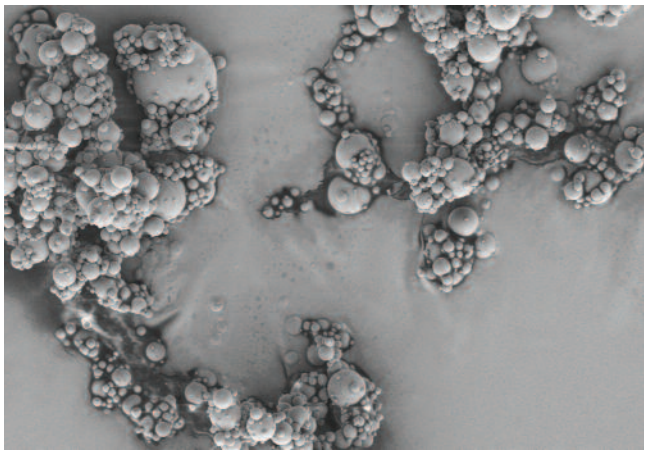
Overcoming challenges in development of nasal vaccines through intelligent particle engineering

Formulation approaches include nano- in microparticles, carrier-based blends or direct coating of the nanoparticles onto a carrier.



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Introduction

Vaccines are used to train the immune system to defend against diseases, which are primarily induced by either bacteria or viruses.[1] Vaccines are mostly administered for prophylactic vaccination against severe diseases such as polio, measles or influenza, however, there is increasing interest in the field of therapeutic vaccination for the treatment of autoimmune diseases or cancer.[2] Nowadays, the main administration route for human vaccines is parenteral, which results in a sound systemic immune response, but requires an injectable formulation. Alternative routes of administration, such as the oral route or inhalation route, make use of the mucosa, which often is the natural entry port for infections by pathogens.

The lymphoid tissue is rich in immune-competent cells such as B-cells and T-cells, dendritic cells (DCs) and macrophages.[3] In clusters (follicles) of the lymphoid tissue, specialized epithelial cells named microfold cells (M-cells), can be found. M-cells mediate uptake of pathogens or particles and presentation to underlying antigen presenting cells (APCs), whereas DCs are distributed throughout the mucosa serving as APCs. In the respiratory tract, follicle-associated lymphoid tissue with M-cells can be found in the tonsils and probably at the bronchial bifurcations,[4] whereas DCs are distributed within the nasal mucosa[5] and can be found all the way down to the alveoli. Consequently, the rich presence of immune-competent cells, moderate environmental conditions with respect to pH and enzymatic challenges, and easy accessibility for self-administration make the respiratory tract an interesting target for vaccine delivery.

In terms of formulation, mucosal administration is not limited to solutions or suspensions, but may also take the form of a gel or a dry powder formulation that can be administered either via a nasal disperser or as a dry powder inhalate. Furthermore, particulate vehicles may mimic pathogens in size and surface characteristics, therefore, they show increased immunogenicity compared to soluble antigens and are well taken up by immune-competent cells.[6] They also can induce cross-presentation of the carried antigen, which improves the immune response.[7] Moreover, they allow antigen stabilization and protection; the antigen can be incorporated and the particle surface may be functionalized to tailor cellular uptake and release. Finally, particulate systems allow local immune processing following uptake. Among particulate vac-

cine vehicles, polymeric particles are the most investigated. A wide range of polymers such as the biodegradable polymers polylactic acid, polyglycolic acid and their copolymers, cationic polymers or biopolymers such as chitosan or alginate may be considered as vehicles for vaccine formulations.[8]

Challenges of formulating inhalable vaccines include the following:

• antigen stabilization;

• achieving optimal particle size for the vaccine vehicle as well as aerodynamic properties of the dry powder formulation; and

• efficient delivery and uptake to ensure processing in immune-competent cells

All of the above challenges are interconnected. Whereas viruses or bacteria as natural pathogens might be very stable in harsh conditions, most antigens used in vaccines are subunits of pathogens or purified proteins. As such, they are sensitive biomolecules and do not tolerate major changes in temperature, dryness or environmental conditions.[9] An injectable dosage of vaccine is normally formulated in liquid form or as a freeze-dried powder to be dissolved in a solvent suitable for injection. Both the vaccine and solvent need to be stored and transported under refrigerated conditions since freezing/thawing or storage at elevated temperatures may lead to aggregation and loss of immunogenicity.

