



Universiteit
Leiden
The Netherlands

Challenges and opportunities in nasal subunit vaccine delivery : mechanistic studies using ovalbumin as a model antigen

Slütter, B.A.

Citation

Slütter, B. A. (2011, January 27). *Challenges and opportunities in nasal subunit vaccine delivery : mechanistic studies using ovalbumin as a model antigen*. Retrieved from <https://hdl.handle.net/1887/16394>

Version: Not Applicable (or Unknown)

License: [Leiden University Non-exclusive license](#)

Downloaded from: <https://hdl.handle.net/1887/16394>

Note: To cite this publication please use the final published version (if applicable).

11

Summary and perspectives

Summary

Nasal vaccination is a promising alternative to classical vaccination via needle injections. The nasal epithelium is equipped with a large number of immune cells, capable of both initiating and providing a protective immune response. Moreover, the nasal cavity is very accessible, allowing simple administration (nasal spray or nose drops), and the proteolytic environment is relatively low (compared to oral) providing a less hostile environment for the administered vaccines. The licensing of the first nasal vaccine (Flumist®) has shown the possibilities nasal vaccination provides. However, the inability so far to develop an effective subunit vaccine that would, unlike Flumist®, also be suitable for young children and elderly has also shown the challenge this administration route comprises (**Chapter 1**).

Vaccine formulation could be the key to successful nasal immunization as one can equip the antigen with the necessary tools to meet the challenges the nasal cavity offers. In order to rationally design nasal vaccine formulations, we will need to know the challenges the antigen will meet. Therefore this thesis has set out 3 aims:

1. To identify the major physiological hurdles subunit antigens have to overcome to elicit an immune response after nasal administration.
2. To develop methods *in vivo* or *in vitro* that allow these hurdles to be studied.
3. To use the obtained knowledge to rationally design nasal vaccine formulations.

The first aim is addressed in **Chapter 2** where the nasal physiology is reviewed and a road map to successful nasal vaccination is presented.

First of all, the nasal cavity has primarily evolved to keep substances out. A mucus layer covering the entire epithelium is replaced every 20 min, thereby removing all its constituents and thereby greatly limiting the time for the antigen to be taken up by epithelium. Formulation of the antigen with muco-adhesive substances like sodium alginate, carbopol, chitosan and N-trimethyl chitosan (TMC) can prolong the nasal residence time of antigen. A second hurdle identified is the passage through the nasal epithelium. Epithelial cells are closely stacked together by tight junctions, which leave little space for intercellular transport of large proteins. The inclusion of a tight junction opener like chitosan in a vaccine formulation can temporarily increase the permeability of the epithelium. The presence of M-cells in the epithelium offers the possibility of transcellular transport. As M-cells preferably transport particulate matter, the use of micro- or nanoparticle is advocated. Finally, when the antigen has passed the epithelium it has to be taken up by dendritic cells (DCs) and processed to elicit

the proper response. Besides the use of particulate systems, the use DC targeting ligands can facilitate endocytosis by DCs. In order to be able to activate T-cells, DCs will have to mature. This can be promoted by the addition of an adjuvant in the formulation. The choice of adjuvant can greatly influence the extent and type of immune response elicited.

The optimal nasal formulation will therefore be multifactorial and can be furnished with distinct functionalities such as mucoadhesive polymers, M-cell or DC targeting ligands and adjuvants. The opportunities in nasal vaccination ask for a concerted approach combining various targeting techniques is advocated.

Methods to investigate the nasal residence time of the antigen, transport by M-cells, the uptake by DCs and the maturation of DCs are discussed in **Chapters 3 and 4**.

