THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Design of solid dosage forms for mucosal vaccination

Investigations on the influence of excipients on product performance

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DESIGN OF SOLID DOSAGE FORMS FOR MUCOSAL VACCINATION
INVESTIGATIONS ON THE INFLUENCE OF EXCIPIENTS ON PRODUCT PERFORMANCE

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Cover: Image showing fictitious vaccine tablets

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ABSTRACT

Most vaccines today are liquid formulations for parental administration. However, there are several drawbacks connected to these vaccines. Since injectable vaccines mainly induce systemic immune responses, they are usually not effective against many important pathogens that affect mucosal surfaces. Furthermore, liquid injectable vaccines need medical personnel for the administration, require cold chain maintenance and occupy large cargo sizes, which all are negative factors especially for vaccine use in developing countries. Solid and preferably mucoadhesive vaccine formulations that are administered via mucosal surfaces may overcome many of these problems. The aim of this thesis was therefore to investigate the design of such formulations with special emphasis on the influence of excipients on i) formulation-related properties such as mucoadhesion and antigen release and ii) antigen-functionality preservation during freeze-dying.

In the first part of the thesis mechanistic and immunological investigations using mucoadhesive hydrophilic matrix tablets as potential formulations for sublingual immunization were performed. The effect of osmotic pressure differences on the adhesiveness of hydrophilic swelling matrix tablets was investigated with a model where the osmotic pressure difference could be tuned with different concentrations of additives. It was found that a decrease in the osmotic pressure difference resulted in a decrease in the adhesive force, i.e. the force required to detach the tablet from a wet surface. Release of the model antigen ovalbumin from hydrophilic matrix tablets providing an extended release and from a fast releasing formulation was characterized. The Bradford Assay used for the protein quantification was found to be disturbed by the hydrophilic polymer Carbopol$^{®}$ and a correction method was established. Sublingual immunizations in BALB/c mice indicated a poor potential of all extended release tablets to evoke intestinal immune responses, whereas an immediate release tablet formulation resulted in high antibody titres. Thus it was concluded that the latter formulation type should be preferred in sublingual immunization. In the second part of the thesis the stabilizing potential of different excipients during freeze-drying was tested using killed whole-cell *Vibrio cholerae* bacteria as a model vaccine for pathogens causing enteric infections. Sucrose showed great potential to avoid bacterial aggregation, preserve important antigen structures and maintained the immunogenicity of the bacteria. The addition of sucrose proved also useful for preparing a fully immunogenic formulation of the commercial oral cholera vaccine Dukoral^{TM} with regard to both antibacterial and antitoxic antibodies.

Keywords: *mucosal vaccination, sublingual vaccine tablet, mucoadhesion, osmotic pressure, hydrophilic matrix tablets, Bradford Assay, protein release, Vibrio cholerae, freeze-drying*

"Coming together is the beginning.

Keeping together is progress.

Working together is success."

Henry Ford

Für meine Familie

LIST OF PUBLICATIONS

This thesis is based on the following scientific papers, referred to by their Roman numerals in the text. The papers are appended at the end of the thesis.

Paper I.   **Osmotic-driven mass transport of water: Impact on the adhesiveness of hydrophilic polymers**
Annika Borde, Anna Bergstrand, Cecilia Gunnarsson, Anette Larsson
Journal of Colloid and Interface Science 2010, 341, 255–260

Paper II.   **Quantification of protein concentration by the Bradford method in the presence of pharmaceutical polymers**
Nils Carlsson, Annika Borde, Sebastian Wölfel, Björn Åkerman, Anette Larsson
Analytical Biochemistry 2011, 411, 116–121

Paper III.   **Effect of protein release rates from tablet formulations on the immune response after sublingual immunization**
Annika Borde, Annelie Ekman, Jan Holmgren, Anette Larsson
European Journal of Pharmaceutical Sciences, 2012, 47, 695-700

Paper IV.   **Preparation and evaluation of a freeze-dried oral killed cholera vaccine formulation**
Annika Borde, Anette Larsson, Jan Holmgren, Erik Nygren
European Journal of Pharmaceutics and Biopharmaceutics, 2011, 79, 508-518

CONTRIBUTION REPORT

Paper I.   Responsible for the main part of the experimental work. Responsible for the interpretation of all results and writing the manuscript with input from the co-authors.

Paper II.   Shared responsibility for the experimental outline and minor responsibility for the analysis of all data. The experimental work was performed together with another PhD student as well as a MSc student under my supervision. Shared responsibility for writing the manuscript.

Paper III.   Responsible for all the experimental work. Responsible for interpretation of all results and writing the manuscript with input from the co-authors.

Paper IV.   Responsible for all the experimental work, except the SEM evaluations which were shared. Main responsibility for analysis and interpretation of all results. Responsible for writing the manuscript with input from the co-authors.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Paper V.   **Increased water transport in PDMS silicone films by addition of excipients**
Annika Borde, Mikael Larsson, Ylva Odelberg, Peter Löwenhielm, Anette Larsson
Acta Biomaterialia, 2012, 8, 579-588

Paper VI.   **Evaluation of carboxymethyl-hexanoyl chitosan as a protein nanocarrier using bovine serum albumin**
Mikael Larsson, Annika Borde, Emma Mattisson, Anette Larsson
Submitted

ABBREVIATIONS

ANOVA	analysis of variance
APC	antigen presenting cell
API	active pharmaceutical ingredient
AUC	area under the curve
B cell	bursa-derived cell
$CaCl_2$	calcium chloride
CB	Coomassie Blue
CT	cholera toxin
CTB	cholera toxin B-subunit
DCs	dendritic cells
ELISA	enzyme-linked immune-sorbent assay
ER tablet	extended release tablet
ETEC	enterotoxigenic *Escherichia coli*
LPS	lipopolysaccharide
LSCM	laser scanning confocal microscopy
MALT	mucosa-associated mucosal tissue
MCC	microcrystalline cellulose
MHC	major histocompatibility complex
NaCl	sodium chloride
PBS	phosphate buffered saline
pIg	polymeric immunoglobulin
pIgA	polymeric IgA
SC	secretory component
SIgA	secretory IgA
T cell	thymus cell
USP	United States Pharmacopeia
V. cholerae	*Vibrio cholerae*
WHO	World Health Organization

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Chapter 1

INTRODUCTION

Human disease is as old as human life. Today many diseases can be treated with medicines and in many cases we are even able to prevent the population from specific diseases by the use of vaccinations. The term vaccination (Latin: *vacca = cow*) dates from the first documented immunization studies in 1796 when the English physician Edward Jenner tested the use of the relatively benign cowpox virus to provide protection against the related and deadly smallpox virus [1]. His experiments led to the discovery that protection against specific diseases can be achieved by exposing the human being to the causative or a related agent for the disease [2]. From then on, the development and use of various vaccines has led to the protection of children and adults from previously lethal diseases. Today the worldwide use of vaccines is considered to be the most effective strategy to prevent, control and even eradicate infectious diseases. One successful example of the latter is the global eradication of smallpox, which was diagnosed the last time in 1978 and was announced to be eradicated in 1980 by the World Health Organization (WHO) [1].

