

REVIEW ARTICLE

CHITOSAN NANOPARTICLES AS DELIVERY SYSTEM FOR NASAL IMMUNISATION

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Summary

Nasal immunisation represents an innovative and perspective route of vaccine administration that provides many benefits compared to the more traditional approaches. Since most infections start on mucosal membranes, the mucosal immunisation provides a rational reason for its application. Mucosal delivery for vaccine administration (for example oral or nasal routes) could stimulate both systemic and mucosal immune responses. However, there are still some limitations that should be solved for a broader utilisation of this approach. There is still the necessity to use strongly immunogenic antigens or appropriate adjuvants for the induction of a strong immune response. The use of nanoparticles in the vaccine development could represent a promising approach for the mucosal vaccine research. Nanoparticles could thus serve as delivery vehicles providing to vaccines their unique properties, such as the antigen stabilisation and protection, serve as an adjuvant and elicit an antigen-specific immune response on the target sites.

Key words: nanoparticle; chitosan; mucosa; vaccine; adjuvant

Introduction

The mucosal surfaces of mammals represent an important line of protection from toxic elements and infectious microbial diseases. Added together, these surfaces cover approximately 300 m² of the area of human body, including gastrointestinal, respiratory and urogenital tracts and also ocular and ear cavities (1). It is estimated that up to 70 % of pathogens entry the body via mucosa, making this site the first line of defence. The number is this high, because the mucosal membranes are thin and permeable barriers to the interior of the body with physiological function in gas exchange (the lungs), food absorption (the gut), sensory activities (the eyes, nose, mouth, and throat), and reproduction (the uterus and vagina) (2, 3). During these natural processes the mucosal surfaces are in a close contact with heterogeneous particles and also represent the first site, where the immune system encounters pathogenic microbes or other harmful substances. Therefore, the protective immune response is induced by an administration of vaccines directly to the mucosa is considered as ideal to prevent mucosal transmitted infections.

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The important part of the mucosal system is its immune defence system. The mucosa is the reservoir of up to 80 % of immune cells in the human body. Their organisation and functionality differs from the systemic immune system. The majority of immune cells are present in a well-organised structure which is called the mucosa-associated lymphoid tissue (MALT). The MALT is situated along the surfaces of all mucosal membranes. This system has its specific subcompartments, which are localized in the gastrointestinal tract (the gut-associated lymphoid tissue, GALT), respiratory tract (the bronchus-associated lymphoid tissue, BALT) or in the nasal cavity (the nasopharynx associated lymphoid tissue, NALT). Functionally, the MALT system could be divided into the inductive and effector sites. The inductive sites are represented by microfold (M) cells and secondary lymphoid tissues with their B-cell follicles. The primary function of inductive sites is the immune response initiation by an uptake of exogenous antigens which are actively transported through the epithelium to the antigen presenting cells (APC's). These cells include dendritic cells (DC's), B-cells, macrophages or follicular cells. Subsequently, the clonally selected B and T-cells migrate into the effector sites after antigen priming, and the local production of the secretory IgA (S-IgA) class is started. The layer of S-IgA covers diffusely the mucosal tissues (4).

Nasal immune system

The nasal cavity serves as a point of entry for many respiratory infections, including bacterial and viral diseases. The primary function of the nasal system is to allow the air to enter lower parts of the respiratory system during respiration. The nasal cavity also contains sensory organs responsible for the sense of smell. So, being in direct physical contact with the external environment, the nasal mucosa routinely filters, moistens, cools and warms the inhaled air to minimize the irritative effects on lower airways. Thus, the local mucosal immune system - NALT - represents an important part that provides local protection (5).

In humans, NALT is represented by the Waldeyer's ring mainly of nasopharyngeal tonsils and adenoids. The nasal cavity is covered by the ciliated epithelium with embedded M cells. These cells are responsible for the recognition and uptake of particulate antigens and their transport through the mucosal membrane via trans-cellular endocytosis. The transported antigens are then immediately processed and presented to APC's by binding to their pattern-recognition receptors. Afterwards, APC's migrate via draining lymphatics to the lymph nodes where the antigen is presented to the immunocompetent cells including naive lymphocytes. Finally, the resulting antigen activated T and B cells then enter the bloodstream and move to the effector sites where the effector arm of the mucosal immune response is performed by IgA producing plasma cells with a support of CD4+ T cells (Th1, Th2 or Th17 subtypes) and regulatory T cells (Treg). The differentiated plasma cells secrete the polymeric IgA immunoglobulin which binds to the immunoglobulin receptor of epithelial cells (EC's). The IgA is then internalised, transported across the EC's and released as the antigen specific secretory IgA on the mucosal surface. Thus, the S-IgA antibodies represent the most abundant antibody type found on the mucosal surface, where they serve as the first line of the immune defence system. The mechanism of S-IgA function is called the immune exclusion when the S-IgA antibodies are particularly important in preventing infection by aggregating the pathogens or harmful substances (6–8).