An *in vitro* model for M-cells, based on intestinal epithelial cells co-cultured with a B-cell line, was assessed for its predictive value in the studies described in **Chapter 3**. Transport by M-cells of ovalbumin (OVA) loaded into TMC nanoparticles was higher than that of unencapsulated OVA or OVA loaded into chitosan nanoparticles. This was confirmed by *ex vivo* confocal fluorescent microscopic inspection of murine jejunum, showing that the M-cell model has predictive value. Moreover, an *in vitro* model for studying antigen uptake by DCs was introduced. Monocytes isolated from human volunteers were cultured into immature DCs, with which uptake of OVA in nanoparticles could be studied. TMC nanoparticles improved the association of OVA with DCs and induced activation of DCs. This correlated with the immunogenicity of OVA-loaded TMC nanoparticles after intraduodenal administration, which was significantly better than that of chitosan particles or a solution of OVA.

In **Chapter 4** a novel method of determining the nasal residence time of antigen using live imaging techniques is introduced. Three different types of nasal vaccine carriers PLGA, PLGA/TMC and TMC nanoparticles were investigated for their ability to decrease the clearance of OVA from the nasal cavity. Only TMC nanoparticles significantly prolonged the nasal residence time. Mice were nasally vaccinated with TMC nanoparticles and compared to the 2 other classes of nanoparticles, which did not prolong the nasal residence time. Interestingly, only the TMC nanoparticles elicited high anti-OVA antibody responses, whereas after intramuscular administration all classes of particles enhanced the immune response.

In contrast, nasal administration of OVA loaded PLGA nanoparticles appeared to result in tolerance rather than immunity (**Chapter 5**), as PLGA vaccinated mice showed a reduction in delayed type hypersensitivity against OVA. Vaccination with PLGA nanoparticles promoted the upregulation of the tolerogenic transcription factor FoxP3 in CD4⁺ T-cell and did not increase the number OVA specific B-cells, which is necessary for an antibody response. Nasal

immunization with OVA loaded TMC nanoparticles resulted in the opposite result, i.e. a large number of OVA specific B-cells was detected in the cervical lymph nodes and spleen.

From the results presented in **Chapters 4 and 5** it can be concluded that not only the ability to prolong the nasal residence time but also other characteristics of nanoparticles greatly influence (the quality of) the immune response elicited after nasal vaccination. Next to their mucoadhesiveness, TMC nanoparticles have other characteristics that make them a more suitable carrier system for nasal vaccination compared to PLGA or PLGA/TMC nanoparticles. Also the rapid release of the contained antigen (promotes uptake by B-cells) and immune stimulatory capacity (counteracting tolerance) are designated as the key characteristics that make TMC nanoparticles potentially successful nasal carrier systems when protective immunity is aimed for.

In **Chapter 6** studies are described in which the formulation of OVA with a delivery system and an adjuvant was investigated. A promising nasal delivery system, cationic liposomes, was used to investigate whether the antigen and adjuvant should be combined in one carrier. Mice were nasally immunized with solutions of OVA and the adjuvant CpG, or with OVA and CpG encapsulated in liposomes. The resulting immune response was measured by determination of anti-OVA antibodies in serum and compared to immunization via other administration routes (transcutaneous with microneedle pre-treatment, intradermal and intranodal). Encapsulation of the CpG and OVA in liposomes had a detrimental effect on the IgG titers after nasal (and transcutaneous) administration compared to co-administration of soluble OVA and CpG, whereas after intradermal or intranodal injection of OVA/CpG-liposomes the immune response was improved compared to soluble OVA and CpG. To gain more insight into the mechanism behind the differences between the responses elicited, the uptake of OVA and CpG by DCs in the draining lymph nodes was investigated after administration of the formulation via the different administration routes. This showed that encapsulation of OVA and CpG in liposomes reduced the amount of antigen and adjuvant reaching the DCs after nasal and transcutaneous administration, whereas after intranodal injection encapsulation had a positive effect on the number of OVA and CpG positive DCs.

These data imply that co-encapsulation of the antigen and adjuvant into a cationic liposome can have a beneficial effect on the antibody response against OVA after parenteral injection. However, the concomitant size increment impairs proper transport of antigen and adjuvant to the lymph node when administered via the nasal or the transcutaneous route.