Instability of a vaccine may be overcome by the addition of stabilizing excipients, such as buffer substances or surfactants.[10] A range of drying techniques including freeze drying, spray drying and combinations thereof, vacuum drying and supercritical fluid drying have also been evaluated for the stabilization of biomolecules or antigens. All offer advantages and disadvantages.[11] As an example, Garmise, et al. showed that for a spray-freeze-dried, whole, inactivated influenza vaccine, drying can lead to a product with increased thermostability compared to a liquid preparation.[12]

The particle size of the vaccine vehicle has implications on the possible uptake route, which can either be via endocytosis through the epithelium and the DCs or by transcytosis using the M-cell route. Uptake by endocytosis is only possible for particles in the nanometer range[13] and shows a certain size-dependent efficacy in terms of the resulting immune response. Very small nanoparticles (~50 nm) are directly transported to the draining lymph node, resulting in a sound systemic immune response,[6] whereas particles larger than 100 nm are taken up and immune-processed locally. It is reported that treatment with smaller nanoparticles (230 nm) resulted in a higher immune response than with larger nanoparticles (700 nm).[14] Other studies have shown that although small particles are taken up to a larger extent, the resulting immune activation is higher for medium-size particles, as they were able to transport a higher amount of antigen per particle. For dendritic cell uptake, sizes between 200 nm and 400 nm seem to be optimal.[15] Transcytosis is possible for particles up to 10 µm, but M-cells will not deliver particles larger than 5 µm to the underlying antigen presenting cells.[16]

Interestingly, it is not only the particle size that influences uptake and immune recognition, but the aspect ratio[17] and surface characteristics of the vaccine vehicle, such as charge and hydrophobicity.[18] Although for uptake the geometric particle size is important, it is the aerodynamic particle size that determines dispersion and deposition in the respiratory tract.[19]

Depending on the desired location for vaccine deposition and delivery, particle size requirements may vary. For a measles vaccine, targeting the complete lower respiratory tract (including the deep lung), an aerodynamic particle size of 1-5 µm might be advantageous.[20] However, for an influenza vaccine, the nasal mucosa might be a good location for immunization and therefore, the particle size must be above 10 µm to avoid a respirable fraction.[21]

Furthermore, although nanoparticles will be needed to allow particulate uptake, nanoparticles will not be dispersed as individual particles, but as larger, uncontrolled agglomerates. Consequently, an additional formulation step will probably be required to process the nanoparticulate vaccine vehicle needed for uptake to larger particle agglomerates in the required aerodynamic size.

The studies presented below show formulation approaches that can meet the challenges of nasal dry powder vaccines.

Methods

Preparation of nanoparticles and further processing. Nanoparticles were produced from chitosan (Chitopharm S, Cognis, Germany) or agarose (AppliChem GmbH, Germany) by ionic gelation or by an alcoholic precipitation step, respectively, as described previously.[22,23] The model antigen was ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO, US), which was incorporated during nanoparticle formation.

Nano- in Microparticles (NiM). The nanoparticle suspension was mixed with an aqueous mannitol solution (Pearlitol, Roquette, LeStrem, France) and then spray dried to microparticles using a Büchi B-290 laboratory spray dryer (Büchi, Flawil, Switzerland).

Carrier-based blend. For the carrier-based blend, the spray dried chitosan NiM were blended with a spray granulated mannitol (Pearlitol 200 SD, Roquette, Lestrem, France) in a sieve fraction of 45-90 µm using the Turbula mixer (Willy A. Bachofen AG, Basel, Switzerland) for 10 minutes, resulting in a blend containing 15% of spray dried product.

Nanosuspension coating. A similar carrier (Pearlitol 100 SD in a sieve fraction of 45-90 µm) was coated directly with a suspension of agarose nanoparticles. The coating was performed in a Ventilus lab coater (InnoJet/Herbert Hüttlin, Steinen, Germany) at a spray rate of 1.5-2.5 g/min and a product temperature of 35-38°C.

Characterization methods

Particle size. The particle size of the nanoparticles, in suspension and after redispersion in an aqueous liquid, was characterized by dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The particle size of the microparticles and blends was determined by laser diffraction using the Sympatec Helos system (Sympatec, Clausthal-Zellerfeld, Germany), either by dry powder dispersion at 3 bar or by using the Sprayer module that directly measures the size of particles or droplets present in the plume formed upon actuation of the corresponding nasal administration device.

Morphology and habit. For the NiM, scanning electron microscopy (SEM, DSM 940, Zeiss, Oberkochen, Germany) was used to determine particle habit and detect agglomerates.

