EXPERT OPINION

Chitosan-based delivery systems for mucosal vaccines

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Introduction: Mucosal vaccine development faces several challenges and opportunities. Critical issues for effective mucosal vaccination include the antigen-retention period that enables interaction with the lymphatic system, choice of adjuvant that is nontoxic and induces the required immune response and possibly an ability to mimic mucosal pathogens. Chitosan-based delivery systems are reviewed here as they address these issues and hence represent the most promising candidates for the delivery of mucosal vaccines.
Areas covered: A comprehensive literature search was conducted, to locate relevant studies published within the last 5 years. Mucosal delivery via nasal and oral routes is evaluated with respect to chitosan type, dosage forms, co-adjuvanting with novel adjuvants and modulation of the immune system.
Expert opinion: It is concluded that chitosan derivatives offer advantageous opportunities such as nanoparticle and surface charge manipulation that facilitate vaccine targeting. Nevertheless, these technologies represent a longer-term goal. By contrast, chitosan (unmodified form) with or without a co-adjuvant has significant toxicology and human data to support safe mucosal administration, and thus has the potential for earlier product introduction into the market.

Keywords: chitosan, chitosan derivatives, mucosal, nanoparticles, nasal, vaccine, oral

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1. Introduction

In recent years, researchers have appreciated the extensive advantages of vaccination via the mucosal route as compared with the traditional parenteral route. However, the development of mucosal vaccines presents a broad set of challenges as well as opportunities. Mucosal surfaces (nasal, respiratory, oropharyngeal, gastrointestinal and urinogenital) are potential routes of infection for pathogenic organisms such as viruses and bacteria, and it is becoming increasingly apparent that local mucosal immune responses are important for protection against disease [1]. Unlike conventional injection, mucosal immunisation has been reported to provide additional antibody-mediated protection against pathogens at the mucosal site of entry. Mucosal tissues also possess both antigen-presenting and antigen-processing cells with the potential to initiate cell-mediated immune responses including immunological memory. Vaccination at one mucosal site can induce immunity at distant mucosal sites via the common mucosal immune system [2].

Mucosal vaccination can be achieved via a number of routes including intranasal, oral, pulmonary, rectal or vaginal. Of these, the nasal and the oral routes are the most accessible and acceptable for vaccine administration. Although the gut has the largest mucosal surface area, this route is disadvantaged by the hostile gastrointestinal environment with a high potential for antigen degradation/denaturation by acid and enzymes, dilution of the vaccine by gastrointestinal contents and elimination by peristaltic movement, thereby requiring high doses of antigen and specialised formulations to overcome the aforementioned barriers to delivery. The nose is an excellent route of vaccine administration, providing a more benign

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Article highlights.

- Of the mucosal vaccination routes, nasal and oral routes are most accessible and acceptable.
- Mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens.
- Chitosan and chitosan derivatives, due to their adjuvanticity and dosage form versatility, offer excellent delivery systems for mucosal vaccines.
- Particulate forms of chitosan and its derivatives should be seen as a long-term goal as the challenges to gain regulatory approval and commercialisation remain significant.
- Unmodified chitosan has great potential for early exploitation for mucosal vaccination.

This box summarises key points contained in the article.

environment with respect to pH and enzymes and hence potentially lower dose requirements, while dose administration is straightforward and convenient to the patient. In addition, the nasal cavity has a large surface area with the local draining lymph nodes lying under the respiratory epithelium and is highly vascularised resulting in venous blood passing directly into the systemic circulation. Furthermore, potent responses in the respiratory and genital tracts can be induced via the common mucosal immune system by intranasal vaccination. However, historically, nasal formulations have been sub-optimal; mucociliary clearance and inefficient uptake of soluble antigens may compromise nasal vaccine delivery, as may respiratory infections or disease.

Research and testing of mucosal vaccines are currently accelerating, stimulated by new information on the mucosal immune system, novel adjuvants, innovative delivery platforms, and increasing awareness of the effect of infectious diseases on long-term health as well as the looming threat of an influenza pandemic [3]. For a mucosal vaccine to be effective, the formulation should ensure stability of the antigen, retain the antigen at the site of delivery for sufficient time to allow interaction with the lymphatic system, stimulate both the innate and cellular systems with or without the use of safe efficacious adjuvants by targeting specific parts of the immune cells and provide long-term immunity against the pathogen. The critical issue in the selection of an adjuvant and/or delivery system is that it not only induces the required immune responses, but also maintains the integrity and the various functions of the mucosal surfaces; it also needs to be safe and meet the standards for quality demanded by regulatory agencies. These criteria have proven to be very challenging to fulfil, and despite highly innovative and ingenious methods being investigated both pre-clinically and clinically, to date mucosal vaccination has not become firmly established as a standard procedure and very few mucosal vaccines have been approved for human use worldwide. Those that are available include oral vaccines against polio virus [4], *Salmonella typhi* [5], *Vibrio cholerae* [5] and rotavirus [6] and nasal vaccines against seasonal influenza [7] and H1N1 swine flu [8]. It is worth noting that none of these mucosal vaccines contain adjuvants or innovative delivery systems.

Mucosal delivery certainly has great potential to provide a more patient-friendly, safe and noninvasive needle-free option of vaccination. Researchers are using a number of approaches to overcome the hurdles and to improve the delivery and efficacy of mucosal vaccines. Various polymers from natural and synthetic origin have been investigated as delivery systems for mucosal vaccines [9]. Such polymers are advantaged in terms of their ability to increase the residence time of antigen at mucosal surfaces, hence enhancing the uptake of the antigen by professional antigen-presenting cells (APCs), and also may have inherent immune adjuvant properties. When choosing an appropriate polymer, considerations are primarily given to toxicity, including irritancy, mucoadhesive ability and antigenicity. Among such delivery systems for mucosal vaccines, chitosan has taken a prominent place. Chitosan is a biodegradable, biocompatible, cationic polysaccharide, which is mucoadhesive, has low toxicity and can be formulated in a number of different ways, such as solution, gel, powder, microparticles and nanoparticles. Furthermore, a number of derivatives have been produced with solubility over a wider pH range, providing more flexibility in matching the optimum formulation pH for antigen stability. A comprehensive literature search was conducted using databases including Pubmed, Medline, Embase, BIOSIS, Pharmaceutical Index and Conference Index, to locate the published studies within the last 5 years for this review, which provides an overview of delivery systems based on chitosan and chitosan derivatives for mucosal vaccines followed by a critical discussion of these systems.

