Summary

Influenza vaccination is the key stone in controlling re-occurring influenza epidemics. The studies described in this thesis evaluated dry-powder formulation of influenza vaccines and explored administration strategies for the development of needle-free dosage forms of influenza vaccines.

Stabilization of influenza vaccines

The first objective of the studies described in this thesis was to study and develop methods for improved influenza vaccine stability. Current inactivated influenza vaccines are mostly formulations composed of whole inactivated virus, virosomes, split virus or subunit antigen, i.e. purified haemagglutinin (HA) and neuraminidase (NA). Today these liquid vaccines have to be handled under refrigerated conditions (2-8°C), with far-reaching consequences like high costs, because of transport and storage issues. Although the logistics of the vaccines is performed under a cold chain regime, accidental storage at elevated temperatures and/or freeze thaw cycles may occur. This may result in deterioration of the vaccine compound. An influenza vaccine that is stable at ambient temperatures and not sensitive to freezing stresses would reduce the dependency on cold-chain facilities and would therefore allow the integration of the vaccine logistics with general drug distribution; especially in developing countries this would be highly attractive. Moreover, this would reduce the risk of vaccine losses caused by "off-label" storage. Overall this would result in enormous annual savings. In addition, a stable vaccine formulation would facilitate stockpiling of potential vaccines against pandemic viruses, which provides an immediate availability and simple distribution of vaccine in a pandemic situation. A potentially successful strategy to stabilize biopharmaceuticals, such as proteins, vaccines and gene delivery systems, is to dry them in the presence of sugars. If dried properly, the biopharmaceutical is incorporated in a glassy matrix of amorphous sugar and thereby stabilized during subsequent storage. Dry-powder formulations, which are less dependent on a cold chain, of two vaccine types (a subunit and a virosomal vaccine) were investigated.

In **Chapter III**, the design of a stable influenza subunit vaccine in the dry state using lyophilization as drying method is presented. It was shown that HA in influenza subunit vaccines is susceptible to freezing and drying stresses, especially at low freezing rates. The use of PBS during lyophilization of subunit vaccine resulted in strong pH changes (due to crystallization of sodium or potassium dibasic phosphate during freezing) leading to conformational changes of HA. The conformational changes of HA during freezing could be prevented by the use of another buffer, hepes (HBS), that does not crystallize and consequently does not result in strong pH changes during freezing. Independent of the buffer used the use of carbohydrates (trehalose, inulin or dextran) as cryo- and lyoprotectants prevented or reduced conformational changes of HA. Subunit vaccine lyophilized with trehalose and inulin was stable for at least 26 weeks at room temperature (20°C). In contrast, vaccine incorporated in a glassy matrix of dextran 56 kD substantially lost its potency during storage for 26 weeks. At elevated temperatures the subunit vac-

cine lyophilized in trehalose was most stable. It was concluded that the use of fast freezing, hepes buffer and the choice for effective carbohydrates (trehalose or inulin) as cryo- and lyoprotectants enables the preparation of a stable subunit vaccine powder by lyophilization.

In **Chapter IV** the formulation of influenza virosomes as a stable dry-powder by freeze-drying (lyophilization) using an amorphous inulin matrix as a stabilizer is presented. In the presence of inulin the structural integrity and fusogenic activity of virosomes were fully preserved during freeze-drying. For example, the immunologic properties of the virosomes, i.e. the HA potency *in vitro* and the immunogenic potential *in vivo*, were maintained during lyophilization in the presence of inulin. In addition, compared to virosomes dispersed in buffer, inulin-formulated virosomes showed a substantially prolonged shelf-life and preservation of the HA potency, upon storage. Also the capacity of virosomes to mediate cellular delivery of macromolecules (e.g. pDNA) was maintained during lyophilization in the presence of inulin and upon subsequent storage. Specifically, when dispersed in buffer, virosomes with encapsulated plasmid DNA lost their transfection activity completely within 6 weeks, whereas their transfection activity was fully preserved for at least 12 weeks after incorporation in an inulin matrix. It was concluded that lyophilization in the presence of inulin as a stabilizing agent, considerably prolonged the shelf-life of influenza virosomes with and without encapsulated macromolecules.

Needle-free dosage forms

The second objective of the studies described in this thesis was to study administration strategies for the development of needle-free dosage forms of influenza vaccines. Current inactivated influenza vaccines are generally administered via the intramuscular (i.m.) or subcutaneous (s.c.) route using needles and syringes. Needle-free delivery, such as mucosal delivery via the respiratory or gastro-intestinal tract, may provide several potential advantages in vaccine delivery, such as eliminated pain at the injection site, easier and faster vaccine distribution and administration, and reduced costs. In addition, an important and promising advantage of mucosal vaccination is that it, in contrast to i.m. vaccines, may result in a local immune response in the respiratory tract. As a result antibodies in the respiratory tract might give protection against influenza infection at the port of entry. In addition, since mucosal IgA responses have been shown to exhibit cross-protective immunity against antigenically distinct viruses, such a mucosal immune response might offer broader protection against drifted, heterologous strains. Unfortunately, despite these potential advantages, until now mucosal vaccination approaches have suffered from several limitations or practical problems related to the use of inadequate or old-fashioned delivery technologies, and thus have frequently resulted in inadequate antibody responses or even in a state of immunological tolerance. Therefore, marketed influenza vaccines, being in the liquid state, are still mainly administered through injection. However, recent developments in the area of vaccine formulation and delivery

technologies now allow efficient delivery of vaccines to specific sites in the human body and therefore provide new opportunities for the use of alternative needle-free dosage forms of influenza vaccines. This thesis addresses some of the issues involved in this development.