Today there are about 40 vaccines on the market and most of them are liquid injectable vaccines. They often provide protection against specific diseases over several years or even long-life. So why design solid formulations for mucosal vaccination, i.e. vaccine administration via mucosal surfaces? People still die and fall ill from a variety of diseases although preventive vaccines against these diseases exist and for many diseases no effective vaccines exist at all. One main reason for the latter is that many of these diseases are caused by infections that start from or affect mucosal surfaces, such as the gastrointestinal tract, respiratory or genital tract. Injectable vaccines elicit systemic immune responses and may be effective at mucosal surfaces that are to some extent permeable for serum-derived antibodies. A mucosal vaccination seems though critical for the protection against infections at mucosal surfaces that are impermeable to antibodies, such as the gastrointestinal tract. An administration of the vaccine via a mucosal surface would instead stimulate the mucosal immune system which protects the different mucosal surfaces and thereby also the body interior. Besides immunological aspects, the injection of a vaccine is painful and includes a risk for the transmission of infections. Furthermore, the often high manufacturing costs, voluminous package sizes as well as cold chain

requirements of liquid injectable formulations cause high health care costs and impede distribution and storage, especially in developing countries, where the need for medical personnel for the administration is a further hindrance. Considering all these aspects, solid formulations for mucosal administration could be a beneficial alternative to meet all these challenges. Solid vaccine formulations for mucosal administration could thus provide several benefits to overcome the mentioned challenges. Pathogens might already be stopped at their portal of entry by the mucosal immune defence. Smaller package sizes and probably better stability properties of a solid formulation would furthermore facilitate transport, distribution and storage and thus the use of the vaccine in mass vaccination campaigns, even in developing countries. The latter is of course not only an advantage for mucosal vaccines but also for injectable ones.

All things considered, it is highly probable that infections by mucosal pathogens, especially those affecting the gastrointestinal tract, can be controlled in all parts of the world if effective mucosal vaccines that are appropriately designed and formulated can be developed. This interdisciplinary work aims to combine the benefits of mucosal immunization and of solid state dosage forms and deals with different issues concerning the influence of excipients in the formulation design.

Chapter 2

AIMS OF THE THESIS

In the design of mucosal vaccines it is important to consider crucial formulation properties in relation to the required functionality and efficacy of the dosage form. The intention of this thesis was to investigate the influence of excipients in the design of solid state vaccine formulations for mucosal administration. The following areas, where excipients are important, were investigated: mucoadhesion mechanism and mucoadhesive properties, protein analysis in the presence of pharmaceutical excipients, controlled protein release from sublingual tablets and the effect on the immune response in mice, and freeze-drying of killed whole-cell bacteria. Many of these factors have been discussed in the literature in different contents whereas others have not been investigated at all so that more detailed and also more applied investigations are required.

The specific aims of the papers included in this thesis were:

- To study the correlation between water mass transport due to osmotic pressure differences and mucoadhesion of dry hydrophilic polymer tablets in order to gain more knowledge about the factors that influence mucoadhesion (Paper I).
- To critically examine the commonly used Bradford Method for protein concentration determination in the presence of different pharmaceutically relevant excipients (Paper II).
- To investigate the influence of protein release rate from tablets on the elicited immune responses after sublingual immunization (Paper III).
- To compare different excipient conditions during freeze-drying of bacteria with respect to the ability to prevent bacterial aggregation, maintain important morphological structures and preserve the oral immunogenicity of freeze-dried bacterial vaccines (Paper IV).

The next two chapters will give a background to these different topics in order to provide an easier understanding of the different studies and results.

Chapter 3

MUCOSAL VACCINATION

'Vaccination' or 'active immunization' involves the administration of an antigen or antigenic material that triggers the development of immunologic defence against a future exposure to this antigen [3]. An antigen (short for antibody generator), sometimes also termed immunogen, is a substance that binds to specific immune receptors and is recognized by the immune system as foreign [4]. Once recognized as foreign, the immune system, which includes tissues, cells and molecules, can elicit an immune response to protect the host against the invading pathogen. After such a response the immune system is trained to defend itself against the pathogen, i.e. it can recognize and efficiently respond to subsequent invasions by the same or related pathogens. This ability is called immunological memory and forms the basis of preventative vaccines. Training the immune system often requires repeated exposure to the antigen and hence the use of booster doses is required in many vaccine regimens.

Although numerous effective vaccines against systemic infections are available, the need for the development of vaccines against pathogens which cause infections of or via mucosal tissues still remains. Most often the mucosal route of vaccination is necessary in order to stimulate the local mucosal immune system. A brief description of the mucosal immune system and different mucosal sites for vaccination are given in the following chapters.

3.1 The mucosal immune system

In the human body specific mucosae line the aerodigestive and urogenital tracts, the eye conjunctiva, the inner ear and the ducts of all exocrine glands. The totally 400 m^2 of mucosal surfaces are in contact with the external environment and often exposed to external microbes and pathogens. Several mechanical and chemical cleaning mechanisms work together with a specialized mucosal innate and adaptive immune system in order to provide protection against the colonization at mucosal tissues and the invasion into the body interior by external pathogens and foreign proteins [5].

Evidence for a common mucosal immune system was already mentioned in 1979 by McDermott and Bienenstock [6]. As extensively discussed and reviewed elsewhere [7, 8], it is today known that lymph nodes draining the

mucosal tissues and aggregates of lymphocytes, so-called mucosa-associated lymphoid tissues (MALT), located below the mucosal surfaces form a highly compartmentalized mucosal immune system that functions independently from the systemic immune system and serves as mucosal inductive site for the initiation of immune responses. Like the systemic lymphoid tissues the MALT is populated by different types of lymphocytes, such as B- and T-lymphocytes (B- and T-cells). The MALT comprises about 80% of all immunoglobulin-producing cells in the human body and its main sites are the gut-associated lymphoid tissues (GALT), which include specialized structures called Peyer's patches in the small intestine, the bronchial-associated lymphoid tissues (BALT) and the nasal-associated lymphoid tissue (NALT) [9-11]. Their common denominator is that they all serve as mucosal inductive sites for the initiation of immune responses.