2. Mechanisms of mucosal immunity

Mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens. Therefore, when formulating a mucosal vaccine, it is absolutely paramount to fully understand and consider the mechanisms of mucosal immunity. These have been extensively discussed previously [1,9,10]. Briefly, the mucosal surfaces protect the body from the outside hostile world by a barrier of epithelial cells, which have associated glands and lymphatic tissues. Mucosal immunity comprises both innate and adaptive defence mechanisms, and the tissues involved can be classified into two groups: inductive and effector sites. The prime inductive sites are nasopharynx-associated (NALT), gut-associated bronchus-associated and urinogenital-associated lymphatic tissues for the nasopharyngeal, gut, upper respiratory tract and urinogenital tissues respectively. These various lymphatic tissues encompass follicle-associated epithelium (FAE) of Peyer's patches, mesenteric lymph nodes or isolated lymphoid follicles, where immune cells encounter the antigen and lead to stimulation of lymphocytes. Lamina propria and the basal epithelial cells represent the effector sites. The epithelial cells function as sensors that detect foreign microbial components via Toll-like receptors (TLRs) and respond by sending cytokine and chemokine messages to underlying dendritic cells (DCs)

and macrophages to trigger innate non-specific defences, including mucins and antimicrobial proteins and also promote adaptive immune responses [11]. In the gastrointestinal tract where bacteria are abundant, epithelial cells along with intraepithelial lymphocytes and phagocytic cells can modulate and dampen signals to prevent undesirable responses to non-threatening nutrients and the normal intestinal flora that could lead to mucosal inflammation. Such a mechanism of tolerance to prevent harmful allergic immune responses to inhaled antigens exists in the nasopharyngeal and respiratory tissues. There has to be a balance between active immunity and tolerance, a point that needs due consideration when developing a mucosal vaccine.

An important characteristic of the mucosal adaptive immune response is the local production and secretion of immunoglobulin A (IgA) antibodies. The secretory IgA (sIgA), unlike other antibody isotypes, is resistant to degradation in the protease-rich milieu of mucosal surfaces and promotes entrapment of antigens or microbes in the mucus, enabling 'immune exclusion'. Furthermore, microbial-specific sIgA might prevent mucosal-cell infection or mediate antibody-dependent cell-mediated cytotoxicity. Although cytotoxic T lymphocytes (CTLs) in mucosal tissues cannot prevent pathogen entry, they might be able to play a role in clearance or containment of both viral and bacterial infections [12,13].

3. Chitosan and chitosan derivatives

Chitosan and chitosan derivatives are cationic polymers, which, due to their structure, have excellent mucoadhesive and absorption-promoting properties. The chemical structure of chitosan and some key derivatives is provided in **Figure 1**. Chitosan is manufactured by alkaline deacetylation of chitin (e.g. derived commercially from exoskeleton of crustaceans or fungi) and is a linear copolymer of β1-4 linked monomers of D-glucosamine and *N*-acetyl-D-glucosamine. The physical and biological properties of chitosan depend on molecular weight and degree of deacetylation [14]. Chitosan is insoluble in water but can be dissolved in acids from which a water-soluble salt can then be isolated (e.g. glutamate, hydrochloride, aspartate, lactate). The pKa of the primary amine group of chitosan is approximately 6.5 and the polymer is largely insoluble above pH 6 [14,15]. This solubility characteristic may present compatibility issues with antigens that are soluble and stable only at neutral pH.

Structural modifications have been made to chitosan to produce derivatives that are soluble at neutral pH yet retain the positive charge and unique properties and activity of chitosan. The majority of chitosan chemical modifications involve the amine groups although derivatisation at the hydroxyl groups is also possible. Many water-soluble derivatives have been prepared by quaternisation [16], introducing hydrophilic groups such as hydroxypropyl, hydroxyethyl, hydroxytrimethyl, hydroxyalkylamino [17,18], thiol [19,20], sulphate [21], succinyl [22], carboxyalkyl groups such as carboxymethyl, carboxyethyl, carboxybutyl or by grafting water-soluble polymers such as polyethylene glycol (pegylation) to the macromolecular chain of chitosan [23,24]. Diquaternary piperazine derivatives of chitosan have also been prepared by coupling a quaternary piperazinium acetic acid into chitosan or by attaching a tertiary 1,4 dimethylpiperazine into *N*-chloroacetyl-6-*O*-triphenyl-methylchitosan [25]. Of all the water-soluble derivatives, *N*-trimethyl and carboxymethyl derivatives of chitosan (TM-CSN, CM-CSN respectively) have been studied most extensively due to their relative ease of synthesis, ampholytic character and ample application possibilities. TM-CSN can be synthesised by a number of different methods thereby enabling various degrees of methylation and water solubility [26]. Soluble TM-CSN has both mucoadhesive properties and excellent absorption-enhancing effects even at neutral pH [27,28]. CM-CSN can be obtained by carboxymethylation of reactive amino and/or hydroxyl groups of chitosan to give three types: N-CM-CSN, O-CM-CSN or N,O-CM-CSN [23].

Chitosan has had diverse uses in pharmaceutical and medical fields [29] and has been reported to have bioactive properties such as antimicrobial, anti-inflammatory, tumour inhibition, antiviral and wound healing [30]. Chitosan has been extensively investigated for its immunogenic activities, especially via the mucosal routes. It should be noted that chitosan is commonly produced from shellfish; biomedical and pharmaceutical grades are very high-purity polysaccharides, the allergenicity risk of which is considered very low.

The main mechanism of chitosan mucoadhesion appears to be electrostatic interaction between the positively charged polymer and negatively charged materials such as cell surfaces and mucus. Mucus contains mucins that comprise significant proportions of sialic acid. At physiological pH, sialic acid carries a net negative charge and, as a consequence, mucins and chitosan can demonstrate strong electrostatic interaction when in solution. On examining mucoadhesion of TM-CSN with different levels of quaternisation, it was found that the presence of quaternary ammonium groups was detrimental to mucoadhesion [31]. The authors concluded that this was due to conformational changes in TM-CSN. Further investigations have shown that although electrostatic attraction is the major mechanism of chitosan mucoadhesion, other factors include hydrogen bonding and hydrophobic effects. Solution pH and presence of other solutes in the solution can change the relative contributions of each physical interaction [15].

A gamma scintigraphy study showed that radiolabelled chitosan solution was cleared significantly more slowly from the nasal cavity of sheep than a control solution [32]. A similar study in man demonstrated that nasal residence was increased in the order chitosan powder > chitosan solution > control [33]. These studies clearly demonstrated that chitosan increases the contact time with the nasal mucosa thereby increasing the potential of enhancing antigen uptake by the APCs.