In vitro release. Release was tested using simulated nasal fluid,[24] pH 6.4 at 34°C, mimicking the conditions in the nasal cavity and using a pH 5.5 phosphate buffer solution at 37°C, mimicking endosomal conditions.[25] 300 mg of NiM were suspended in 10 mL of dissolution media and incubated in a shaking water bath at the respective temperatures. 300 µL samples were taken at the respective sampling points and protein quantification was performed by UV measurement at 230 nm using a standard calibration curve.

Nasal dispersion device. For formulation dispersion, the Powder UDS device from Aptar (Aptar Pharma, Louveciennes, France) was used. This is a single-use, single dose device, where the dose is held in a protected dose chamber placed centrally in the nasal adapter (Figure 1).

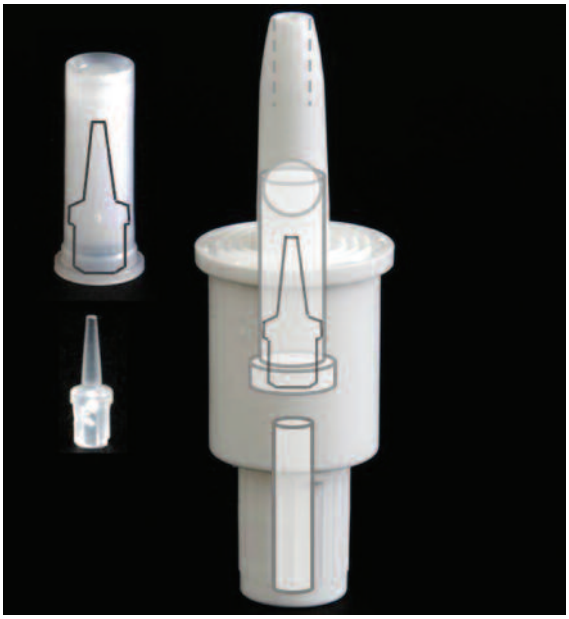
Nasal deposition. Deposition studies[26] were carried out using a nasal cast model (Boehringer Ingelheim, Ingelheim, Germany),[27] based on a CT scan of a human male nasal cavity. The formulation was administered into the coated nasal cast model, operated with and without simulated inspiration air flow (15 L/min and 0 L/min), respectively, using the Powder UDS device. The nasal cast segments were rinsed and protein content was determined by BCA assay (Micro-BCA assay kit, Thermo Scientific, Rockford, IL, USA).

Results and discussion

The nanoparticle preparation reproducibly resulted in particles of about 260 nm, as confirmed independently for the agarose particles by transmission electron microscopy (TEM). In addition, the samples showed a polydispersity index (PDI) below 0.25. As both polymers are biopolymers with certain variability in molecular weight, a very narrow monomodal particle size distribution with a PDI < 0.1 is not expected. The nanoparticles were stabilized in the microparticulate mannitol matrix by spray drying and could be easily redispersed by dissolving the matrix. The resulting particle size was slightly smaller than the original particles in the case of chitosan (~200 nm), whereas they were slightly larger in the case of agarose (~ 300 nm), but no major aggregates were detected. This observation is suspected to be due to shrinking of the particles upon drying and differences in the degree of swelling upon redispersion because agarose is more hydrophilic than chitosan. The particles were able to stabilize the model antigen ovalbumin

Figure 1

Image and schematic of the Powder UDS depicting the central container bearing the single dose and rod for actuation.



(OVA) during spray drying without degradation as shown by SDS-PAGE electrophoresis and the antigen could be redissolved from the dried particles in the native state as confirmed by circular dichroism (data not shown). OVA is a good model antigen for vaccine preparations as it is safe to handle and can be used for *in vivo* characterization in a mouse model. In order to further evaluate stabilization in terms of activity, additional studies using a model antigen such as lactate dehydrogenase or haemagglutinin would be needed, which would also allow *in vitro* activity testing.

The microparticles obtained after spray drying with mannitol as the matrix component had a size in the low micrometer range (x_{50} = 2.2 µm for agarose and x_{50} = 5.55 µm for chitosan nano- in microparticles). The SEM images show highly aggregated particles for the chitosan batch (Figure 2). Nonetheless, regarding the aerodynamic behavior of the chitosan NiM, it is very probable that nasal administration could result in a large, undesired, post-nasal fraction.[28] Therefore, a carrier-based blend was produced. Laser diffraction measurements showed that no measurable fine particle fraction was detected in the particle size distribution when the blend was dispersed by means of the Powder UDS device (Figure 3). This demonstrates that blending the chitosan NiM agglomerates with a rough carrier, such as the spray granulated mannitol, results in an interactive mixture that has a low tendency to separate upon dispersion. Consequently, the NiM remain on the carrier and will be deposited together with the carrier. As the carrier used is in a size range that does not permit it to pass through the nasal cavity,[26] the resulting carrier-based formulation will deposit in the nose only.