Antigens that enter our body are either taken up by absorptive epithelial cells or through specialized epithelial cells mechanisms. An example of a specialized uptake mechanism is the transport of antigens in the gut, where specialized microfold cells (M-cells) discovered in 1974 by Owen and Jones [12] line the Peyer's patches and facilitate antigen transport across the epithelium [13]. Having been identified as "dangerous" the antigen is captured and processed by so called antigen-presenting cells (APCs) of the innate immune system, mainly dendritic cells (DCs), B lymphocytes (B cells) and macrophages [14]. They load peptide fragments into either major histocompatibility complex (MHC)-I or MHC-II molecules on their surface for display of the antigenic material to adjacent $CD 4^+$ or $CD 8^+$ T lymphocytes (T cells), depending on the type of antigen [14]. The activated mucosal lymphocytes, both B and T cells, then move through the lymphatic system and enter the systemic circulation to finally end up at specific mucosal sites where differentiation into either effector or memory cells takes place [15]. As a result of this adaptive immune response increased antigenic specificity and memory are obtained. Figure 1 illustrates possible mechanisms for the uptake and processing of an exogenous antigen at the sublingual mucosa.
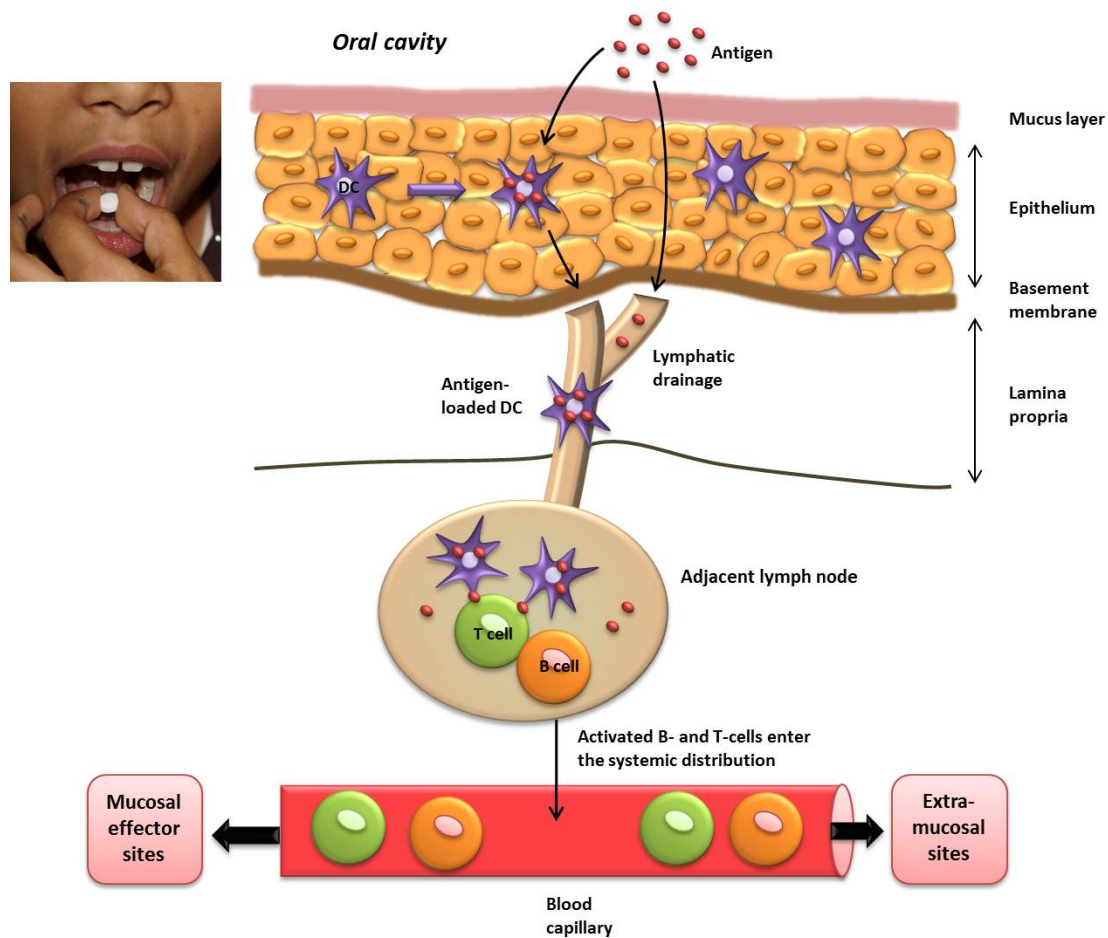
Figure 1. Schematic drawing that illustrates the uptake and processing of exogenous antigens at the sublingual mucosa. When an exogenous antigen is identified as "dangerous" antigen-presenting cells (APCs), such as dendritic cells (DCs), are recruited to capture antigen (as indicated by the purple arrow). The APCs and probably also some free antigen migrate via the draining lymph to lymph nodes, where they become active APCs that stimulate T and B cells for immune responses. The activated T and B cells can subsequently via the lymph enter the systemic circulation and migrate to either mucosal effector sites or extramucosal sites.

The dominant antibody type in external secretions, such as saliva or intestinal fluid, is IgA, mainly in the form of secretory IgA (SIgA). SIgA is a dimer of two monomeric IgA molecules that are covalently linked by a joining chain (J chain) molecule and non-covalently complexed with a so-called secretory component (SC) molecule produced by mucosal epithelial cells. The formation of this complex molecule can briefly be described as follows. The dimeric, or polymeric, IgA (pIgA) part is synthesized by plasma cells in the lamina propria of mucosal tissues. After binding to a transmembrane glycoprotein, called the polymeric immunoglobulin (pIg) receptor, on the basolateral side of the epithelial cells pIgA is transported by transcytosis to the apical side of the epithelium. There it is released from the cells into the mucosal secretions as final SIgA through a proteolytic cleavage of the transmembrane part of the pIg receptor leaving the rest of the receptor, the SC, associated with the pIgA [16].

In the often protease rich mucosal secretions the protease resistance of the SC molecule allows SIgA to survive for a long time [17]. Numerous studies have provided evidence for the protective role of SIgA [18-22]. The different mechanisms of protection include the ability of SIgA to hinder pathogens from penetration through and adhesion to the mucosa [23, 24], inhibition of multiplication and colonization as well as penetration of pathogens across epithelia [25, 26], neutralization of their toxins [27] and enhancement of non-specific defence mechanisms [28]. In addition, it has been found that SIgA can also limit the person-to-person transmission of pathogens [29].

3.2 Advantages of mucosal vaccination

Mucosal vaccination has gained a lot of interest in the recent years [30-32]. The mucosal route of vaccination offers several advantages. The needle-free administration increases patient compliance, decreases the risk for blood-borne infections due to the injection and reduces health care costs due to the ease of administration which does not require specialists. All these factors are especially of advantage in low income countries.

However, the main reason for using the mucosal route of immunization is the fact that, besides systemic immunity, the local mucosal immune system can also be stimulated which offers great possibilities as many infections are acquired at mucosal surfaces. Examples are gastrointestinal infections caused by *Vibrio cholerae* (*V. cholerae*), *Salmonellae* spp. or *Shigella* spp., respiratory infections caused by influenza virus or *Mycoplasma pneumoniae* or sexually transmitted genital infections caused by *Chlamydia trachomatis* or human immunodeficiency virus (HIV) [33]. For infections at mucosal sites with a good permeability for serum antibodies such as the lower respiratory tract and the female genital mucosae an injectable vaccine may still provide sufficient efficacy. However, when the infection occurs at a mucosal site with low or no antibody permeability to serum-derived antibodies that are induced upon parenteral immunization, a local mucosal administration of the antigen is most often crucial to achieve efficient protection. This is for example the case for gastrointestinal infections caused by *V. cholerae* or Enterotoxigenic *Escherichia coli* (ETEC) [33].

As recently reviewed by Holmgren and Czerkinsky [31], the effects after mucosal immunization are highly compartmentalized, even between regions within the same mucosal organ as for example the gastrointestinal tract. For example, oral immunization leads to considerable antibody levels in the

salivary glands, the stomach and the small intestine but elicits only poor responses in distal segments of the gut, whereas rectal immunization provokes the strongest local antibody responses in the rectum [34-36]. Since the mucosal tissues are interconnected via circulating lymphocytes, a local administration of vaccine may even initiate responses at other mucosal sites in the body [37]. However, the best response is thought to be induced at the mucosal site initially exposed to the antigen. Given the high degree of compartmentalization of the mucosal immune system, different factors have to be taken into account in order to select the appropriate route or maybe a combination of immunization routes when designing mucosal vaccines. Besides the most likely site of challenge where a protective immunity is desired, the advantages and disadvantages of the different administration sites also have to be considered.

3.3 Choice of mucosal vaccination route

The oral or nasal mucosa are traditional mucosal sites for the administration of vaccines, but also other mucosal routes such as the rectal, vaginal or transcutaneous and recently also the sublingual route have been investigated for inducing mucosal immunity. The objective of this thesis was not to study or compare different mucosal administration sites for vaccine delivery. Nonetheless, a brief comparison of the most common mucosal routes for immunization will be given since all investigations of my work were aimed for the development of improved mucosal vaccine delivery systems.

Oral immunization can induce substantial immune responses in the gastrointestinal tract, but also in the mammary and salivary glands [36, 38-40]. One successful example of a licensed oral vaccine is the oral cholera vaccine (Dukoral^{TM}). However, it is a challenge to develop oral vaccines with sufficient efficacy. A main problem in oral immunization is the degradation and destruction of antigens by several enzymes present in the gastrointestinal tract as well as the acidic environment in the stomach so that delivery systems that can sufficiently protect the antigen are required. In developing countries the efficacy of oral vaccines is additionally restricted by chronic environmental enteropathy, especially in children, which causes disturbances in the digestion and malabsorption due to overgrowth of the intestinal flora and local inflammation [5].