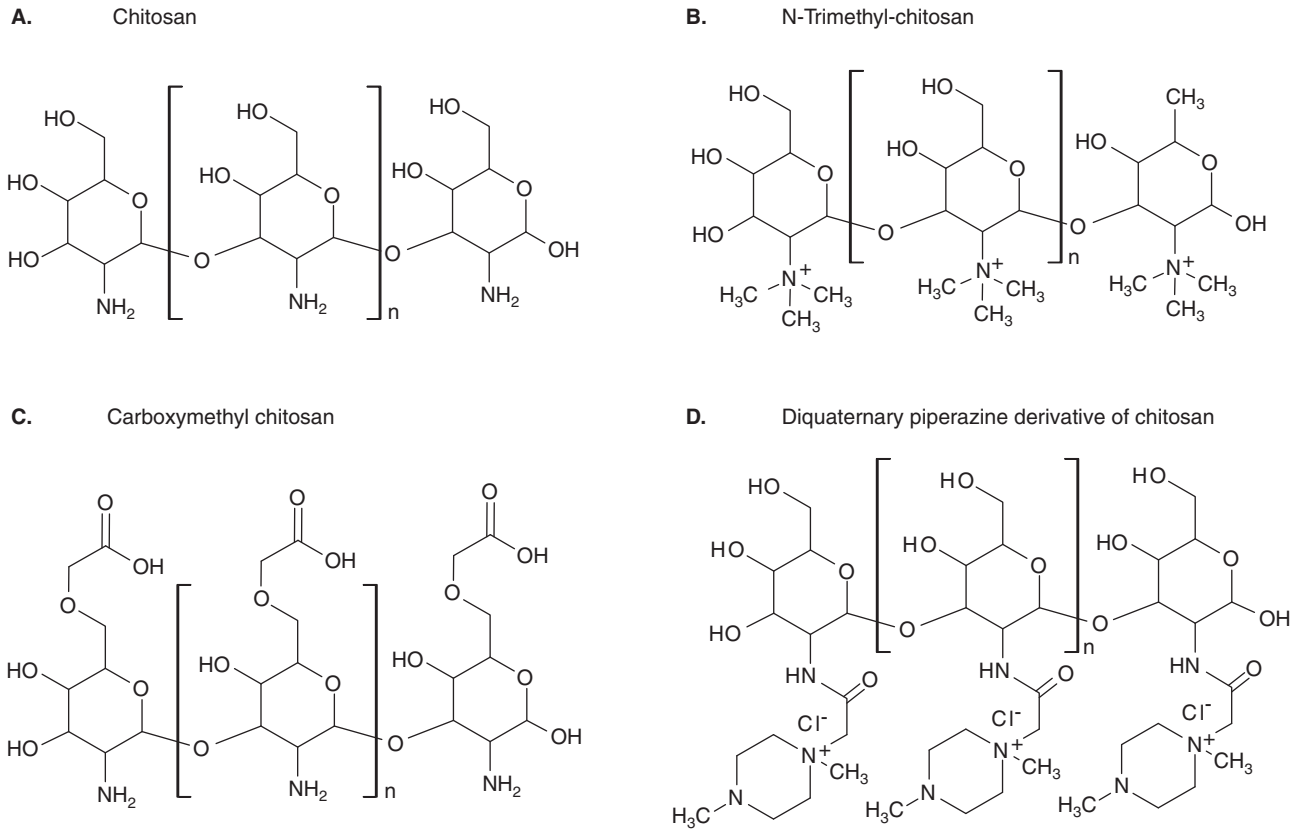

Figure 1. Chemical structure of chitosan and the key derivatives. A. Chitosan. **B.** *N*-Trimethyl-chitosan. **C.** Carboxymethyl chitosan. **D.** Diquaternary piperazine derivative of chitosan.

The absorption-promoting effect of chitosan may be due not only to improved adhesion between the vaccine formulation and the mucosal tissues (e.g. nasal, gastrointestinal), but also to a transient effect of chitosan on paracellular transport processes. Investigations in cell culture (CaCo-2) as well as animal models appear to demonstrate that chitosan can modify paracellular transport [34,35]. A recent study has also demonstrated that propyl chitosan chloride with α,β glycerophosphate as a gel form enhances transepithelial transport via paracellular routes [36]. Immunohistological studies have shown that chitosan can open the tight junctions between cells through an effect upon F-actin filaments [37]. Furthermore, chitosan has been demonstrated to have adjuvant properties (humoral and cellular immune responses) following parenteral administration of inactivated H5 influenza vaccine. In this study, chitosan was shown to stimulate proliferative and cytotoxic activity of splenic mononuclear leucocytes in mice and promote an increase in the numbers of CD32, CD3/NKa (major histocompatibility complex (MHC) class II) and H-2Db (MHC I) cells. Also chitosan did not induce IgE antibodies or antibodies against chitosan, therefore, clearly indicating chitosan is a promising adjuvant [38].

Chitosan in base or salt forms is commercially available from a number of suppliers in several grades of purity and molecular weight. There is a monograph for chitosan chloride in the European Pharmacopoeia (EP2011) and a drug master file Type IV has been filed by one manufacturer with the US Food and Drug Administration. By contrast, most chitosan derivatives are currently only experimental materials, and although TM-CSN and CM-CSN are commercially available with high purity, they have not yet been approved for human medical use by regulatory authorities.

4. Mucosal delivery systems based on chitosan and its derivatives

Chitosan and its derivatives have been extensively reviewed for their role in vaccine delivery via the mucosal routes including a succinct summary of published work on chitosan particulates by Şenel [9,10,39,40]. Recent studies (within last 5 years) on the application of chitosan and its derivatives as mucosal delivery systems for various antigens in different dosage forms are summarised in **Table 1**. It is interesting to note that the extent of responses produced in humoral or cellular arms of the immune system can be modulated by using chitosan of different molecular weights, chitosan derivatives, microparticles, nanoparticles, nanoparticle mixtures prepared from

Table 1. Summary of studies carried out using chitosan-based mucosal vaccine delivery systems in the last 5 years.

Antigen	Chitosan type	Dosage form	Adjuvant	Vaccine recipient	Vaccination route	Study type	Brief outcome of investigation	Ref.
Anthrax PA-recombinant	Chitosan	Dry powder	MPL	Rabbit	Intranasal	*In vivo*	Serum IgG response	[85]
Bordetella bronchi-septica dermonecro-toxin	Chitosan MW: 100 kDa	Microspheres (5 – 6 μm)		Mice	Intranasal	*In vivo*	Serum IgG response IgA response observed in serum, saliva and nasal wash	[66]
Bordetella bronchi-septica dermonecro-toxin	Chitosan MW: 10 kDa	Microspheres		Pigs (colostrum-deprived)	Intranasal	*In vivo*	Serum IgG and nasal wash IgA responses. Following the challenge, signs of infection in the vaccinated animals were sixfold lower than the non-vaccinated animals	[96]
Bovine herpesvirus 1	Chitosan Chitopharm S, M and L	Microparticle and Gel				*In vitro*	Cytotoxicity: both microparticles had no toxic effect at a concentration of 1 mg/ml on MDBK cells. Cellular uptake: particles taken up by MDBK cells were mainly around the nucleus, while the large aggregates were not taken up but were adsorbed to the surface Infectivity: the antigen maintained its potency as all loaded particles caused infectivity of the cells	[69]
Coxsackievirus B3	Chitosan	Microparticle	LTN gene – T cell-attractive-chemokine	Mice	Intranasal	*In vivo*	**Co-immunisation with pLTN** significantly enhanced neutralising antibodies in serum and intestinal mucosa. Promoted systemic and mucosal Th1 and $CD8^+$ CTL immune responses. Reduced myocardial viral load. Profound subsidence of myocarditis. Increased survival rate	[88]
Diphtheria toxoid	TM-CSN	Microparticulate powder		Guinea pigs	Pulmonary	*In vivo*	Serum IgG (IgG1 and IgG2) response	[56]
Foot-and-mouth disease DNA antigen	Chitosan MW: 170 kDa	Nanoparticles 255 nm	Provax IL-15	Mice and guinea pigs	Intranasal	*In vivo*	Serum IgG and lung/nasal wash IgA responses. Cellular response (IL-4 and IFN-γ in $CD4^+$ cells and IFN- γ in $CD8^+$ cells)	[91]
Foot-and-mouth disease-inactivated antigen	Chitosan Chitopharm L and M Protasan UP Cl 213	Gel		Guinea pigs	Intranasal	*In vivo*	Serum IgG and nasal wash IgA	[97]
H. pylori	Chitosan	Solution particles		Mice	Oral	*In vivo*	Serum IgG1 and IgG2a indicating both Th1- and Th2-type responses. Cellular response (IFN-γ, IL-12, IL-10 and IL-4	[49]
HIV DNA-based	Chitosan	Solution		Human	Intranasal (part of a trial testing intramuscular and intravaginal routes)	*In vivo* Clinical	Awaiting results as the study is currently ongoing	[98]

Adapted from [39,40].