The peristaltic movements of nasal tract help to remove the aggregated clumps from epithelial surfaces. The cellular compartment of the immune system also belongs to the defence mechanisms on the effector sites. Namely, cytotoxic T cells (CTL's) and macrophages that help directly eliminate pathogens from the mucosa or the other T cells subsets as Th1, Th2 and Th17, which serve as regulators of the immune response by the cytokine production (Figure 1) (9, 10).

Mucosal delivery system

The administration of vaccines via the nasal mucosa is a novel perspective which offers many benefits opposite to the commonly used routes of immunisation, such as the intramuscular, subcutaneous or intradermal applications. First of all, there is no necessity for using syringes. This easy application results in better compliance and accessibility for larger population. Another important advantage of this approach is that the nasal vaccine administration could induce strong mucosal and systemic immune responses not only in the respiratory tract, but also in the genitourinary system and weak local immunity in the gastrointestinal tract. This immune interconnection at different mucosal sites represents the concept of mucosal immunity, where diverse mucosal epithelium tissues utilize similar migration

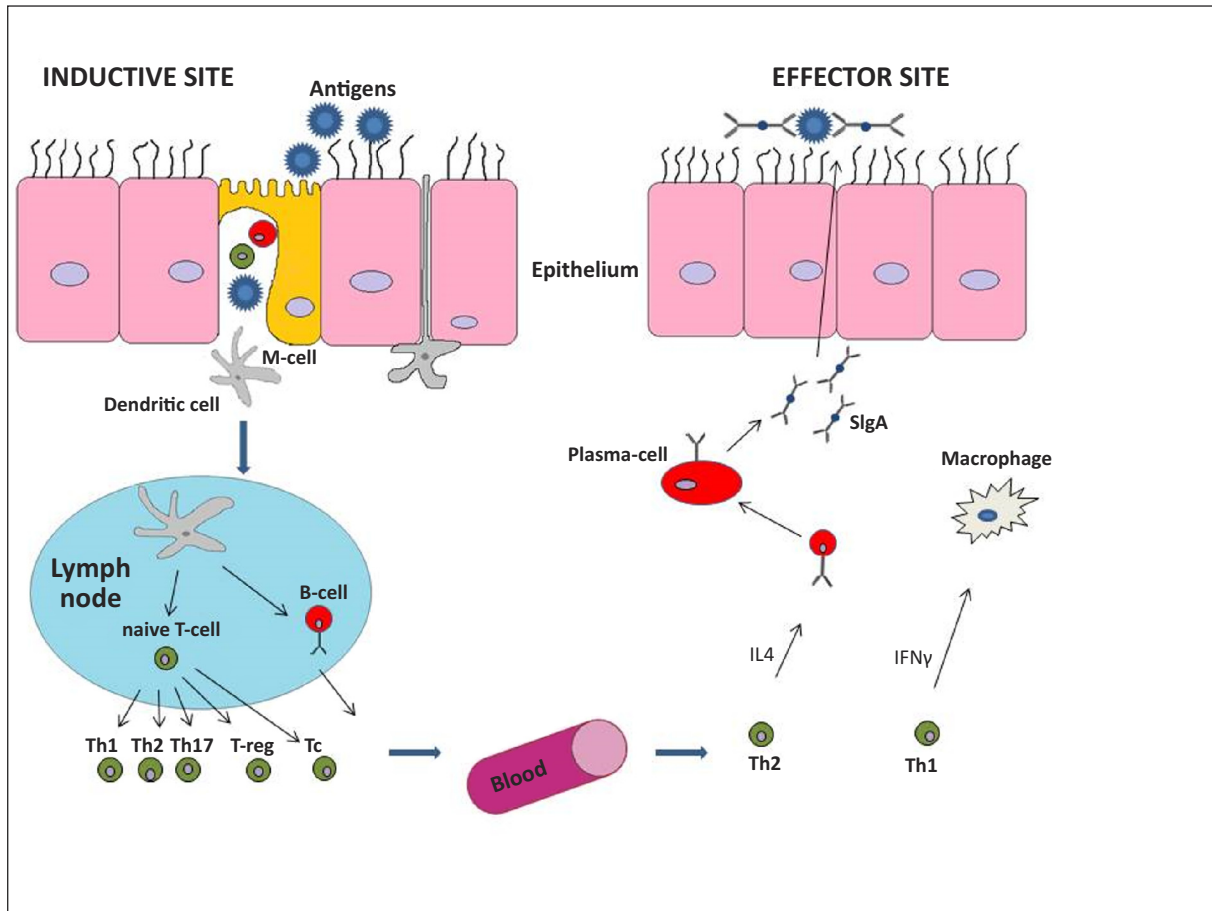


Figure 1. Concept of mucosal immune system.

characterizing patterns on the cell surface of primed T or B cells. The example of this phenomenon is CCL28, a ligand for CCR10 receptor on the surface of B cells, that is expressed in epithelial cells in the gut, lung, breast, vaginal, and salivary gland. Recently, it has been shown that intranasal immunization induces expressions of high levels of CCR10 and $\alpha_4\beta_1$ -integrin by IgA-secreting B cells, allowing them to strongly migrate to the respiratory and genito-urinary tracts expressing the corresponding ligands CCL28 and VCAM1 (11). On the contrary, B cells induced by the intestinal inductive sites such as Peyer's patches, express CCR9 and $\alpha_4\beta_7$ -integrin, so they preferentially migrate to the intestinal tracts expressing their ligands CCL25 and MAdCAM-1 (12).

The induction of the immune response following the mucosal immunisation depends on many factors, such as the nature of used antigens, route of administration and presence of immunomodulators. The main limitation is the poor immunogenicity of many antigens. The mucosal administration therefore requires additional materials to potentiate the vaccination effect. There is an ample evidence of adjuvant systems for the parenteral application, but not for the mucosal application. For