The respiratory tract including the highly permeable nasal mucosa seems a natural target for vaccines against severe infectious diseases of the respiratory tract, such as influenza and pneumonia, since strong antibody responses can be

induced in the mucosae of the airways and in nasal secretions. More surprising, the nasal mucosa has also been found to evoke antibody responses in cervical and vaginal secretions, thus providing a potential for vaccines against sexually transmitted infections, e.g. HIV [41]. However, there are some disadvantages of nasal immunization that should be considered. The nasal cavity provides a large absorption area [42], but natural differences in membrane permeability or diseases affecting the mucosal surface, such as allergic rhinitis, can lead to a high variation in the antigen uptake and thus to differences in the effectiveness of nasally administered vaccines. The volume and thus the dose of antigen that can be sprayed into the nose at once is limited [43]. Furthermore, it is known that antigens and adjuvants can be translocated to the olfactory bulb of the brain upon intranasal administration [44]. This retrograde transport to the brain can of course be an advantage for drugs with a desired effect in the central nervous system, but it raises severe concerns about safety issues for human nasal vaccines.

The sublingual mucosa, i.e. the mucosa under the tongue, has been studied for a long time and found to be advantageous as delivery route for low-molecular-weight drugs due to its easy accessibility and fast uptake of the active pharmaceutical ingredient (API) into the bloodstream [45]. During the last decade, this route has also received increasing attention as a delivery site for mucosal vaccines and immunotherapeutics. Several studies with different antigens have shown the efficacy of sublingual immunization [46-51] and sublingual immunotherapy has been successfully investigated and used with increasing frequency in allergy treatment [52-56]. The sublingual mucosal epithelium in humans is comprised of a non-keratinized, stratified, squamous epithelium located on top of a basement membrane. This membrane forms the boundary to the underlying lamina propria, which contains of a dense network of dendritic-like cells that are responsible for antigen capturing, as well as numerous mononuclear leucocytes, whereas MALT is not present [48, 57]. An induction of both mucosal and systemic immune responses is possible after sublingual vaccination with an exceptionally broad dissemination of effector cells to different mucosal surfaces including the gastrointestinal and the respiratory tracts and most interestingly also the genital mucosa [47, 49-51]. Thus, sublingual vaccination may offer an alternative to the nasal or oral route of immunization with various advantages. Although the human sublingual epithelium is thicker than that of the nasal cavity and the surface area of the sublingual mucosa is smaller than in the nose [58, 59], antigens are quickly absorbed via the sublingual mucosa without passing through the gastrointestinal tract so that enzymatic degradation, as occurs in oral

administration, is avoided [45]. Unresponsiveness of oral vaccines connected to chronic environmental enteropathy which is a huge problem in children of developing countries might possibly be circumvented by sublingual immunization. Furthermore, in comparison with nasal vaccination, the production of convenient delivery systems for sublingual vaccine delivery is not as challenging. Tablets or mucoadhesive films can for example be given sublingually, whereas a special delivery device such as an aerosol dispenser is often required for nasal administration. Moreover, and importantly in comparison to nasal immunization, sublingually administered antigens or adjuvants have not been found to be translocated to the olfactory bulb in the brain [60]. Thus, sublingual vaccines seem to be safer than intranasal vaccines from that point of view.

Taken everything into consideration, the sublingual route of immunization provides several advantages over traditional mucosal routes and has therefore become an attractive site for vaccination studies against different infections in the gastrointestinal tract, respiratory and female reproductive tract, although its effectiveness in humans remains to be determined. In this work, the effect of protein release rates in correlation to the immune response was studied for this vaccination route in order to gain knowledge about suitable tablet characteristics for potential sublingual vaccine candidates. The oral route was used in Paper IV to study the immunogenicity of freeze-dried whole-cell *V. cholerae* bacteria since a commercially available oral vaccine based on these bacteria was used as a model system.

3.4 Challenges in mucosal immunization

Despite the various advantages of mucosal immunization mentioned before, the question still crops up why there are only a few mucosal vaccines approved for human use on the market compared to over 30 registered injectable vaccines? Of course, the evaluation of the immunogenicity of a mucosal vaccine in humans is not as simple as for injectable ones since it is considerably easier to measure the systemic antibody response in the blood than to measure the mucosal immune response. However, a major problem is that proteins often behave as poor immunogens when delivered to mucosal surfaces. For a significant immune response, the antigen has to be exposed to the immune system in a sufficient amount, often much higher than needed in parenteral administration. After administration to a mucus membrane, the antigen has therefore to be transported across the epithelium to come into contact with APCs. The production of mucus as a natural protection barrier to keep

pathogens away from the epithelium, as well as dilution due to salivation and mechanical stress in the oral cavity, can however limit antigen exposure and uptake and thus antigen accessibility for the immune [61]. In addition, the high molecular weight of proteins is often a limiting factor, resulting in only small amounts of antigen crossing the intact mucosa to reach the immune competent cells [62]. In mucosal tissues with MALTs, this is less of a problem as specialized mechanisms are often available to deliver the antigen for example via transcytosis to APCs and lymphocytes located at the basolateral side [14]. However, even here the exposure is limited since only small areas are associated with these specialized regions. A problem which occurs more or less in all cases of mucosal delivery is the degradation of the antigen prior to or during the absorption process by mucosal and luminal enzymes. This is particularly a problem in the gastrointestinal tract, especially when the protein is delivered unprotected [63]. A further difficulty lies in the fact that many proteins, such as food proteins, are actively tolerated by the mucosal immune system [61]. Therefore, the co-administration of so called adjuvants is often necessary to activate APCs and/or other innate signalling pathways in the epithelial cells in order to enhance the immune response against the antigen. The development of safe and efficient adjuvants and appropriate delivery systems that can provide protection, increase antigen uptake and enhance the immune response to an antigen will thus be necessary in order to overcome the challenges one has to deal with in mucosal vaccination.

3.5 Oral killed whole-cell cholera vaccine as model for mucosal vaccines against enteric diseases

Enteric infections that cause billions of disease episodes and millions of deaths every year in developing countries, especially in children, and that are also a common cause of illness in travellers to affected regions continue to be a global health problem [64]. Vaccines are only available against some of these diseases and there is a great need for the further development of mucosal vaccine formulations against the many different disease causing agents. Cholera is a typical example of an enteric disease for which a protective vaccine exists and where the use of a mucosal vaccine is most efficient since cholera is caused by a non-invasive pathogen that remains at the apical side of the mucosal epithelia in the gastrointestinal tract. In spite of the available vaccines against cholera, it still causes high morbidity and mortality in parts of the world and continues to be one of the main causes of diarrhoeal diseases in many developing countries where it is a significant negative factor for the economic development. Cholera

is endemic in over 50 countries, mainly in Asia and Africa and the prevalence of cholera shows no tendency to decrease. In 2011, a 85 % increase compared to 2010 was reported to the WHO, with a total of 589 854 cases worldwide including 7816 deaths [65]. Cholera is caused by the bacterium *V. cholerae* and in 98 % of all cases by a single serogroup (O1) and biotype (El Tor) [66]. Cholera is transmitted via the faecal-oral route. *V. cholerae* bacteria enter our body via contaminated water, which is particularly a problem in countries with poor sanitation and hygiene conditions, and via contaminated food, e.g. undercooked seafood [67]. The bacterium colonizes the mucosa in the upper part of the small intestine where it starts to produce the pathogenic cholera toxin (CT). The action of CT on the intestinal epithelium provokes severe and life-threatening watery diarrhoea. The rate of diarrhoea can often reach up to one liter per hour, thus causing acute dehydration [67].