Although the agarose NiM have a smaller mean particle size than chitosan NiM, their aerodynamic characteristics are different and consequently not much material was found in the post-nasal fraction upon dry powder administration with the Powder UDS to the nasal cast model (Figure 4). These particles are predominantly deposited in the nasal vestibule, which is the narrowest part of the nose, whereas the particles passing the vestibule are mainly deposited in the middle and upper turbinates. This is the area where the narrow nasal adapter of the Powder UDS device aims upon actuation. The deposition profile is only slightly changed when simulated inspiration airflow is applied to the model, resulting in a shift towards the posterior parts of the nose and also increasing the post-nasal fraction. To interpret this profile, an ideal deposition profile was calculated,[23] which

Figure 2

Scanning electron micrograph images of chitosan NiM (left) and agarose NiM (right); magnification 500x (left) and 1000x (right).

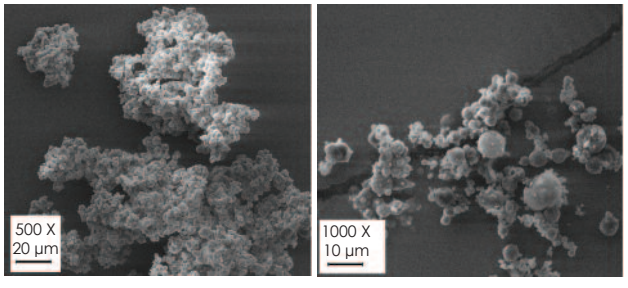


Figure 3

Particle size distribution of chitosan NiM (green) and chitosan NiM-mannitol carrier blend (yellow) upon dispersion with Powder UDS device, measured by laser diffraction.[22]

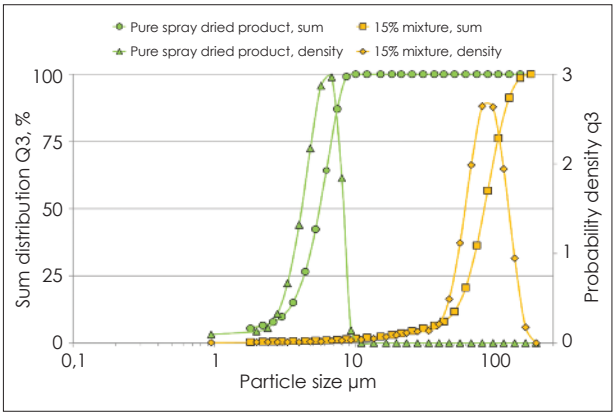
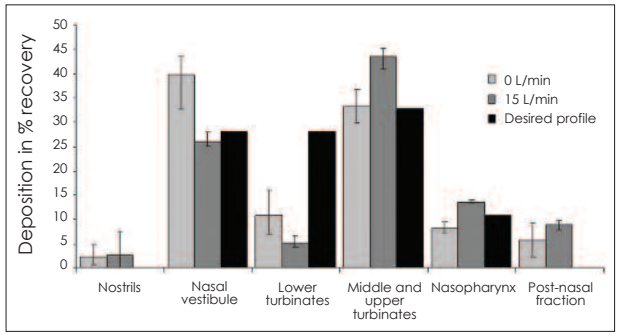


Figure 4

***In vitro* nasal deposition profile of agarose NiM at 0 and 15L/min, compared to an ideal deposition profile[23] (n=3, error bars represent min-max). Reproduced with permission of VCU/RDD Online.**



aims at an even distribution on the inner surface of the model, excluding the nostrils and taking into account the differences in surface area for the different parts of the nasal cast model. Comparing this ideal profile with the measured deposition of agarose NiM, it can be seen that the deposition in the lower turbinates should ideally be increased and vestibule deposition decreased, whereas the other parts have acceptable deposition. It might be arguable whether a higher deposition in the lower turbinates is indeed preferable, as this part of the nose is cleared more rapidly than the upper turbinates[29] and therefore, the formulation would have less time for matrix dissolution and nanoparticle uptake.