CM-CSN: Carboxymethyl chitosan; CpG: Cytosine-phosphate-guanine nucleotide; CTB: Cholera toxin B subunit CTL: Cytotoxic T lymphocyte; CVB3: Coxsackievirus B3; DC: Dendritic cell; DNA: Deoxyribonucleic acid; DQ: Degree of quaternisation; HA: Hyaluronic acid; HI: Haemagglutinin inhibition; HIV: Human immunodeficiency virus; HTCC: N-(2-hydroxy-3-trimethyl ammonium) propyl Chitosan chloride; IFN: Interferon; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IL: Interleukin; LPS: Lipopolysaccharide; LTN: Lymphotactin; MDP: Muramyl dipeptide; MPL: Monophosphoryl lipid; MW: Molecular weight; NP: Nanoparticle; ODN: Synthetic oligonucleotides; OVA: Ovalbumin; PAM: PAM(3)CSK(4); PCL: Poly-(ε-caprolactone); PEG: Polyethylene glycol; PLGA: Poly(D,L-lactide-co-glycolide); Th1: T helper cell type 1; Th2: T helper cell type 2; TLR: Toll-like receptor; TM-CSN: Trimethyl chitosan; TPP: Pentasodium tripolyphosphate; WIV: Whole influenza virus.

Table 1. Summary of studies carried out using chitosan-based mucosal vaccine delivery systems in the last 5 years (continued).

Antigen	Chitosan type	Dosage form	Adjuvant	Vaccine recipient	Vaccination route	Study type	Brief outcome of investigation	Ref.
Hepatitis B surface antigen – plasmid DNA	Glycol Chitosan	Chitosan-coated liposomes 775 nm		Mice	Intranasal	*In vivo*	Serum IgG response. IgA response in saliva nasal and vaginal wash. Cellular response (IL-2 and IFN-γ))	[92]
Hepatitis B surface Antigen – recombinant	Chitosan	Nanoparticles – alginate coated 300 – 600 nm	CpG ODN	Mice	Oral	*In vivo*	Serum IgG and IgA in intestinal washings. Serum IgG1 and IgG2a indicating mainly Th2-type pre-boost and Th1-type post-boost. Cellular response (CD69 expression in $CD8^+$ and $CD4^+$ T cells)	[81]
Hepatitis B surface Antigen – recombinant	Chitosan	Alginate-coated Chitosan nanoparticles 300 – 600 nm	CpG ODN	Mice	Intranasal	*In vivo*	Positive IgG response (IgG1/IgG2a > 1). Positive IgA response in faeces, nasal and vaginal wash. Positive cellular response (IFN-γ))	[87]
Hepatitis B surface Antigen – recombinant	Chitosan	PLGA microparticle coated with CSN		Mice	Intranasal	*In vivo*	Coated and uncoated microparticle deposition in NALT, seen under fluorescence microscopy. Coated microparticles showed marked increase in anti-HBsAg titre compared with un-coated	[99]
Influenza virus, whole-inactivated	TM-CSN: different DQ, o-methylation, acetylation	Solution		Mice	Intranasal	*In vitro*	All formulations induced strong serum IgG, IgG1 and IgG2a/c responses than WIV alone. TM-CSN with DQ of 56% resulted in significantly IgG2a/c:IgG1 implying a bias towards Th2-type response. All showed protection against aerosolised virus challenge	[45]
Influenza virus, whole-inactivated	TM-CSN	Solution		Mice	Intranasal	*In vitro*	TMC-whole influenza virus showed close interaction with epithelial surfaces of naso- and maxilloturbinates. Showed minimal toxicity in terms of ciliary beat frequency.	[53]
Recombinant influenza H1N1 HA protein	Chitosan	PCL nanoparticles coated with chitosan		Mice	Intranasal	*In vitro* *In vivo*	Nanoparticles with positive surface charge and antigen entrapment efficiency of 74.8%. Immune responses (HI, IgG, IgG1, IgG2a titres and nasal/lung wash IgA) were enhanced by CSN-PCL vaccination. Th1 and Th2 responses were balanced	[100]
Influenza, H5N1 subunit	Chitosan glutamate	Solution		Mice	Intranasal	*In vivo*	Increased haemagglutination inhibition and single radial haemolysis against homologous vaccine strain and drifted H5 strains. Mixed Th1 and Th2 cytokine response with increased $CD4^+$ T cells ($IFN\gamma^+$, $IL2^+$ and $IFN\alpha^+$)	[46]

Adapted from [39,40].

CM-CSN: Carboxymethyl chitosan; CpG: Cytosine-phosphate-guanine nucleotide; CTB: Cholera toxin B subunit CTL: Cytotoxic T lymphocyte; CVB3: Coxsackievirus B3; DC: Dendritic cell; DNA: Deoxyribonucleic acid; DQ: Degree of quaternisation; HA: Hyaluronic acid; HI: Haemagglutinin inhibition; HIV: Human immunodeficiency virus; HTCC: N-(2-hydroxy-3-trimethyl ammonium) propyl Chitosan chloride; IFN: Interferon; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IL: Interleukin; LPS: Lipopolysaccharide; LTN: Lymphotactin; MDP: Muramyl dipeptide; MPL: Monophosphoryl lipid; MW: Molecular weight; NP: Nanoparticle; ODN: Synthetic oligonucleotides; OVA: Ovalbumin; PAM: PAM(3)CSK(4); PCL: Poly-(ε-caprolactone); PEG: Polyethylene glycol; PLGA: Poly(D,L-lactide-co-glycolide); Th1: T helper cell type 1; Th2: T helper cell type 2; TLR: Toll-like receptor; TM-CSN: Trimethyl chitosan; TPP: Pentasodium tripolyphosphate; WIV: Whole influenza virus.

Table 1. Summary of studies carried out using chitosan-based mucosal vaccine delivery systems in the last 5 years (continued).