example, alum, the adjuvant that was frequently used in human vaccines, is a poor inducer of the mucosal immunity (13). However, the mucosal epithelium expresses many immune receptors including Toll-like receptors (TLRs). Therefore, bacterial flagellin (TLR5 ligand), poly I:C (TLR3 ligand) and unmethylated CpG oligonucleotides (TLR9 ligand) are effective mucosal adjuvants (14). In addition, the cholera toxin and the heat-labile enterotoxin of *Escherichia coli* act as mucosal adjuvants stimulating dendritic cells. On the other hand, their use in humans has been restricted by their toxicity. Biocompatible polymers, such as chitosan, are also effective mucosal adjuvants. Chitosan binds to mannose receptors on macrophages, activates complement and stimulates cytokine production. Moreover, nanoparticles are promising adjuvants in new vaccines. Many different compounds such as poly amino acids, polysaccharides, biodegradable polymers and polystyrene

are used to prepare nanoparticles (14). The application of nanoparticles in the mucosal vaccine delivery has a number of benefits. They improve the ability to cross mucosal barriers, enhance the uptake by APCs and prolong the availability of the antigen to interact with APCs (15). The chitosan nanoparticles can thus be an excellent candidate, in conjunction with other immunostimulating compounds such as CpG oligonucleotides, the mast cell-activating compound C48/80 and the monophosphoryl lipid A, for the nasal immunization (16,17).

Chitosan

Chitosan, a linear polysaccharide composed of glucosamine and N-acetylglucosamine units linked by β (1 \rightarrow 4) glycosidic links, represents a saccharidic substance, which is most extensively tested as a carrier in the development of nasal vaccines. Chitosan is obtained by a deacetylation of the natural polysaccharide chitin. The acetamido group at the position of C-2 of the chitin is replaced with an amine group. The degree of deacetylation of chitosan significantly affects its chemical and biological properties (18). The dissociation constant (pK_a) of the primary amine of chitosan is ~ 6.5 according to the degree of N-deacetylation, thus at $pH > 6.5$ chitosan is deprotonated, which causes its insolubility in water (19). On the other hand, in dilute acid solutions the functional amino groups are protonated, the polysaccharide is converted into a polycation, allowing its dissolution. Based on differences in their molecular weight, three main types of chitosan are recognized: the high molecular weight chitosan (700 to 1000 kDa), the low molecular weight polymer (less than 15 kDa) and the medium molecular weight chitosan (20).