The first developed vaccines against cholera were killed injectable whole-cell vaccines that were used worldwide [68]. However, these vaccines were not cost-effective since they provided only limited and short-lived protection even after annual booster doses and the immunizations were associated with adverse reactions such as local inflammatory reactions [69]. For these reasons the WHO has long stopped recommending the use of these injectable cholera vaccines. Instead oral cholera vaccines (OCV) with stronger efficacy and negligible side effects have been developed. Until recently, the only commercially available mucosal cholera vaccine with a demonstrated efficacy of up to 90 % has been the OCV Dukoral^{TM} [68]. The vaccine is based on a killed whole-cell mixture of *V. cholerae* (three different strains that are inactivated by two different methods) combined with purified recombinant CTB and it has been shown that the protective efficacy is based on the local intestinal production of both antibacterial and antitoxic SIgA antibodies [70]. In 2009 a second OCV was licensed that contains similar whole-cell bacterial components as the Dukoral^{TM} vaccine but lacks CTB. The immunogenicity of both *V. cholerea* and CTB can easily be estimated by different *in vitro* and *in vivo* methods. Due to the known efficacy of the Dukoral^{TM} vaccine and the fact that several tools for *in vitro* and *in vivo* evaluations are available, it was used as a model vaccine for the evaluation of excipients as stabilizers during freeze-drying of killed whole-cell bacteria in Paper IV. Initial investigations were performed on formalin-killed *V. cholerae* O1 bacteria of the Inaba serotype (strain JS 1569), a derivative of the toxin-depleted oral vaccine strain CVD103 that was used in a live-attenuated oral vaccine which is no longer produced [71]. The most promising freeze-drying conditions were then applied to the Dukoral^{TM} vaccine.

3.6 *In vivo* evaluation of mucosal vaccines

3.6.1 Murine model for mucosal vaccination

Within biomedical research animal testing is still the best proof-of-concept method prior to clinical trials and it is as important as clinical testing of new therapies before they are approved for widespread use on humans. Which animal model one choses depends on the goals of the study, robustness of the animal for the experiments, costs, ease of handling, the availability of immunological reagents as well as facilities for housing and how familiar the laboratory is with a specific species. In studies where a high number of animals are needed for the statistical outcome of the testing, mice are ideal animals. They have short life cycles, are inexpensive and relatively easy to purchase and maintain and are therefore extensively used for the discovery and manufacturing of new vaccines [72].

The majority of all animal studies on mucosal immunization have been performed using mice [73]. However, as with all animal models, there are some limitations connected to the murine model. When mice are used for investigations of sublingually administered antigens one has to consider that, in contrast to humans, the epithelial cells at the floor of the mouth are keratinized, so that the uptake of antigens might be different to humans [50]. In order to be able to immunize mice, they may need to be anaesthetized for a short time, which might also have an impact on the immune response. In Paper III and IV mucosal immunizations in female BALB/c mice were performed in order to evaluate the different formulations *in vivo*. All animals were used one week after delivery at the age of about 8 weeks and were marked individually prior to the experiments. For the administration of the test formulations all the mice were slightly anaesthetized. Booster immunizations were performed as described in the respective papers in order to achieve sufficient immune responses and to reduce the inter-mouse variability in responses.

3.6.2 Cholera toxin as adjuvant in mucosal immunization

As mentioned above, several antigens and pathogens evoke only weak adaptive immune responses upon mucosal administration, so that the use an adjuvant is necessary. CT produced by *V. cholerae* bacteria, which was identified over 50 years ago by De [74] and Dutta et al. [75] as the toxic substance causing cholera disease, is, together with the closely related *Escherichia coli* heat-labile enterotoxin (LT), the best studied and most potent adjuvant known for use in

mucosal immunization. Both CT and LT have been shown in several animal models to strongly potentiate the immunogenicity of several antigens when co-administered [76-79]. The most important effect is probably an increased antigen presentation by dendritic cells (DCs), macrophages and B cells [80]. However, the underlying mechanisms of CT adjuvanticity are still not fully understood.

CT has a molecular mass of about 85 kDa and consists of a single active A-subunit (CTA) which is non-covalently bound to a five-membered B-subunit ring (CTB) [81]. After binding of the CTB subunits to GM1 ganglioside receptors on the surface of intestinal epithelium cells, CT is endocytosed and dissociates into its subunits. Subsequently, the CTA subunit activates the adenylate-cyclase enzyme which leads to an increased production of intracellular adenosine monophosphate. This, in turn, leads to the secretion of electrolytes and water into the lumen of the small intestine causing diarrhoea and rapid dehydration, i.e. cholera disease [81-83]. Even just small quantities of CT are highly toxic in humans and cannot be used as adjuvant for human vaccines [84]. Therefore, several approaches to develop genetically modified derivatives with reduced or no toxic activity but retained mucosal adjuvant properties have been undertaken in many laboratories.

In the sublingual immunizations in Paper III CT adjuvant was co-administered with the model soluble protein antigen ovalbumin (OVA) since CT has proven to induce strong antibody responses against sublingually delivered OVA [48]. Since the experiments were only aimed at understanding the general functionality for the development of sublingual tablets, the toxicity of CT in humans could be neglected.

3.6.3 Sublingual immunizations

For the investigations in Paper III on the effect of protein release in sublingual immunization the model protein ovalbumin (OVA) was incorporated in different tablet formulations. OVA is a phosphorylated glycoprotein made up of 385 amino acids with a molecular weight of 42.7 kDa [85] and is a commonly used model soluble protein antigen in immunology research for studies of immune response. Earlier studies have shown the ability of this model antigen to induce mucosal antibody responses in mice, both when administered alone or along with CT adjuvant [48].

The administration of the sublingual tablets was carried out by holding the tongue of the slightly anaesthetized mice with a forceps with small plastic tubes on the tips and pressing the tablet with another forceps against the ventral side of the tongue. An anaesthesia with isofluorane leads to a reduced production of saliva in mice which was found to impede the mucoadhesion of the tablets. Therefore a subcutaneous dose of pilocarpine-HCl was co-administered which has been shown to stimulate mouse salivary secretion [86]. Since chewing can lead to an initial loosening of the tablet, food and water was not provided until 30 minutes after administration. Each mouse was also kept in a cage with a black bottom so that spewed out tablets could be observed. The retention of pure Carbopol® tablets was tested in a group of 5 test mice which were anaesthetized a second time 2 hours after administration. The tongue of each mouse was carefully lifted to inspect the status of the tablet (Figure 2).

Figure 2. Photograph of a C57/Bl6 mouse during the ocular inspection of a mucoadhesive tablet 2 hours after sublingual administration. The arrow indicates the swollen tablet at the ventral side of the tongue.

3.6.4 Peroral immunizations

Since an oral killed whole-cell vaccine was used as the model vaccine in the work in Paper IV, peroral, or more specifically intragastric, immunizations were performed with all freeze-dried vaccine preparations to be compared with the original vaccine. The freeze-dried preparations were re-suspended in buffer prior to administration. The formulations were administered using special feeding needles with silicon tips that were inserted into the stomach under a light anaesthesia. In order to neutralize the acidic environment of the stomach all preparations were co-administered with a neutralizing sodium bicarbonate solution.

3.6.5 Analysis of immune responses

Both systemic and mucosal immune responses are of interest for the evaluation of the immunogenicity of a vaccine. For the analysis of the systemic responses serum IgG and/or IgM antibody titres are usually determined in serum samples. For the estimation of mucosal immune responses IgA antibody titres are measured in the tissues chosen depending on the administration route and the type of disease. In Paper III and IV, blood samples for the final analysis of the systemic immune responses were taken from the subclavian vein of anaesthetized mice and the sera were analysed for IgG+IgM antibody levels. Since both sublingual and peroral immunization evokes strong immune responses in the gastrointestinal tract, intestinal IgA antibody responses were examined. In Paper III tissue extracts of the small intestine were prepared using a modified version of the perfusion-extraction technique [87]. In Paper IV supernatants from homogenized and centrifuged fecal pellets [88] as well as tissue extracts were used for the investigation of intestinal IgA levels.