Whereas **Chapters 3-5** describe TMC nanoparticles as a very promising nasal delivery system for subunit antigen, **Chapter 6** indicates that liposomal carriers may have difficulties

penetrating the nasal epithelium, because of their relatively large size. This would imply that a smaller entity that still has the same characteristics as TMC nanoparticles would be an even better choice for nasal delivery. In **Chapter 7** studies are presented in which such a possibility was investigated, as it introduces the conjugation of an antigen, OVA, to TMC as an alternative to nanoparticles for subunit vaccination. The size of these constructs was significantly smaller than that of TMC nanoparticles (30 nm vs. 300 nm). OVA was covalently linked to TMC using thiol chemistry (SPDP method [1]). It was found that with the SPDP method a reducible covalent bond between TMC and OVA could be introduced, without disrupting the protein's antigenicity and structure. Moreover, TMC-OVA conjugates were shown to be very comparable to TMC nanoparticles regarding their co-localization of OVA and TMC and their interaction with DCs. Uptake of TMC-OVA conjugate by DCs was similar to the uptake of TMC/OVA nanoparticles, i.e. over 5-fold increase compared to a solution of OVA and TMC. Mice intramuscularly immunized with TMC-OVA conjugate produced about 1000-fold higher OVA specific IgG titers than mice immunized with OVA and about 100-fold higher than mice receiving a physical mixture of TMC and OVA. These antibody titers were even slightly elevated compared to the titers obtained with TMC/OVA nanoparticles.

Just like TMC/TPP/OVA nanoparticles, TMC-OVA nanoconjugate prolonged the nasal residence time of the antigen (**Chapter 8**). The immunogenicity of TMC-OVA nanoconjugate was assessed after nasal vaccination and compared with that of TMC/TPP/OVA nanoparticles, solutions of OVA and a physical mixture of TMC and OVA. Mice nasally immunized with TMC-OVA conjugate produced high levels of secretory IgA in nasal washes and higher titers of OVA-specific IgG than mice immunized with any of the other formulations. The improved performance of TMC-OVA conjugates might be attributed to better penetration of the nasal epithelium. *In vitro* the conjugates diffused significantly better through a monolayer of lung carcinoma (Calu-3) cells than TMC/TPP/OVA nanoparticles did. Moreover, nasal immunization of mice with the conjugate resulted in significantly more OVA positive DCs in the cervical lymph nodes as compared to TMC/TPP/OVA nanoparticles. In conclusion, the TMC-antigen nanoconjugate improves nasal delivery and immunogenicity of the antigen. This suggests that efficient co-delivery of antigen and adjuvant to DCs, rather than a particulate form of the antigen/adjuvant combination, is decisive for the immunogenicity of the antigen.

A second way to improve TMC nanoparticles as nasal delivery system is to combine it with an adjuvant, which is investigated in the studies described in **Chapter 9 and 10**. In previous chapters it has been shown that TMC/TPP nanoparticles effectively induce antibody responses. However, in some cases a strong cellular response is highly desirable, e.g. for

vaccination against intracellular bacteria (e.g. *M. tuberculosis*) or viruses (e.g. HIV, Influenza A). Therefore in **Chapter 9** the composition of TMC nanoparticles has been altered. Whereas TMC was physically crosslinked with the strongly negatively charged molecule tripolyphosphate (TPP) in earlier chapters, here TMC was crosslinked with CpG DNA, an adjuvant known to provoke a cell mediated immune response. TMC/CpG/OVA nanoparticles showed similar physicochemical characteristics as TMC/TPP/OVA nanoparticles in terms of particle size (ca. 380 nm), zeta potential (+21 mV) and antigen release characteristics. Nasal administration of TMC/CpG/OVA and TMC/TPP/OVA nanoparticles to mice resulted in comparable serum IgG levels (ca. 1000 fold higher than those induced by unadjuvanted OVA) and local secretory IgA levels. Moreover, TMC/CpG/OVA nanoparticles induced a 10 fold higher IgG2a response than TMC/TPP/OVA nanoparticles and increased the number of OVA specific IFN-gamma-producing T-cells in the spleen. This shows that nasally administered OVA loaded TMC nanoparticles, containing CpG as adjuvant and crosslinker, are capable of provoking strong humoral and mucosal responses as well as Th1 type cellular immune responses and are therefore an all-round vaccine delivery system.