Antigen	Chitosan type	Dosage form	Adjuvant	Vaccine recipient	Vaccination route	Study type	Brief outcome of investigation	Ref.
Influenza, H5N1 split antigen	HTCC)/α, β-glycerophosphate	Gel	MF59	Mice	Intranasal	*In vitro* *In vivo*	Enhanced transepithelial transport via paracellular routes. Antigen-specific systemic and mucosal IgA responses. Boosted cytokines IFN-γ and IL-4. Promoted antigen-specific central and effector memory $CD8^+$ in NALT	[36]
Neospora caninum tachyzoites – rNcPDI antigen	Chitosan	Nanogel with alginate or alginate-mannose surface		Mice	Intranasal	*In vivo*	The intranasally treated animals with the nanogel were protected (9 of 10 mice) following challenge with Neospora caninum tachyzoites. Significant reduction of cerebral parasite numbers following intranasal vaccination except for groups vaccinated with alginate-mannose nanogels.	[83]
Newcastle disease	Chitosan	Solution		Chicken	Oculo-nasal	*In vivo*	Enhanced antigen-specific cell-mediated immune response (IFN-γ). No systemic, lachrymal and digestive – antibody-mediated response	[47]
Newcastle disease –rHVT-ND	Chitosan	Solution		Chicken	Oculo-nasal	*In vivo*	rHVT-ND/live ND-chitosan provided best protection against mortality and morbidity and the strongest reduction of virus shedding	[48]
Norovirus virus-like particles	Chitosan glutamate	Powder	MPL	Human	Intranasal	Clinical	Dose-dependent increase in serum anti-norovirus IgA (4.8-fold) and IgG (9.1-fold) antibodies plus HI titres. Significant increase in IgA antibody secreting cell response. Overall, a safe vaccine with no vaccine-related serious adverse events. Protection against both norovirus-associated viral gastroenteritis and infection following a challenge against homologous virus	[55]
Ovalbumin	Chitosan MW: ~ 70 kDa	Multiple emulsion (w/o/w) 19 – 20 μm	Squalene oil	Mice	Oral and intranasal	*In vivo*	Serum IgG and IgA in faeces	[77]
Ovalbumin	Chitosan TM-CSN	Nanoparticles		Mice	Intra-duodenal	*In vitro* and *ex vivo* *In vivo*	**TM-CSN NPs but not Chitosan NPs:** Increased M-cell dependent uptake. Enhanced association of OVA with DCs. Had adjuvant effect on DCs Significantly higher immune responses compared with OVA alone Potential for oral immunisation	[70]

Adapted from [39,40].

CM-CSN: Carboxymethyl chitosan; CpG: Cytosine-phosphate-guanine nucleotide; CTB: Cholera toxin B subunit CTL: Cytotoxic T lymphocyte; CVB3: Coxsackievirus B3; DC: Dendritic cell; DNA: Deoxyribonucleic acid; DQ: Degree of quaternisation; HA: Hyaluronic acid; HI: Haemagglutinin inhibition; HIV: Human immunodeficiency virus; HTCC: N-(2-hydroxy-3-trimethyl ammonium) propyl Chitosan chloride; IFN: Interferon; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IL: Interleukin; LPS: Lipopolysaccharide; LTN: Lymphotactin; MDP: Muramyl dipeptide; MPL: Monophosphoryl lipid; MW: Molecular weight; NP: Nanoparticle; ODN: Synthetic oligonucleotides; OVA: Ovalbumin; PAM: PAM(3)CSK(4); PCL: Poly-(ε-caprolactone); PEG: Polyethylene glycol; PLGA: Poly(D,L-lactide-co-glycolide); Th1: T helper cell type 1; Th2: T helper cell type 2; TLR: Toll-like receptor; TM-CSN: Trimethyl chitosan; TPP: Pentasodium tripolyphosphate; WIV: Whole influenza virus.

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Ovalbumin	TM-CSN PLGA PLGA/TM-CSN	Nanoparticles		Mice	Intranasal	*In vitro* *In vivo*	Rapid release of OVA from TM-CSN NPs, no burst release of PLGA NP and medium release of PLGA/TM-CSN NPs. Less OVA damaged during encapsulation in TM-CSN NPs compared with PLGA or PLGA/TM-CSN NPs. Toxicity: all NPs showed no significant decrease in cilia beat frequency. Compared with OVA solution, TM-CSN NPs showed significant decrease in clearance rate in the nasal cavity. TM-CSN and PLGA/TM-CSN NPs not taken up by monocyte-derived DCs, but TM-CSN NPs induced DC maturation. TM-CSN NPs resulted in significantly higher immune responses (IgG and IgA) as compared with PLGA NPs or PLGA/TM-CSN NPs	[73]
Ovalbumin	TM-CSN	Nanoparticles Cross-linked with either TPP or CpG (TLR-9 ligand)		Mice	Intranasal	*In vivo*	OVA/TM-CSN/TPP induces strong humoral response with Th2 bias. OVA/TM-CSN/CpG also induces equivalent humoral response but 10-fold higher IgG2a than OVA/TM-CSN/TPP. OVA/TM-CSN/CpG also enhanced OVA-specific IFN-γ T cells	[101]
Ovalbumin, Hsp70-mB29a peptide	PLGA PLGA/TM-CSN TM-CSN-TPP	Nanoparticles		Mice	Intranasal	*In vitro* and *in vivo*	All nanoparticles enhanced antigen presentation by dendritic cells as shown by increased $CD4^+$ T cells. Only PLGA particles induced immunoregulatory response and suppressed OVA-specific Th-1 mediated delayed hypersensitivity reaction. TM-CSN-TPP induced humoral immunity and enhanced generation of OVA-specific B cells. Following intranasal immunisation with Hsp70-mB29a peptide-loaded PLGA particles suppressed proteoglycan-induced arthritis, thereby significant reduction of disease. Differential regulation shown by nanoparticles to modulate nasal immune responses	[74]
Ovalbumin	TM-CSN thiolated	Nanocomplex with thiolated HA/ Nanocomplexes treated with Maleimide PEG		Mice	Intranasal and intradermal	*In vivo*	Following intranasal or intradermal vaccination, OVA loaded-stabilised TM-CSN – S – S – HA produced superior immunogenicity in terms of serum IgG. PEGylation totally abolished the beneficial effects of stabilisation for intranasal administration and no enhanced immune responses after intradermal vaccination	[102]

Adapted from [39,40].