Within the agarose NiM formulation, only a certain part of the OVA added during nanoparticle preparation (0.5 mg OVA per mg of agarose) is directly associated with the nanoparticles, resulting in an antigen load of 6.6% (m/m), whereas the majority is incorporated as free antigen within the mannitol matrix. This allows rapid presence of soluble antigen as soon as the matrix dissolves, whereas the nanoparticles will carry their load into the cell upon internalization. In an OVA release study, it was observed that the mannitol matrix dissolves within 30 minutes in simulated nasal fluid (pH 6.4 at 34°C) releasing about 80% OVA from the formulation. The remaining antigen is associated with the nanoparticles and is not released in this media (Figure 5), which is in good agreement with the determination of antigen load. If OVA release is tested in a phosphate buffered system of pH 5.5 at 37°C, mimicking the endosomal pH, 100% OVA is released from the NiM formulation within 30 minutes, indicating that the antigen will be completely released from the nanoparticles after uptake and translocation to an endosomal compartment. Intracellular release is necessary in order to allow immune recognition and processing. Preliminary uptake experiments in BMDC (bone marrow-derived dendritic cells) indicate that the nanoparticles were well taken up upon NiM dissolution and may activate dendritic cells as seen from cellular markers.[30]

Another formulation approach used direct coating of the nanosuspension onto a larger carrier, which should facilitate nose-exclusive deposition. Here, possible further processing steps (blending or similar) are avoided. Product temperature was kept below 40°C during the coating process so as not to harm the protein which, together with the challenge of avoiding dissolution of the mannitol carrier in the aqueous coating media, resulted in long process times. As a result, the particle size distribution was reduced to smaller particles (Figure 6). Even though the initially-chosen carrier size should not allow nasal passage, the product gave a certain post-nasal fraction which was due to the diminution process during coating. This can be explained by partial dissolution and therefore diminution of the carrier, as well as the formation of nanoparticle agglomerates that were dried from the coating suspension without being coated on a carrier particle. Nonetheless, intact nanoparticles could be measured after redispersion of the coated product. Further process optimization is needed to overcome these issues in order to become a feasible alternative.

Figure 5

Cumulative release of OVA from agarose NiM in simulated nasal fluid (SNF, pH 6.4) and at endosomal pH (phosphate buffer, PBS, pH 5.5), error bars are standard error of the mean.

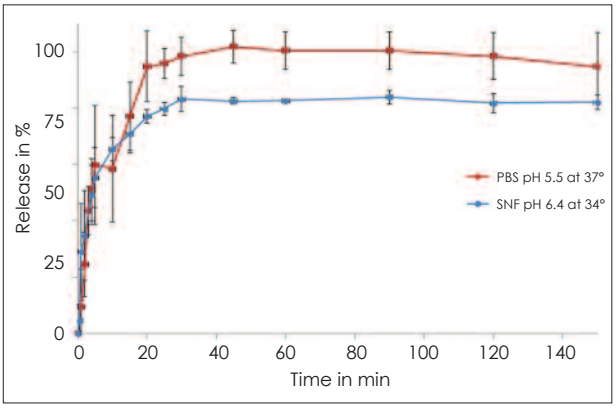
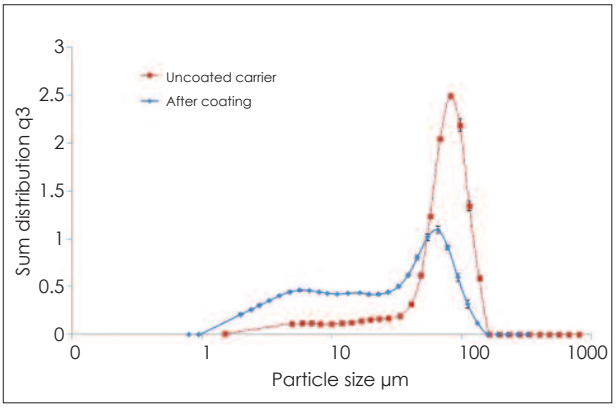


Figure 6

Particle size distribution before and after the coating process, measured by laser diffraction (dry dispersion).



Conclusion

To overcome the challenges in formulating inhaled vaccines, it is necessary to ensure antigen stability. This may be facilitated by immobilization in a polymeric matrix, which can be dried and may show increased thermostability. The primary particles should be formulated in a size that allows particulate uptake, has some immunogenic features and releases the antigen upon uptake. Finally, these particles need to be formulated to a respirable formulation with the correct aerodynamic properties for the targeted deposition site and chosen device. It was shown that these requirements can be addressed by employing formulation approaches including nano- in microparticles, carrier-based blends or direct coating of the nanoparticles onto a carrier.

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