Finally in **Chapter 10**, next to CpG various other adjuvants are described for inclusion in TMC nanoparticles. Toll like Receptor (TLR) ligands (including lipopolysaccharide (LPS), PAM₃CSK₄ and CpG DNA), a NOD-like receptor-2 ligand (muramyl dipeptide (MDP)) and a GM1 ganglioside receptor ligand (cholera toxin B subunit) were encapsulated with OVA in TMC nanoparticles by ionic crosslinking with TPP. Physical characteristics like the nanoparticles' size, zeta potential and loading efficiency were determined to ensure that these parameters were similar between all particles. The effectiveness of the adjuvant loaded OVA-containing TMC particles was assessed *in vivo* by nasal vaccination of Balb/c mice using intradermal vaccination as a control. LPS loaded nanoparticles elicited the strongest IgG titers after nasal as well as intradermal vaccination. Moreover, LPS loaded nanoparticles induced higher sIgA levels than unadjuvanted TMC nanoparticles. Distinct differences between administration routes were observed: IgG titers after nasal immunization with MDP loaded particles were increased; nanoparticles with CpG showed decreased IgG levels compared to plain TMC particles; CpG loaded TMC particles after intradermal administration induced higher IgG and IgG2a titers; and MDP did not have an addition effect at all. This study shows that the inclusion of an adjuvant in OVA loaded TMC nanoparticles can significantly enhance the immune response. The selection of the adjuvant is not arbitrary and depends on the route of administration and the type of response required.

Perspectives

This thesis has set out to identify the limiting steps in nasal vaccination, study those limitations and find possible solutions to tackle the hurdles associated with these issues. In the next paragraphs several important aspects of nasal vaccine design are discussed and recommendations for future development are provided.

The antigen determines the formulation

The core of each vaccine formulation is the antigen. It is therefore not surprising that the rational design of a nasal vaccine should be based on the physicochemical and biological/immunological characteristics of the antigen, as well as the source (e.g. bacterium, tumor cell) from which it is derived. The model antigen in this thesis (OVA) is a water soluble negatively charged protein, with little mucoadhesive and immune stimulating properties. For instance, TMC nanoparticles were shown to be excellent nasal carriers for this antigen as the mucoadhesive and immunostimulatory characteristics of TMC compensated for the poor characteristics of OVA in this respect. Moreover, OVA easily complexes with the positively charged TMC polymers into nanoparticles, leading to a high encapsulation efficiency and, as shown in **Chapter 10**, adjuvants can be co-encapsulated to further increase and/or modulate the immune response. Notwithstanding these favorable characteristics of TMC as a nasal adjuvant for OVA, TMC may not be the ideal adjuvant for every antigen. Positively charged antigens are difficult to associate with TMC nanoparticles, leading to a lower encapsulation efficiency (unpublished results) and thus loss of costly antigen. Similarly, antigens with large lipophilic domains, such as hepatitis B surface antigen or hemagglutinin and neuraminidase, may profit more from formulation in liposomes as these membrane proteins can be incorporated in the liposomal bilayer, thereby mimicking more closely the natural way these antigens are presented to the immune system.

In some cases the antigen itself already has mucoadhesive or immune stimulating properties. For instance, Hageraars et al. [2] used whole inactivated influenza virus (WIV) and showed that co-administration with TMC did not prolong the nasal residence time of the antigen, as the plain antigen already resided in the nasal cavity for more than 4 hours. Although formulation of WIV with TMC did improve the immune response, one could argue that focusing on immune potentiation rather than mucoadhesion may be a better approach to improve the immune response to this antigen.

Finally, each vaccine should elicit a tailored immune response that is strongly dependent on the pathogen (or disease) to be combated. To repel pathogens that reside in the bodies' interstitial spaces, antibodies can be instrumental, making a humoral response desirable.