Chitosan exhibits a number of positive features such as the biocompatibility, biodegradability and low toxicity. Moreover, it possesses a significant antimicrobial activity and mucoadhesive property. Chitosan enhances the penetration by opening the tight junctions of the epithelium and thus promotes the permeability of cells to drugs of interest (21). It forms a complex with the negatively charged mucin by ionic or hydrogen bonding and through hydrophobic interactions. Mucoadhesive properties of chitosan result in a prolonged contact time between the antigen and absorption surface (22). In addition, chitosan has the ability to form fibers, gels and films. All of these properties have made chitosan an excellent candidate in a number of applications (23).

To improve chitosan solubility under physiological conditions, many derivatives of chitosan have been developed (17). Both functional groups of chitosan, amino and hydroxyl groups, can serve as active sites for modification. For example, the amine group of chitosan can be methylated to form trimethyl chitosan (TMC) that is widely exploited for mucosal vaccine applications. It has been shown that TMC nanoparticles induce strong mucosal immunity against hepatitis B virus (24) and group A *Streptococcus* (15) following nasal administration. Furthermore, chitosan reacts with sulphates, citrates and phosphates that can enhance its stability (25). Moreover, the mucoadhesive properties of chitosan can be further improved when nanoparticles are formed using thiolated chitosan (26).

Chitosan nanoparticles

Chitosan nanoparticles (CS-NPs) were first described by Ohya (27). At least five methods are presently available to synthesize CS-NPs. These include the ionotropic gelation, microemulsion, emulsification solvent diffusion, polyelectrolyte complex and reverse micellar method (28). Out of these mentioned techniques, the ionotropic gelation and polyelectrolyte complex are the most widely used methods.

Ionotropic gelation

This is a simple method that utilizes electrostatic interactions between the positively charged amino group of chitosan and the negatively charged group of a polyanion, such as tripolyphosphate or sodium sulfate. Chitosan is dissolved in the acetic acid, then the polyanion is added and nanoparticles are formed during mechanical stirring at room temperature. Jonassen et al. observed an increase in particle size when increasing the chitosan concentration and the ratio of polymer to polyanion (29). The size, zeta potential and antigen loading efficiency of the CS-NPs are dependent on various factors such as chitosan, crosslinker and antigen concentrations, pH of the media and the homogenization speed (30). The simplicity of the technique and aqueous environment are the main advantages of the ionotropic gelation. Disadvantages of this method include a poor stability in acidic conditions and a difficulty to entrap antigens with high molecular weight (31).

Polyelectrolyte complex

Polyelectrolyte complexes are formed spontaneously by adding an anionic solution (for example the DNA solution) to the cationic polymer (chitosan dissolved in the acetic acid solution), under mechanical stirring at room temperature resulting in charge neutralization (32).

The microemulsion method

In this method, a surfactant is dissolved in an organic solvent (hexane). Then chitosan in an acetic solution and a crosslinker (glutaraldehyde) are added to the mixture under continuous stirring. To complete the crosslinking process between chitosan and glutaraldehyde the mixture is stirred overnight. Then, the organic solvent is removed by evaporation and the excess surfactant is removed by precipitation (33).

The emulsification solvent diffusion method

This method is suitable for hydrophobic drugs showing high portion of drug entrapment. The injection of an organic phase into chitosan solution with a stabilizing agent (e.g. poloxamer) leads to the formation of an emulsion. Nanoparticles are formed as a result of the diffusion of organic solvent into the water (34).

The reverse micellar method

Nanoparticles of a very narrow size range are obtained with this method. The reverse micelles are formed by the addition of the chitosan aqueous solution to an organic solvent containing a surfactant followed by constant vortex mixing (35).

