levels of gene expression and IgG titers.

6 Parenteral Delivery

Non-invasive immunization is a top priority for public health agencies due to the fact that the current immunization practices are unsafe, particularly in developing countries because of the widespread reuse of non-sterile syringes. However, parenteral delivery is still the most valid route. Hence, studies are also being carried out using chitosan for parenteral delivery (Table 1). The efficiency of chitosan-encapsulated DNA-based respiratory syncytial virus (RSV) vaccine was investigated after intravenous injection to mice [73]. The nanoparticles were found to be nontoxic to cells when used at concentrations $\leq$400 μg/mL. Immunohistochemical

and real-time polymerase chain reaction results showed a higher level of RSV protein expression in mouse tissues when nanoparticles were injected intravenously than after injection of naked DNA.

Borges et al. [84] have developed a nanoparticulate delivery system composed of a chitosan core to which HBsAg was adsorbed and subsequently coated with sodium alginate. A potent adjuvant, CpG ODN 1826, that was shown to induce a Th1-type immune response was also associated with the nanoparticles [74]. A high anti-HBsAg IgG titer (2271 ± 120 mIU/mL), with the majority of antibodies being of Th2 type, was observed with the coated nanoparticles. However, regarding cellular immune response, no significant differences were observed for antigen-specific splenocyte proliferation or for the secretion of IFN-γ and IL-4, when compared to the control group. An increase in IgG titers was obtained in the presence CpG ODN 1826 that was not statistically different from the group without the immunopotentiator, whereas a significant increase in the IgG2a/IgG1 ratio of the IFN-γ production by the splenocytes stimulated with the HBV antigen was observed [74]. Chitosan nanoparticles co-administered with the CpG ODN were reported to result in a mixed Th1/Th2-type immune response.

Chitosan microspheres to deliver a fusion protein, Ag85B–MPT64–Mtb8.4 (AMM), from three *Mycobacterium tuberculosis* genes were prepared and C57BL/6 mice were immunized at weeks 1, 3 and 5 subcutaneously with the AMM-loaded chitosan microspheres (6 μm), with AMM in incomplete Freund's adjuvant (IFA), or with AMM in PBS [75]. Splenocytes immunized with AMM loaded chitosan microspheres were found to produce higher levels of IFN-γ compared to that of control. The levels of Ag85B-specific IgG (H+L), IgG1, and IgG2a were also higher than those with AMM in PBS.

7 Concluding Remarks

Chitosan and its derivatives are very promising both as adjuvants and as delivery systems for vaccine delivery, especially in micro- and nanoparticulate forms. Amongst the non-invasive routes studied for antigen delivery, the nasal route seems to be the most suitable, particularly because of ease of administration, absence of degrading enzymes, and safety. However, it is important to maintain contact of the antigen with the nasal mucosa in order to guarantee an efficient antigen uptake. The bioadhesive property of chitosan provides prolonged contact of the antigen and hence a superior uptake, which results in an enhanced immune response. Furthermore, owing to its positive surface charge, chitosan increases the interaction of the antigen with the tissue, which also contributes to improvement of the immune response. These two latter properties make chitosan favorable for vaccine delivery. To date, there are very few studies reported on chitosan-based delivery systems for vaccine delivery in humans. Nevertheless, the results obtained are very promising which encourages researchers to continue their investigations in this field.

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