When intracellular bacteria or viruses are concerned, the help of cytotoxic T-lymphocytes (CTLs) and other leukocytes involved in the cellular immune response is required. A mucosal (sIgA mediated) response can prohibit pathogens that invade our bodies via mucosal surfaces from colonizing these epithelia and may therefore be very useful for the protective efficacy of a vaccine as well. This should be kept in mind when formulating an antigen for nasal administration. As can be concluded from this thesis, TMC increases sIgA production and causes a Th2 type response after nasal administration and may therefore be a good choice if a humoral immune response against respiratory or intestinal pathogens is required. This would make TMC based formulations potentially useful for future nasal vaccination against for instance diphtheria, influenza and polio.

TMC is however not likely to be the “weapon of choice” if a cellular response is required, for instance against herpes, HIV, malaria or RSV infection. The use of a different antigen delivery system that is more capable of eliciting cellular response, like ISCOMs or the addition of an adjuvant that can enhance the T-cell mediated immunity should be considered.

Nasal residence time, a critical parameter

Nasally applying an antigen that is cleared from the nasal cavity within minutes seems a waste of vaccine if the antigen does not get a chance to be absorbed into the nasal epithelium. The pivotal role of a prolonged residence in the nasal cavity is supported by various studies that have shown increased antibody response after nasal administration of antigen with mucoadhesive substances. A recent study by Nochi et al. [3] even shows that when the antigen is present up to days after administration (using a mucoadhesive nanogel), no additional adjuvant is needed to boost the immune response, emphasizing the potential of a long nasal residence time. In this thesis the importance of delaying nasal clearance is underlined (**Chapter 4**). Moreover, it is not hard to accomplish, as simple co-administration of TMC already caused a significant increase in the nasal residence time of OVA (**Chapter 8**). Similarly, studies in which mucoadhesives like chitosan or carbopol were co-administered with the antigen describe a similar mode of action [4, 5]. Therefore, the inclusion of a mucoadhesive polymer in a vaccine formulation seems one of the simplest ways to improve the efficacy of a nasal vaccine.

Small is beautiful

Particulate antigens have been associated with higher immune responses as compared to soluble antigens [6]. Particles offer the distinct advantage of being efficiently phagocytosed by DCs and transcytosed by M-cells. Moreover, multimeric antigen presentation can improve the

antigen specific uptake by B-cells. Indeed, particles can have a positive effect on the immune response after vaccination. However, careful consideration to the particle characteristics is recommended. As can be concluded from **Chapter 6** the size of the particle is an important point to consider. Nanoparticles are generally more effective than microparticles, but small nanoparticles (100 nm) were not more effective than 500 nm particles. As the particles in this size range all greatly exceed the maximum diameter of a tight junction in the nasal epithelial, we can assume their transport to the subepithelium to be mainly dependent on active transport by M-cells. Therefore equipping particles with an M-cell targeting ligand or selection of particles that naturally have a strong affinity for M-cells is more likely to improve M-cell transport than further reduction in particle diameter. Alternatively, a drastic size reduction might improve the passive, intercellular uptake by the nasal epithelium. In **Chapter 8** it is shown that TMC-OVA conjugates (size ca. 30 nm) cross the nasal epithelium more effectively than TMC/OVA nanoparticles and consequently induce a higher immune response. In conclusion, nasal vaccination could benefit from particulate formulations, however with respect to passing the epithelial barrier a diameter as small as possible is preferred to facilitate passive .

Adjuvants

The arsenal of adjuvants at our disposal is steadily growing. Whereas for more than a century alum was the only approved adjuvant for human use, recently new adjuvants like squalene emulsions (e.g., MF-59) and non toxic variants of LPS (e.g., MPL) were licensed for the European market. Increasing knowledge on the activation of the immune system (specifically the activation of APCs) has speeded up this process, as it has explained the mode action of several adjuvants from which the mechanism was unknown until recently (e.g., alum, MDP and LPS), which is a prerequisite for approval by the American Food and Drug Administration. Moreover, the observation that APC maturation can be triggered via specific pathogen recognition receptors (PRRs, e.g. Toll-like receptors and NOD-like receptors) has led to the identification of new ligands that could act as adjuvants. As the signaling cascade resulting from activation of PRRs is becoming more clear, in the near future it may be possible to select the proper adjuvant according to the nature of the vaccine and the type of immune response required .