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Ovalbumin	TM-CSN	Nanoparticles	TLR ligands: LPS, PAM 3CSK4. CpG DNA NOD-like receptor ligand: MDP and GM1 receptor ligand: CTB	Mice	Intranasal Intradermal	*In vivo*	**Induction of serum IgG, IgG1, IgG2a and nasal wash IgA when compared with non-adjuvanted:** Higher with LPS and MDP No change with CTB, PAM 3CSK4 or CpG. **Induction of serum IgG, IgG1, IgG2a when compared with non-adjuvanted:** Higher with CpG and LPS	[103]
Pneumococcal surface antigen expressing DNA	Chitosan	Nanoparticles		Mice	Intranasal	*In vivo*	Serum IgG response Balanced IgG1/IgG2a ratio, enhanced IFN-γ and IL-17A indicating induction of cellular immune responses. IgA in mucosal lavages. Fewer pneumococci recovered from nasopharynx following intranasal challenge with ATCC 6303 (serotype 3)	[93]
Tetanus toxoid	Chitosan MW: 600 kDa	Microparticles		Mice	Oral	*In vivo*	Serum IgG and IgA in faeces and intestinal wash	[78]
Tetanus toxoid	Chitosan glutamate Protasan UPG 113 (CS) MW: 150 kDa CM-CSN/ TM-CSN	Nanoparticles CSN: 300 – 400 nm CM-CSN: 40 – 90 nm TM–CSN: 300 – 400 nm		Mice	Intranasal	*In vivo*	Serum IgG response (IgG2a/IgG1 < 1). No mucosal response in terms of nasal and vaginal wash IgA	[104]
Tetanus toxoid	Chitosan CM-CSN/ TM-CSN	Nanoparticles of TM-CSN, CM-CSN and TM-CSN/CM-CSN complex		Mice	Intranasal	*In vitro* *In vivo*	**Cellular toxicity:** CM-CSN > TM-CSN/CM-CSN > chitosan > TM-CSN. Uptake by murine macrophages: positively charged chitosan and TM-CSN nanoparticles higher than negatively charged CM-CSN nanoparticles. TM-CSN/CM-CSN complex induced both IgG1 and IgG2a, but the response was Th2 dominant. The complex demonstrated adjuvant effect	[72]
Tetanus toxoid	Chitosan	Chitosan-reduced gold Nanoparticles			Oral	*In vivo*	Mucosal response obtained	[79]
Urease	TM-CSN	Nanoparticles 180 – 330 nm		Mice	Oral	*In vivo*	Positive serum IgG response. Positive IgA response in gastric and intranasal wash	[80]

Adapted from [39,40].

CM-CSN: Carboxymethyl chitosan; CpG: Cytosine-phosphate-guanine nucleotide; CTB: Cholera toxin B subunit CTL: Cytotoxic T lymphocyte; CVB3: Coxsackievirus B3; DC: Dendritic cell; DNA: Deoxyribonucleic acid; DQ: Degree of quaternisation; HA: Hyaluronic acid; HI: Haemagglutinin inhibition; HIV: Human immunodeficiency virus; HTCC: N-(2-hydroxy-3-trimethyl ammonium) propyl Chitosan chloride; IFN: Interferon; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IL: Interleukin; LPS: Lipopolysaccharide; LTN: Lymphotactin; MDP: Muramyl dipeptide; MPL: Monophosphoryl lipid; MW: Molecular weight; NP: Nanoparticle; ODN: Synthetic oligonucleotides; OVA: Ovalbumin; PAM: PAM(3)CSK(4); PCL: Poly-(ε-caprolactone); PEG: Polyethylene glycol; PLGA: Poly(D,L-lactide-co-glycolide); Th1: T helper cell type 1; Th2: T helper cell type 2; TLR: Toll-like receptor; TM-CSN: Trimethyl chitosan; TPP: Pentasodium tripolyphosphate; WIV: Whole influenza virus.

different derivatives or coated with materials such as alginate, as well as the additional use of a chemically distinct adjuvant. Dependent on the adjuvant used, different arms of the immune system can be targeted via stimulation of specific receptors (e.g. Toll-like receptors, nucleotide-binding oligomerisation domain (NOD)-like receptors, RIG-1 (retinoic acid-inducible gene 1)-like receptors, c-type lectin receptors, integrins) on APCs, thereby eliciting antigen-specific T cells either favouring the bias towards humoral (T helper cell type 2 (Th2)) or cellular (T helper cell type 1 (Th1)) responses [41]. The last 5 years have seen a majority of the reported investigations being directed at evaluation of the immune responses in pre-clinical studies, with some *in vitro* studies, and only two clinical studies (one ongoing). Although there are potentially a number of mucosal routes, intranasal has been the most widely reported followed by oral.

The following sections describe mucosal vaccination studies in terms of the type and physical form of the chitosan used. Early work with chitosan as a mucosal delivery system was carried out mainly on solution or powder formulations [37]. Within the last few years, there has been greater emphasis on the use of more sophisticated delivery systems, such as particulate or gel forms of chitosan. The use of co-adjuvants is of significant potential and is also discussed below.

4.1 Aqueous solutions

Chitosan solution formulations have been shown to significantly enhance the serum and mucosal immune responses to many antigens including pertussis, diphtheria (CRM_{197}), influenza, Newcastle disease and *Helicobacter pylori* in preclinical studies [42-49]. Chitosan solution seems to elicit mixed Th1 and Th2 and has shown to significantly increase HI, SRH and neutralising antibodies [46,50]. Preliminary studies have shown that following intranasal vaccination of mice with H5N1 antigen solution formulations, both chitosan and TM-CSN significantly enhanced the cellular immune responses, and seroprotective responses, with chitosan performing marginally better than TM-CSN (unpublished data). Phase I/II clinical studies have also demonstrated chitosan to be an effective nasal delivery system for vaccines. Administration of nasal solutions of trivalent seasonal influenza vaccine formulated with chitosan generated serum HI titres that met the accepted criteria of European Medicines Agency's Committee for Medicinal Products for human use (CHMP) [51]. An enhancement of serum HI titres was demonstrated in further studies and a meta-analysis showed that overall (> 600 subjects), CHMP criteria were met in subjects at significant risk of infection (i.e. when individuals who had significant pre-existing immunity were excluded).

A nasal pandemic influenza (H5N1) vaccine using the chitosan delivery system is currently being investigated under the European Commission FP7 programme [52]. Chitosan and TM-CSN as solution delivery systems have been shown to promote moderate dendritic cell maturation while TM-CSN was also effective in augmenting TLR agonist-induced pro-inflammatory cytokines, including IL-12 family members that are involved in directing Th1 and Th17 responses (unpublished data). It has also been shown that whole influenza virus formulated with TM-CSN has much closer interaction with the epithelial surface, with the potential to generate enhanced uptake and induction of immune responses; furthermore, the study demonstrated that TM-CSN and the whole influenza virus as a solution formulation induced minimal local toxicity in terms of ciliary beat frequency in the nasal cavity [53].

4.2 Powders

Chitosan, principally in salt form, has also been investigated as a dry powder that would have the potential to enable a thermally stable vaccine and thereby avoid a cold chain for distribution. Studies using powder formulations of diphtheria, anthrax and norovirus have shown that chitosan can outperform solutions in terms of eliciting humoral responses [43,54-56]. A Phase I/II study showed that a single nasal immunisation with a powder formulation of diphtheria antigen with chitosan was well tolerated and significantly boosted antitoxin neutralising activity (twofold) in healthy volunteers, which could be further boosted (more than twofold) by a second immunisation. The neutralising activity far exceeded the accepted protective levels of 0.1 IU/ml and was equivalent to that induced by standard diphtheria intramuscular vaccine [57], clearly indicating that a single low-dose nasal vaccination could produce protection against diphtheria.