All samples were analyzed by enzyme-linked immuno-sorbent assays (ELISA). ELISA is the most commonly used biochemical technique in immunology to detect antibodies or antigen in a sample and is based on an antigen-antibody reaction followed by enzyme-coupling [89], as illustrated in Figure 3 for the commonly used "indirect" assay.
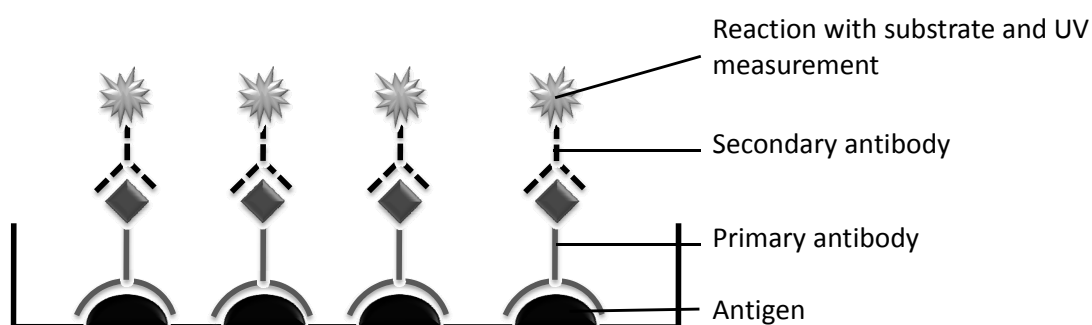

Figure 3. Illustration of the "indirect" ELISA method for the detection of antigen or antibody.

In the "indirect" assay, which was mainly used in this work, antigen is adhered to a surface and a specific primary antibody to the antigen is then added. In Paper III and IV the antibody to be analyzed in the samples from the immunizations was added in this step. Then a second specific, enzyme-linked antibody is applied that can bind to the primary antibody and finally a substrate to the enzyme is added. The colour change of the substrate due to the reaction

with the enzyme can be measured with a spectrophotometer and the detected absorbance can be directly correlated to the amount of primary antibody bound to the antigen. In order to be able to quantitatively determine the antibody amount in a sample, a standard curve of known primary antibody concentrations is included in the assay [89]. The systemic and mucosal antibody responses in all immunized mice in Papers III and IV were analyzed on microtiter plates by "indirect" ELISA as described in detail in the respective papers.

Chapter 4

SOLID DOSAGE FORMS FOR MUCOSAL VACCINATION

The first chapters in this thesis highlight the benefits but also the challenges of mucosal immunization faced by immunologists and make clear that the design of suitable formulations is complex and of utmost importance in order to overcome some of the problems. So this begs the question as to what properties such a formulation should have and how we can use pharmaceutical excipients to design solid dosage forms that are suitable and effective in mucosal vaccination. The following chapters describe and explore different contents where excipients can be used and where it is essential to understand their role and impact during development and manufacturing. A background of the main areas of interest is given together with a description of the essential methods and materials of each study. More detailed experimental descriptions can be found in the respective papers.

4.1 Use of excipients in solid state formulations

Pharmaceutical formulations in the solid state can for example be powders, granules, capsules, films or tablets. All these formulations are normally prepared from mixtures of the API and so-called pharmaceutical excipients that help attain the desired properties. Tablets are the most common solid dosage form and comprise today about 40 % of all dosage forms on the market. They are easy and relatively cheap to produce and are often described as convenient for self-administration since they can easily be handled by the patient [90-92]. A tablet is prepared by compressing a powder where shape and size are controlled by the die and the punch used for compression. Even multiple-layer tablets can be compressed with the API in both or in one of the layers. After preparation, tablets can be coated with solutions of sugars or polymers. Such coatings can, for example, mask the taste of the API or can color the tablet for easier recognition and distinguishing, for example when several tablets have to be taken by a patient. The coating can even modify the release properties of the tablet. Especially important for normally sensitive antigenic material is the fact that the antigen can be protected in the tablet matrix from destructive influences in the environment during distribution and storage, such as humidity, and in the body after intake, such as enzymes or gastric acid [91].

Depending on the API, the administration site and the desired effect in the body different formulation aspects have to be taken into account where the use of the right excipients is essential. If the promising sublingual route is to be used for vaccination a solid vaccine might be easier to keep under the tongue than a solution or a gel, which is easily washed away or swallowed. The use of mucoadhesive excipients can here provide additional advantages. A tablet can be designed to release the API immediately or prolonged over a defined period of time. Furthermore, for the preparation of a solid vaccine formulation dried antigenic material needs to be produced. Since proteins and other biological materials often are labile to drying processes, the use of stabilizing excipients might be crucial.

4.2 Mucoadhesive tablets

4.2.1 Mechanism of mucoadhesion

When a vaccine is to be administered via a mucosal surface, it can be helpful to keep the vaccine at the site of administration for a sufficient time, so that all antigenic material can be taken up and processed. Especially when the vaccine is given via the mucosal surfaces of the oral cavity, there is a risk that the vaccine is swallowed before all antigen is released and taken up. Here mucoadhesive agents can be used to prepare formulations that adhere to mucosal surfaces. A widely explored group of mucoadhesive agents that can be used in dry formulations like tablets are hydrophilic polymers including chitosan, Carbopol®, carboxymethylcellulose, hydroxypropyl- and hydroxypropyl methylcellulose [93]. These polymers contain a high number of hydrogen bond forming groups and the adhesive properties are initiated by applying moisture.

The process of mucoadhesion is referred to as bioadhesive interactions with mucosal surfaces [94]. For the establishment of a mucoadhesive bond, close contact between the formulation and mucus is required. Different bonds are discussed to cause adhesion: ionic, covalent, hydrogen, van-der-Waals or hydrophobic bonds [95]. The detailed mechanisms behind mucoadhesion and factors influencing the strength of a mucoadhesive bond are still not completely understood and might vary depending on the *in vivo* situation and the type of formulation, but in all cases the adhesion process between a mucoadhesive material and a mucous membrane occurs in two steps [95]. The first one is the contact stage where a close contact is established and the second one is the

consolidation stage where different interactions strengthen the adhesive joint. Two main mechanisms are discussed in the literature: the interpenetration theory and the dehydration theory [95]. The interpenetration theory explains the mucoadhesion process on a macromolecular level. After contact and wetting of the mucoadhesive polymer the mucin glycoproteins and the polymer chains can diffuse across the interface and adhesive forces are developed due to interaction and entanglement of the chains.

However, no study really proves this theory. For a dry solid formulation the often rapidly developing mucoadhesive forces cannot just be explained by this theory, since the interpenetration of polymer chains can only occur when the polymers are sufficiently hydrated. Here, the dehydration theory provides a more likely explanation for the initial adhesion. This theory means there is a movement of water from the mucosa into the polymer formulation resulting in the hydration of the polymer and a local dehydration in the mucus layer. The local lack of water in the mucus leads to a negative pressure and thus a "sucking" of the formulation to the mucosa [96, 97]. Figure 4 (adapted from [95]) shows an illustration of this theory.
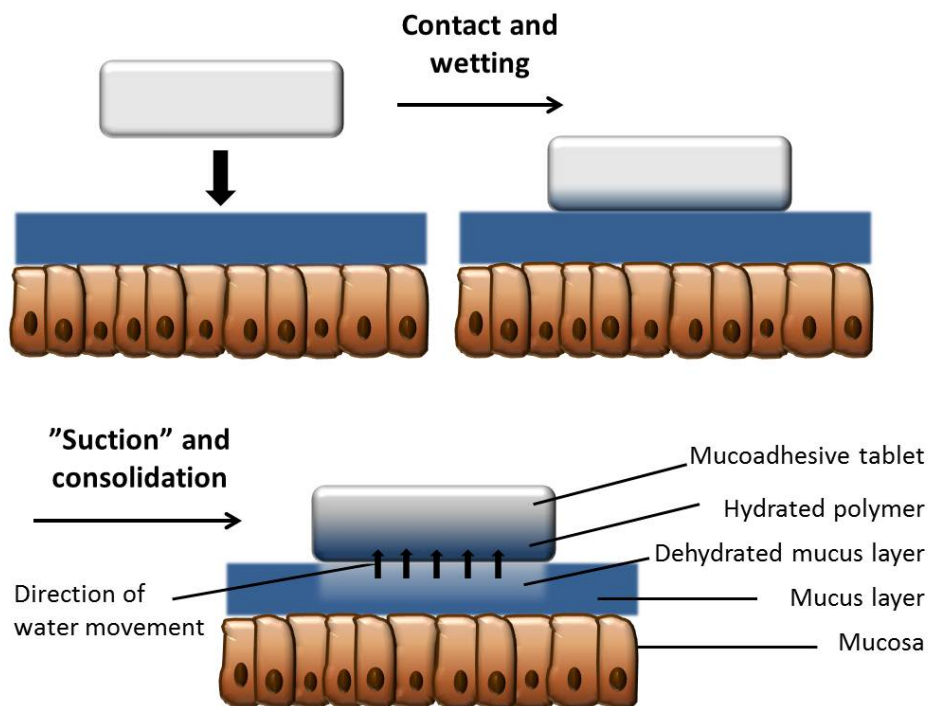