Interactions with antigens

An attachment of an antigen to the nanoparticles is achieved either by a simple but effective adsorption method, or more complex methods, such as chemical conjugation or encapsulation (36). The adsorption of an antigen onto a nanoparticle is based on the charge or hydrophobic interaction. The antigen of interest is preferably adsorbed to the particle surface. The interaction is relatively weak, which may lead to a desorption of the antigen from the particle surface. In addition, the antigen may be degraded by enzymes from the body fluids. A polysaccharide polymer, like sodium alginate can be used to coat nanoparticles with the antigen and thus, protect it from the enzymatic degradation (37). On the contrary, the encapsulation and chemical conjugation ensure a stronger interaction between the antigen and the nanoparticle. Antigens are encapsulated into particles during the synthesis process of the nanoparticles. The release of the antigen occurs when the nanoparticle is decomposed (36). In the chemical conjugation, the antigen is chemically cross-linked to the nanoparticle surface allowing for the presentation of antigens to antigen presenting cells similarly as during a natural infection (38).

Chitosan nanoparticles in the nasal vaccine delivery

Several recent studies have shown that CS nanoparticles represent an eligible antigen delivery system for the mucosal administration of a vaccine, especially by the route of nasal application. It has been reported that both cellular and humoral immune responses were induced after intranasal immunization with CS-NPs (16,39,40). Table 1 summarizes the studies, in which CS-NPs were used for nasal vaccines. CS-NPs enhance the absorption of the antigen from the nasal mucosal membrane and increase the immune response due to the prolonged interaction with antigen presenting cells. Mangal et al. have shown that the clearance of CS-NPs is decreased in the nasal mucosa, thus leading to the transport across the epithelial membrane and uptake by M cells (41). The internalization mechanisms of nanoparticles depend on the particle size, charge and shape (42). Smaller particles (20-200 nm) are preferentially taken up by dendritic cells while larger particles (0,5-5 µm) are taken up by macrophages (43). Additionally, surface charge plays a significant role in the interaction with antigen presenting cells. It has been shown that positively charged particles exhibit better mucoadhesive properties than negatively charged particles (44). The transport of nanoparticles through the mucosal membrane is suggested to be mediated by caveolin-1, clathrin and the micropinocytosis into M cells (45-47).

Table 1. Chitosan nanoparticles used in nasal vaccine delivery.

Antigen/pathogen	Animal model	Immune response
<i>Influenzavirus</i> , hemagglutinin	Mice	Significant IFN- γ secreting cells in spleen. Systemic and mucosal antibody response (48).
<i>Mycobacterium tuberculosis</i> , Hsp65	Mice	Induction of secretory IgA in lung. Th1 immune response in spleen (49).
Hepatitis B surface antigen	Mice	IgG and secretory IgA response (24).
Group A <i>Streptococcus</i>	Mice	Systemic and mucosal immune response (50).
<i>Escherichia coli</i> O157:H7	Mice	Systemic IgG and IgA response. Significant secretory IgA detection (51).
<i>Brucella abortus</i> , malate dehydrogenase	Mice	Th2-related immune response. Increased IgA production (52).
<i>Chlamydia psittaci</i> antigens	Mice	High levels of IgG and sIgA antibodies (39).
<i>Neisseria meningitidis</i> , NMB0315	Mice	Bactericidal antibodies in serum (53).
<i>Bacillus anthracis</i> , protective antigen + activator C48/80	Mice	Induction of mucosal immunity (16).

Conclusions

The nasal route of vaccination represents a novel and perspective alternative to more conventional parenteral injection. The nasal cavity is highly accessible and offers a non-invasive approach of the vaccine administration. In addition, intra-nasal vaccines induce both mucosal and systemic immune responses. The elicited mucosal IgA antibodies may bind the pathogens thus controlling them at the primary site of entry. However, the nasal route of the simple antigen administration has some limitations such as the rapid clearance in the mucosal membrane and a poor immunogenic response (17). These hurdles can be overcome by the incorporation of the suitable adjuvants and the application of nanoparticles as the mucosal vaccine delivery system. Nanoparticles represent promising materials to carry immunogens. Once vaccine antigens are incorporated into the nanoparticles they can be protected from biodegradation. Another advantage of nanoparticles is the possibility to transport different biomolecules, e.g. adjuvants and antigens. Chitosan represents a useful material for the preparation of the nanoparticles. It exhibits a low toxicity, biocompatibility and mucoadhesive properties (40). In conclusion, chitosan nanoparticles are considered as an attractive tool for mucosal vaccine delivery systems.

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Disclosure statements

The authors declare no conflict of interest.

Adherence to Ethical Standards

Not applicable.

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