4.3 Particulates

Microparticles and nanoparticles made from chitosan or its derivatives are currently being extensively researched for mucosal delivery of vaccines. Depending on the preparation process, nanoparticles can be nanospheres having a monolithic type structure (matrix) or nanocapsules that have a membrane-wall structure with antigen entrapped in the core or adsorbed onto the exterior [58]. Since it is often difficult to establish whether particles are of a matrix or membrane type, the term 'nanoparticles' is adopted to describe both types. Nanoparticles can be prepared both from water-insoluble or water-soluble polymers using several methods: ionotropic gelation, microemulsion, emulsification-solvent diffusion, polyelectrolyte complex, complex coacervation and co-precipitation [10,59,60]. Of these, the most widely developed methods, ionotropic gelation and polyelectrolyte complex formation, do not use organic solvents or high shear force, thereby maximising antigen stability. Both methods are based on electrostatic interaction between the positive (amine) group of chitosan and the negative group of the polyanion (e.g. tripolyphosphate, alginate, dextran sulphate, DNA) [61-63]. Hence by altering the ratio of chitosan to the stabiliser, the size and surface charge can be manipulated, which could ultimately affect the target vaccination site as well as its interaction with the specific effector cells [64,65]. The entrapment efficiency is dependent on the pKa and solubility

of the entrapped antigen. The antigen is mostly found to be associated with chitosan via electrostatic interaction, hydrogen bonding and hydrophobic interaction.

Microparticles and nanoparticles have the capability to entrap and retain antigens in local lymph nodes and, being particulate, mimic natural pathogens by being readily engulfed by APCs, thereby leading to a cascade of subsequent reactions to produce the appropriate immune (humoral and cellular) responses. Formulating the antigen as a nanoparticle may enable protection from hydrolytic enzymes or low pH in the gastrointestinal mucosa. Furthermore, nanoparticles have the advantage of enabling the incorporation of a co-adjuvant in addition to the antigen, thereby further enhancing specific immune responses with regard to bias towards humoral or cellular immunity.

When used for antigen delivery, the size and surface charge of microparticles or nanoparticles appear to be key determinants of immune response. In general, particles smaller than 10 μm have been shown to improve immune responses significantly, allowing antigen uptake by the mucosal-associated lymphoid tissues [66,67]. Nagamoto and colleagues reported that ovalbumin-loaded chitosan particles with mean diameter of 0.4 and 1 μm showed a significantly higher production of IgA than 3 μm particles when intranasally administered to rats [68]. Bovine herpes virus (BHV)-loaded chitosan particles (~ 5 μm) were shown to be taken up by Madin-Darby bovine kidney epithelial (MDBK) cells, while larger aggregates were adsorbed only to the surface [69]. Microfold (M) cells present in FAE of Peyer's patches are known for their transcytotic transport capacity and may be critical for effective antigen transport to subepithelial cells [1]. Using Caco-2 cells and Raji-B cells, which induce the generation of M cells, it was demonstrated that transport of ovalbumin-loaded nanoparticles of chitosan and TM-CSN was significantly increased in the presence of M cells. Overall the transport of TM-CSN particles was higher than that of chitosan nanoparticles [70]. Since particle charge is known to have a significant effect on cellular uptake of nanoparticles, the above observation in *ex vivo* studies was possibly due to TM-CSN having a higher positive surface charge at physiological pH, whereas chitosan loses charge at this pH because of deprotonation of the primary amine groups [71]. Another study clearly showed that uptake of tetanus toxoid loaded nanoparticles by murine macrophages was higher for positively charged chitosan and TM-CSN than negatively charged CM-CSN [72]. Researchers have demonstrated that nanoparticles enhance association of antigen with DCs and increase their maturation [73,74], thereby exhibiting their adjuvant effect, illustrating the suitability of nanoparticles as mucosal delivery systems via nasal or gastrointestinal routes.

Novel chitosan vaccine nanoparticles have been investigated by a number of methods to assess the stability and integrity of the antigen. With respect to the influence of chitosan molecular weight, a study has shown that a greater amount of tetanus toxoid was released at 2 h from nanoparticles as the chitosan molecular weight decreased; the antigen was in active form for extended periods of time [75]. Release of ovalbumin from nanoparticles monitored by optical density showed antigen burst effect for TM-CSN nanoparticles; this could enable promotion of ovalbumin specific B cells. The release profile of antigen could be modified by coating chitosan nanoparticles with alginate, which also improved stability of the nanoparticles [76]. Using sodium dodecyl sulphate, polyacrylamide gel electrophoresis followed by Western blotting showed that majority of the antigen from TM-CSN nanoparticles retained its integrity but not from poly(lactic-co-glycolic acid) (PLGA) or PLGA/TM-CSN nanoparticles [73]. BHV from chitosan particles and gel formulations was observed to infect MDBK cells, clearly indicating that the virus maintained its potency [69].

Studies have shown that the nasal clearance rate of ovalbumin-loaded TM-CSN nanoparticles is significantly decreased compared with ovalbumin solution [73]. Ovalbumin-loaded TM-CSN nanoparticles or gel did not significantly decrease ciliary beat frequency of human nasal epithelial cell cultures as compared with ovalbumin solution alone. Also CSN microparticles had no toxic effect on cell viability and proliferation of MDBK cells [69].

The oral route for vaccination has certainly proven to be very challenging as discussed earlier, despite employing particulate delivery systems that would mimic natural pathogens. Although several *in vitro* studies have demonstrated significant uptake of particles by APCs, macrophages and M cells of Peyer's patches, very few oral studies have been performed. Orally administered microparticles or nanoparticles of chitosan-incorporating antigens have been shown to induce antigen-specific serum IgG, IgG1, IgG2a and local mucosal IgA antibodies in intestinal washes, gastric washes or faeces [77-80]. Furthermore, these microparticles elicit cellular responses in terms of expression of $CD8^+$, $CD4^+$ T cells and cytokines such as interleukin (IL)-10, IL-4, interferon (IFN)-γ and IL-12 [49,81]. Attempts have also been made to improve the delivery system by coating the chitosan nanoparticles with alginate or Eudragit® (methacrylate copolymer) [81,82].

4.4 Gel formulations

The concept of *in situ* gelling to enable longer residence time in the nasal cavity has recently been described. A hydrogel prepared from *N*-(2-hydroxy-3-trimethylammonium) propyl chitosan chloride and α, β-glycerophosphate is a free-flowing solution at room temperature but gels rapidly at body temperature. H5N1 influenza vaccine formulated with this thermal-sensitive hydrogel delivery system induced greater systemic and mucosal antibodies compared with MF59-adjuvanted H5N1 split antigen following intranasal vaccination. Moreover, the hydrogel-based formulation promoted the antigen-specific $CD8^+$ T cells in the NALT, thereby indicating the potential of an adjuvant-free H5N1 vaccine [36]. Chitosan nanogels coated with alginate were shown to provide significant protection following challenge with Neospora caninum

tachyzoites as well as significant reduction of cerebral parasite number in intranasally treated mice [83].

4.5 Co-adjuvants

Adjuvants can pose a major challenge to researchers with regard to achieving the required effect while avoiding reactogenicity or toxicity [84]. Co-adjuvanting of mucoadhesives with potent adjuvants is one of the possible ways of overcoming this hurdle as materials such as chitosan and its derivatives have been shown to express adjuvant effects themselves and also have the potential to reduce the dose of a co-adjuvant, thereby minimising any toxicity. Hence, the synergy between a mucoadhesive and an adjuvant could optimise efficacy of a vaccine.