Figure 4. Schematic illustration of the different steps in the mucoadhesion process and the development of the adhesive joint according to the dehydration theory (adapted from [95]).

4.2.2 *In vitro* evaluation of mucoadhesion in correlation to osmotic pressure

In order to achieve good mucoadhesive properties it is important to understand how excipients affect the mucoadhesive behaviour of a formulation. Water transport into the mucoadhesive tablet seems to be crucial for the adhesion of solid state formulations to the wet mucosa. Since a difference in the osmotic pressure is a driving force for water transport it should have a significant influence on the extent of mucoadhesion. Hence, excipients that alter the osmotic pressure in the formulation would also affect the adhesive behaviour of a tablet.

The hypothesis that osmotic pressure differences can be correlated with the adhesiveness of dry hydrophilic tablets was therefore investigated in Paper I with tablets based on the hydrophilic polymer Carbopol®. Carbopol®, which is the trade-name for carbomer, was chosen since it is one of the best known mucoadhesives that is pharmaceutically approved. It consists of hydrophilic cross-linked polyacrylic acid (Figure 5) which easily swells and forms a gel when it comes into contact with water. This property might be the main reason for the extensive mucoadhesive behavior since the adhesiveness of Carbopol® tablets can directly be correlated to the amount of swelling (Paper I). Another theory of the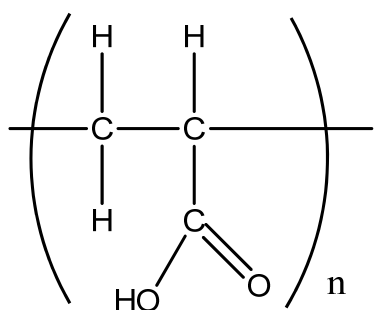 mucoadhesive behavior of Carbopol® describes the phenomenon whereby carboxyl groups interact and orientate towards the mucosa and form hydrogen bonds [98]. Carbomers are commonly used as thickeners, suspending agents and emulsifying agents [99]. As Carbopol® is a hydrophilic swelling polymer it can also be used to modify the velocity of drug release from tablet formulations which was also of interest in this thesis (Paper III).

Figure 5. Structure of polyacrylic acid.

Different parameters have been used to assess *in vitro* mucoadhesive properties including molecular interactions and the adhesive force. The latter was applied for the aim of the studies in Paper I, i.e. the force required for the detachment of a formulation was measured. Either tissues, mucin solutions or other systems that imitate a mucosa can be used to replicate a mucosal surface [100, 101]. Tissue samples might be the best alternative from a biological point of view, however, it is often hard to fix tissue samples in a way that makes a detachment of tablets possible and moreover, the reproducibility is often poor. Therefore, an agarose gel was used as a model surface in this work and Carbopol® tablets

of 12 mm diameter served as a model formulation. Sodium chloride (NaCl) and mannitol were added in different concentrations to the agarose solution before the gels were made in order to modify the osmotic pressure difference between the gel and the tablet. To be able to keep the agarose gel in place during the adhesion measurements, they were directly manufactured in steel vessels. Force-displacement measurements were performed using a Texture Analyzer TA-HD*i* (Stable Micro Systems). The tablet was attached to the upper movable probe and after lowering it onto the model surface they were kept in contact at a constant force for a specified time. During the following detachment of the tablet in vertical direction the force required was recorded as a function of both time and distance (probe displacement). From the resulting force-time or force-distance plot the maximum adhesive force (N) can be determined and if it is of interest the latter can also be used to determine the adhesive work (Nmm) from the area under the curve (AUC) as illustrated in Figure 6.
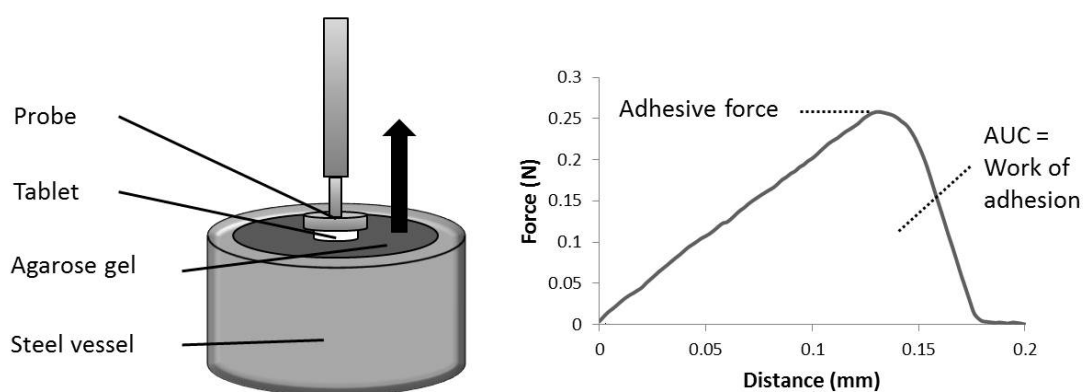

Figure 6. Schematic drawing of the upper probe with the attached tablet which is lowered onto the agarose gel. The force required for the detachment of the tablet (the probe is raised again, as indicated by the arrow) is recorded as a function of the probe displacement and the time. From the data the adhesive force and the adhesive work can be obtained as illustrated in the force-distance graph.

In all cases the swollen tablets were also re-weighed immediately after each run in order to measure the amount of water taken up by the tablet. The agarose surface was inspected after each measurement to ensure that the failure in the adhesive joint did not occur in the agarose gel.

4.3 Modified protein release from sublingual tablet formulations

As mentioned above, tablets are the most prevalent solid dosage form. However, many people, especially children, have difficulties in swallowing a tablet which impedes patient compliance. Sublingual tablets against different diseases have therefore been developed during recent years in order to facilitate intake and increase patient compliance. Considering the benefits of sublingual immunization discussed in Chapter 3, it seems therefore interesting and beneficial to develop a sublingual vaccine tablet and the question is how the antigen should be released from such a formulation. An extended release would result in exposure of the antigen for a prolonged period of time which could probably be advantageous. However, all sublingual formulations containing low molecular APIs that are on the market are based on fast dissolving tablets and in contrast to the buccal route (which is the administration via the mucosa lining the cheeks) the use of extended release tablets (ER tablets) for sublingual administration seems to be quiet unexplored [91]. Therefore it was of interest to investigate the use of hydrophilic matrix tablets as extended release delivery systems for sublingual vaccination and compare their properties with an immediate release tablet (IR tablet).