In **Chapter V** it was investigated to which part of the gastro-intestinal (GI) tract, the upper part or the lower part, an oral influenza vaccine should be targeted to result in an effective immune response in mice. Our study demonstrated that without adjuvant substantial systemic but low respiratory mucosal immune responses were induced in mice after delivery of influenza subunit vaccine to the upper GI-tract (intragastric) or the lower GI-tract (intracolonic). In order to enhance the immune responses of the immunizations, *E.Coli* heat-labile enterotoxin (LT) was added as a model for a strong adjuvant. LT, indeed, enhanced the immune responses of the intragastric and intracolonic immunizations significantly. Interestingly, intracolonic administration of vaccine with adjuvant also resulted in enhanced cellular immune responses and the desired Th1-skewing of these responses. Intragastrically administration of the adjuvanted vaccine also increased T-helper responses. However Th1-skewing was absent. The differences in cellular immunity between the LT-adjuvanted groups were in correspondence with the IgG subtype profile. While IgG1 secretion was increased by LT in intragastric immunized mice, this increase was not found in intracolonic immunized mice. It was concluded, that the right combination of strong mucosal adjuvant (e.g. LT) and antigen delivery site (e.g. the lower part of the gastro-intestinal tract) might result in effective vaccination via the oral route.

In **Chapter VI** pulmonary vaccination with a new influenza subunit vaccine powder was evaluated. Vaccine powder was produced by spray-freeze drying (SFD) using inulin as stabilizer. The new powder formulation of subunit influenza vaccine described in this chapter, has the potential to be used for vaccination by dry powder inhalation in humans. First, it was shown that the vaccine antigen, HA, retained its structural and antigenic properties after SFD using inulin as stabilizer. Secondly, the SFD formulation is suitable for vaccine delivery into the lungs by inhalation. The SFD formulation consisted of large porous particles having an average aerodynamic diameter of 5.3 μm with a broad size distribution (2-12 μm) facilitating vaccine deposition over the large surface area of the lungs. As a result the SFD particles are capable to be deposited throughout the entire lung, including the lower airways, which is supported by the FPF of the SFD formulation (38%) measured with cascade impactor analysis.

It was demonstrates that pulmonary administration of the influenza subunit vaccine powder, induced strong cell-mediated as well as systemic and mucosal humoral immune responses in Balb/c mice. These responses were superior to those elicited by conventional i.m. vaccination or pulmonary vaccination with a liquid aerosolized subunit vaccine. The superiority of the SFD vaccine powder compared to the aerosolized liquid subunit vaccine might be ascribed to: the lower respiratory tract deposition of the powder and increased residence time in the lungs. This increased residence time can be the result of a lower respiratory tract deposition as well as an increased viscosity at the site of deposition caused by the inulin.

With respect to the cell-mediated immune responses, pulmonary delivery of the SFD formulation resulted in higher numbers of IFNγ- and IL4-producing T-helper cells than the conventional i.m. injection of influenza subunit vaccine. Moreover, the phenotype of these immune responses was more balanced. The relative high contribution of Th1 cells is important, since they are superior to Th2 cells in providing protection against viral infection and can provide a certain degree of cross-protective immunity.
In conclusion, this study demonstrated that the combination of SFD antigen powder and pulmonary antigen delivery improves the immunogenic potential of (influenza subunit) antigen. Vaccination with an SFD subunit vaccine powder by inhalation might be feasible and could be an alternative to conventional parenteral vaccine administration.

Conclusions

In this thesis formulation and delivery strategies are presented that finally may result in a stabilized influenza vaccine that is administered via a non-parenteral route.
Incorporation of vaccines in amorphous glassy sugar matrices has been shown to have the potential to solve the problems associated with the cold chain requirement of liquid vaccines. These solid vaccine powders are interesting starting materials for the development of non-parenteral dosage forms for influenza vaccines. Various strategies, such as oral or pulmonary delivery may evolve in successful non-parenteral dosage forms for influenza vaccines. Critical re-evaluation of old clinical studies, the use of new (up-to-date) delivery technologies, site specific vaccine delivery together with new adjuvants may facilitate the development of such needle-free influenza vaccines.
Such vaccination strategies, based on stable influenza vaccine powders, may lead not only to influenza vaccinations that provide broader protection against new emerging influenza viruses, but also would facilitate vaccination of people in "hard to reach" areas with a temperature-resistant self-administerable and needle-free influenza vaccine.