For an intranasal dry powder vaccine containing anthrax antigen and monophosphoryl lipid (MPL), a TLR4 agonist, it was demonstrated that the presence of ChiSys® (a proprietary delivery system based on chitosan), significantly enhanced the adjuvant effect of MPL on protective antigen-specific antibodies in rabbits; a significant protection of rabbits was noted against lethal anthrax spore challenge 9 weeks after a single immunisation [54,85].

A nasal vaccine against norovirus is currently under investigation, which also utilises the co-adjuvanting concept. A Phase I/II clinical study investigating intranasal powder norovirus vaccine comprising virus-like particles with MPL and ChiSys showed the vaccine to be well tolerated and safe with no vaccine-related serious adverse events. A dose-dependent increase in circulating serum anti-norovirus IgA (4.8-fold) and IgG (9.1-fold) antibodies compared with control (adjuvant/excipient alone) was observed as well as an increase in HI titres, a measure of functional antibodies that may contribute to protection against the virus. Priming via the nasal immune mucosa was also demonstrated by IgA antibody secreting cell (ASC) responses in the peripheral blood. 100% of subjects in the highest (100 μg) dose group had measurable IgA ASCs after just one dose. Following a challenge with homologous norovirus, the subjects showed protection against both norovirus-associated viral gastroenteritis and infection [55,86].

Hepatitis surface antigen formulated with alginate-coated chitosan nanoparticles associated with an immunopotentiator, synthetic oligonucleotide (ODN)-containing immunostimulatory cytosine-phosphate-guanine (CpG) motif (TLR9 agonist), was shown to elicit mixed Th1 and Th2 immune responses following oral or intranasal vaccination [87]. Lymphotactin gene (T-cell-attractive chemokine) incorporated in chitosan microparticles significantly enhanced neutralising antibodies in serum and intestinal mucosa, reduced myocardial viral load of coxsackievirus B3 and increased the survival rate of mice following nasal administration [88].

Chitosan has also been shown to improve the adjuvanticity of secondary immunomodulators such as muramyl di-peptide (MDP), which is a nucleotide-binding oligomerisation domain-containing protein 2 (NOD-2) agonist known to elicit cell-mediated immunity, and cholera toxin subunit B (CTB), which is a potent mucosal adjuvant that induces strong humoral responses following nasally administered recombinant *H. pylori* urease in mice, thereby indicating that MDP and CTB possibly work independently and antagonistically in elicitation of mucosal responses [89]. CpG-ODN and squalene oil have been described as co-adjuvants for enhancing the immune response to orally administered hepatitis B surface antigen-containing chitosan nanoparticles [81].

4.6 Delivery of DNA vaccines

Chimeric and DNA vaccines are currently being developed to tackle continuous genetic drift of viruses; antibodies are being raised to specific proteins involved in immunity to infection. The supercoiled structure and negative charge of DNA hinders the entrapment efficiency and stability of the vaccine and presents a delivery challenge. Several investigators have used chitosan to complex with DNA antigens in the form of nanoparticles or microparticles for intranasal delivery. It has been shown that particles carrying no charge tend to aggregate and are unstable, whereas nanoparticles containing chitosan lead to stable DNA formulations [37].

Following nasal inoculation of chitosan-DNA plasmid expressing epitopes of respiratory syncytial virus (RSV), protective CTL responses were observed in mice [90]. Intranasal delivery of nanoparticles of chitosan and plasmid DNA-expressing antigens (e.g. foot and mouth disease, hepatitis B surface antigen, pneumococcal surface antigen) has also clearly demonstrated induction of antigen-specific serum IgG, mucosal lavage IgA and cellular immune responses as evaluated by expression of cytokines (e.g. IFN-γ, IL-4, IL-2, IL17A) in $CD4^+$ and $CD8^+$ T cells [91,92]. Moreover, a study using pDNA-expressing pneumococcal surface antigen demonstrated protection against the disease following an intranasal challenge with ATCC630 (serotype 3) as fewer pneumococci were recovered from the nasopharynx [93]. Hence DNA vaccines have been shown to be proficient in triggering both humoral and cellular immune responses and may provide faster and more easily scaled-up alternatives to vaccine manufacture.

5. Expert opinion

The continuous emergence of new pathogens and evolution of resistance of microorganisms to antimicrobial drugs emphasise the importance of developing efficient vaccination strategies capable of generating defence against the invading pathogen as well as producing long-lasting effective immunity. In recent years, the prospect of vaccination via mucosal routes has been much researched. Mucosal immunisation is capable of inducing both mucosal (local) and systemic immune responses and, since it avoids the use of needles, is more acceptable to people and has the potential for rapid mass global vaccination. The ability to administer mucosal vaccines in a solid (powder) form may enhance stability and allow avoidance of a cold chain. Despite these advantages,

researchers have faced many hurdles to improve the delivery and efficacy of mucosal vaccines.

Among the various innovative technologies investigated, chitosan appears to be the most promising candidate as an adjuvant and delivery agent for mucosal vaccines and the enhancement of immune responses has been widely reported. Despite chitosan (in unmodified form) and its salts being available commercially in high-purity grades, along with significant toxicology and human exposure data to support safe mucosal administration, it is currently not a constituent of an approved pharmaceutical product. Furthermore, chitosan is unlikely to achieve the status of a universal adjuvant as it may be incompatible and/or ineffective with some antigens.

In vivo studies would indicate that nasal administration is a preferred route of mucosal immunisation in terms of effectiveness and patient acceptability. Although oral vaccine administration would be viewed as 'ideal', difficulties in delivering effective amounts of antigen to target sites in the gastrointestinal tract appear to preclude its use for the majority of applications. There may be limited opportunities for rectal or vaginal delivery of vaccines, for example, HIV immunisation [94].

The ability to chemically modify chitosan to dictate structural architecture and physicochemical properties, such as charge and solubility, thereby enabling optimum fit to the antigen, route of delivery and physical nature of the formulation (e.g. nanoparticle) offers much future potential. Chitosan derivatives may be able to incorporate specific ligands (antigens, adjuvants, moieties such as mannose) and thus be designed to specifically interact with preferred cell types and open up new possibilities in the field of vaccine targeting. However, there are many challenges to gaining regulatory approval and commercialising such novel materials and as a consequence their introduction into vaccine products should be seen as a longer-term goal.

Although there have been some examples of nasal vaccines showing good effectiveness through the use of chitosan alone, a single 'universal' immunostimulant or delivery system will most likely not be sufficient in many instances to produce the broad and long-lasting immunity that is required. An effective adjuvant system is likely to require synergy between one or more immunostimulants and the delivery vehicle. The use of unmodified chitosan in combination with established adjuvants, as illustrated by the nasal norovirus vaccine under development utilising chitosan and MPL, will offer a lower risk (and lower cost) approach and has the potential for earlier introduction into medical practice to the benefit of people.

Finally, the use of mucosal vaccination is already well established in domestic and farm animals [95] and opportunities exist for early exploitation of enhanced (chitosan-based) delivery systems in these applications.

Declaration of interest

All authors are employees of Archimedes Development Ltd.

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