As has been pointed out in **Chapter 10**, the use of adjuvants can be very beneficial for nasal vaccination, but not every adjuvant is a good nasal adjuvant. Whether or not an adjuvant is a good choice for nasal use, will depend on the type of response required (e.g. antibodies or cytotoxic T-lymphocytes) and on the dose of adjuvant required. In concurrence with the

“danger model” adjuvants are supposed to be dangerous goods and consequently there is only a small margin between immune potentiation and toxicity. Therefore not all adjuvants are suitable because their effective dose is a toxic one. For instance, heat labile enterotoxin (LT) was successfully used as an adjuvant with virosomes, but after nasal administration LT was presumably taken up by olfactory neurons, eventually leading to Bell’s palsy [7]. Whether an adjuvant will have a strong positive effect on the immune response raised by a nasal vaccine likely depends on how easily the adjuvant is absorbed into the nasal epithelium and on the expression of its complementary receptor on nasal DCs. For instance, in **Chapter 9**, CpG was a more effective as an adjuvant when formulated in the TMC nanoparticles as a delivery system. Interestingly, mice that received 2x 20 µg CpG in TMC nanoparticles elicited strong IgG and sIgA titers, whereas mice that received 2x 10 µg CpG (**Chapter 10**) in the same particle formulation did not develop an antibody response. This suggests that activation of the receptor for CpG (TLR-9) on DCs in the nose may not be that easy to establish. In this respect TLR-4 ligand (MPL) or MDP (a NOD-like receptor 2 ligand) in combination with TMC particles may be better candidates for nasal vaccination. Literature on PRR expression on nasal DCs is scarce, making it very difficult to select the right adjuvant *a priori*. However, the high dose of CpG was well tolerated by the mice, making CpG still a promising candidate adjuvant for nasal vaccination. Nonetheless, increasing knowledge on the expression pattern of PRR on nasal DCs would be very helpful for the rational design of nasal vaccination including the choice of ‘the right’ adjuvant.

Combine and conquer

The major challenge in formulation of nasal vaccine formulation is to manage the interplay between antigen, delivery vehicle and adjuvant in such a way that the optimal response is obtained. The research described in this thesis addressed this challenge and has identified some “must do’s”, if formulation with soluble antigens such as OVA is concerned.

Must do’s:

- Add a mucoadhesive to overcome the short residence time.
- Use small entities, to improve penetration through the nasal epithelium.
- Add an adjuvant to overcome nasal tolerance.
- Co-localize antigen and adjuvant to achieve better DC uptake/maturation.

In accordance with these 4 “must do’s”, also a certain “degree of freedom” was observed when OVA was formulated with TMC. This could be important, as it may simplify the design of the formulation.

Degrees of freedom:

- The mucoadhesive can be administered in conjunction with the delivery system or in a free form.
- Co-localization of adjuvant and antigen does not necessarily have to be achieved through the use of particles but can also be effectuated by conjugation of antigen and adjuvant.

Combining these rules of thumb, it seems that a small, co-localized antigen-adjuvant entity (like a conjugate or a nanoparticle <50nm) formulated in a solution with a mucoadhesive (mucoadhesive in a free form) could be a very promising approach. Based on the results in this thesis, in general a conjugate between an antigen with MPL, would be an interesting choice as LPS (of which MPL is a derivate) turned out to be the best adjuvant for OVA in **Chapter 10**. MPL is a less toxic variant of LPS and already licensed for human use. With the addition of TMC (as a mucoadhesive and additional adjuvant) antigen-LPS conjugates may provide effective future nasal subunit vaccines.

Into the clinic, bears on the road

Even if such nasal vaccine formulation with a relevant antigen is successful in a laboratory setting transferring it to the clinic successfully, will be large effort. The lack of correlation between mice and man is one of the first hurdles to take. Mice have been instrumental in the mechanistical aspects of nasal vaccination in this thesis, but will never be able to fully predict the immune response in humans. For instance, a very obvious difference between mice and man is the size of the nasal epithelium. Relatively the nasal epithelium of mice 4 times larger than the human epithelium, which could cause an overestimation of the absorbance and residence time of the antigen in mice compared to humans. Furthermore, the murine immune system is different than the human immune system, like the expression patterns of PRRs and the secretion of different antibody subtypes. Finally, many pathogens do not cause disease in mice. Mouse strains susceptible for these pathogens are being developed, but still these models have their limitations. Mice can be useful to test for local toxicity of the vaccine. TMC for instance was well tolerated by all mice in this study and has recently been applied to pigs with no resulting damage to the nasal epithelium (unpublished data). This, in combination

with the high antibody titers in mice, may be convincing enough to start a first small clinical trial.

Furthermore it seems imperative to keep the formulation simple. From the experience with the one nasal vaccine on the market (Flumist) we know nasal administration is well perceived by the public and especially by children [8]. However, this positive perception is only sustained if the costs are low. The first year Flumist was introduced only 500,000 vaccines were sold, whereas a year later when the price was drastically lowered the sales quadrupled [9]. A formulation can therefore only be commercially interesting if the antigen and adjuvant are readily available and the formulation is cheap, reliable and scalable. Also in this respect, the addition of TMC to the formulation is very feasible. The material from which TMC is derived, chitin, is the second most abundant polymer on earth and therefore readily available. Although it is a very heterogeneous substance, synthesis routes to standardize the production of TMC have already been established [10]. TMC is therefore also in this respect a very promising vaccine adjuvant.

Finally, the pharmaceutical form will be very important. A nasal spray seems an obvious choice, however this would require the vaccine to be in solution. Vaccine solutions are generally unstable and require cold storage and imply a short shelf life. A product in dry powder form is much more stable and could therefore be more easily distributed; also to countries where maintaining the cold chain is not self-evident. Lyophilization of OVA without damaging its antigenic epitopes has been shown in this thesis and for various other antigens this technique has also been successfully applied. If a simple and cheap delivery device can be developed to apply the vaccine as a powder or to reconstitute the vaccine just before application, nasal vaccination may become the new standard in vaccination.

References:

1. van Dijk-Wolthuis, W.N., et al., *A versatile method for the conjugation of proteins and peptides to poly[2-(dimethylamino)ethyl methacrylate]*. Bioconjug Chem, 1999. **10**(4): p. 687-92.
2. Hagenaaers, N., et al., *Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine*. J Control Release, 2010. **144**(1): p. 17-24.
3. Nochi, T., et al., *Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines*. Nat Mater, 2010. **9**(7): p. 572-8.
4. Soane, R.J., et al., *Clearance characteristics of chitosan based formulations in the sheep nasal cavity*. Int J Pharm, 2001. **217**(1-2): p. 183-91.
5. Bromberg, L.E., *Enhanced nasal retention of hydrophobically modified polyelectrolytes*. J Pharm Pharmacol, 2001. **53**(1): p. 109-14.
6. O'Hagan, D.T. and R. Rappuoli, *Novel approaches to vaccine delivery*. Pharm Res, 2004. **21**(9): p. 1519-30.
7. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland*. N Engl J Med, 2004. **350**(9): p. 896-903.
8. Flood, E.M., et al., *Children's Perceptions of Influenza Illness and Preferences for Influenza Vaccine*. Journal of Pediatric Health Care. **In Press, Corrected Proof**.
9. Wikipedia.org. *FluMist*. 2010; Available from: http://en.wikipedia.org/wiki/Nasal-spray_flu_vaccine.
10. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan*. Biomaterials, 2008. **29**(27): p. 3642-9.