4.3.1 Excipients to control the release profile

The basis of a pharmaceutical tablet is often a substance that serves as a bulking agent. In order to obtain a fast dissolving tablet that releases the API more or less immediately disintegrating excipients with the ability to rapidly break the tablet into small fragments can be incorporated in the tablet powder blend [92]. If a longer exposition is desired different technologies are available to design a tablet with an extended release of the API. A very common method is the embedding of the API in a slowly eroding matrix of hydrophilic polymers. Upon contact with water a hydration of the polymer occurs leading to gel formation. As mentioned in the previous chapter, many of these polymers also provide mucoadhesive properties [95] which would be advantageous for a sublingual tablet. The release rate of the API from a hydrophilic matrix tablet is affected by the dissolution and diffusion of the API, the diffusion path length and the rate of matrix erosion [102]. In the case of vaccine tablets the antigenic material is normally of macromolecular nature. The diffusion of the API will thus be extremely slow so that erosion will be the most likely release mechanism.

In order to examine the ability to evoke immune responses with sublingually administered tablets different formulations were prepared and characterized *in vitro* and subsequently tested in mice (Paper III). Since the main aim was to investigate if a slow releasing formulation could provide advantages over an IR tablet by providing an exposure of small antigen amounts over a prolonged time, three different ER tablets were prepared containing OVA as a soluble model protein antigen and the formulations were compared with an IR tablet formulation. The matrices of the three ER tablets were composed of (i) hydroxypropyl methylcellulose (HPMC), (ii) HPMC mixed with mannitol and (iii) Carbopol[®]; the IR tablet contained a mixture of microcrystalline cellulose (MCC) and lactose. Lactose is a widely used pharmaceutical excipient that can be used as a tablet and capsule filler or diluent and among the different disintegrants that are available MCC was chosen for that study since it is a widely used excipient in oral formulations [99]. HPMC is one of the most commonly used hydrophilic polymers for release-rate controlling swellable matrix tablets and consists of a cellulose ether substituted with methoxy and hydroxypropoxy groups (Figure 7). Like Carbopol[®] it is also used in other pharmaceutical contents as a thickening or stabilizing agent or for coatings [99]. All experiments in this work are based on HPMC 90SH 100 000 (Ph. Eur. type 2208) which has a methoxyl content (w/w) of 22-24 % and a hydroxypropoxyl content (w/w) of 8-12 % and a viscosity grade of 100 000 mPa*s [103]. Mannitol, which is otherwise most often used as a bulking agent, has been found to increase the rate of water transport into tablets and thus the dissolution rate of the tablet [99, 104]. It is a hexahydric alcohol with a high solubility (aqueous solubility 240 mg/ml) and was used to modify the release of protein from HPMC tablets so that different release profiles were obtained.
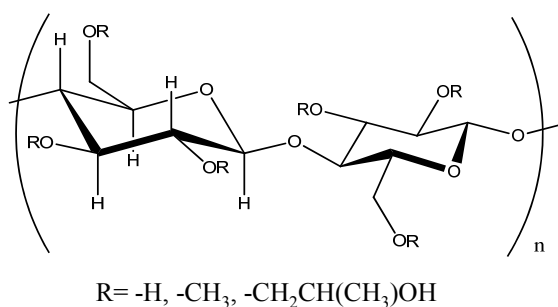

R= -H, -CH$_3$, -CH$_2$CH(CH$_3$)OH

Figure 7. Structure of hydroxylpropyl methylcellulose (HPMC).

Since all formulations were aimed to be tested *in vivo* on mice and it was found that HPMC tablets did not adhere sufficiently under the murine tongue, all tablets for the *in vivo* examinations were manufactured as two-layer tablets with a matrix layer containing the antigen and a mucoadhesive layer composed of pure Carbopol[®] (Figure 8). The tablets for the *in vitro* characterizations were made without this second mucoadhesive layer.
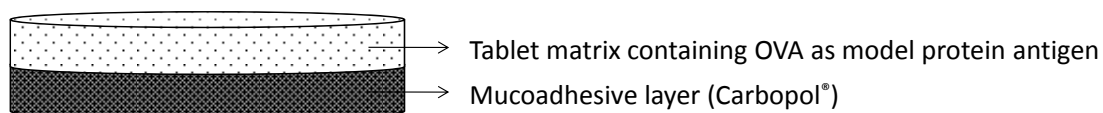
Figure 8. Illustration of a two-layer tablet consisting of a mucoadhesive layer and a protein releasing layer.

4.3.2 Characterization of protein release

Different standardized methods are available to study the release from tablets, a.o. by the United States Pharmacopeia (USP). However, no standard method exists that provides a good correlation for the *in vivo* situation of sublingually delivered tablets. All protein release characterizations in Paper III were therefore performed with a standard procedure for orally administered tablets using a modified variant of the USP II dissolution model. A stirring paddle was put in the release medium and instead of having the tablet free in the vessel it was fixed in a stationary non-rotating basket above the paddle (Figure 9). This modification leads to more reproducible hydrodynamic conditions [105] and the tablet cannot adhere to the vessel or float in the bottom or at the surface of the medium, so that hydration occurs from all sides at all times. When this method is used it is however important to ensure that the tablet does not swell to such an extent that its size becomes larger than the basket, since this would most likely lead to a disruption of the gel layer.
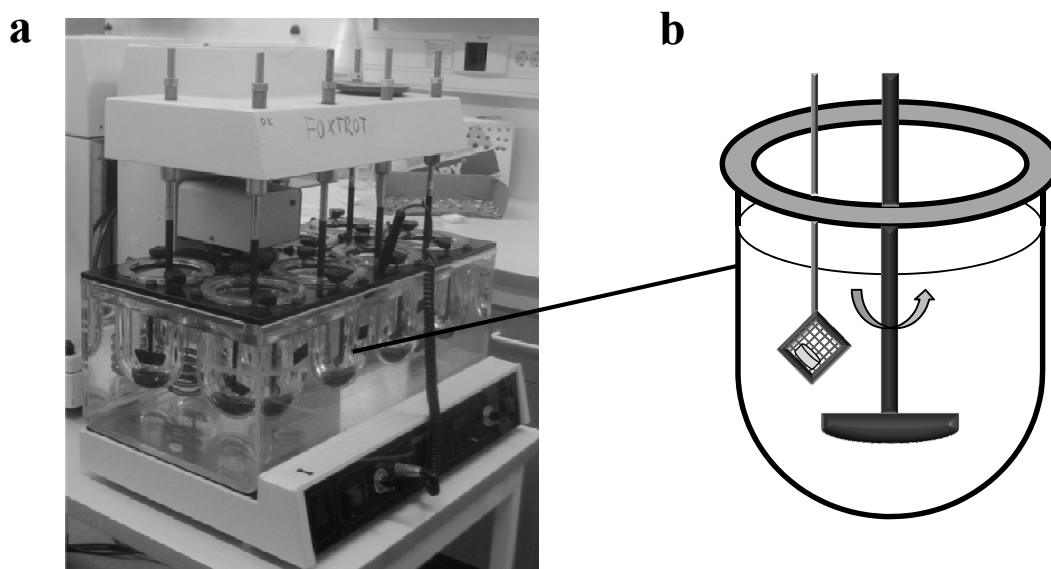

Figure 9. (a) Photograph of a release study setup using a temperate dissolution bath with USP vessels. (b) Schematic illustration of a USP vessel with tablet basket.

4.3.3 Protein analysis with Bradford Assay

In order to determine the concentration of protein in the samples containing OVA within the protein release study, the widely used Bradford Assay was used. This spectroscopic assay allows a fast determination with high sensitivity and specificity for proteins and can, depending on the protein concentration, even be performed with small sample volumes. The method is based on a complex reaction between protein and the dye Coomassie blue (CB). CB has been used as a colorimetric reagent for the detection and quantitation of protein since 1976 [106]. Depending on the pH in the surrounding medium CB can occur in three different forms (anionic, cationic or neutral) with different UV/Vis absorption spectra. Thus, the chemical environment influences the UV/Vis absorption spectrum and the maximum level of absorption shifts to different wavelengths and reveals different colours under changing conditions (Figure 10) [107].
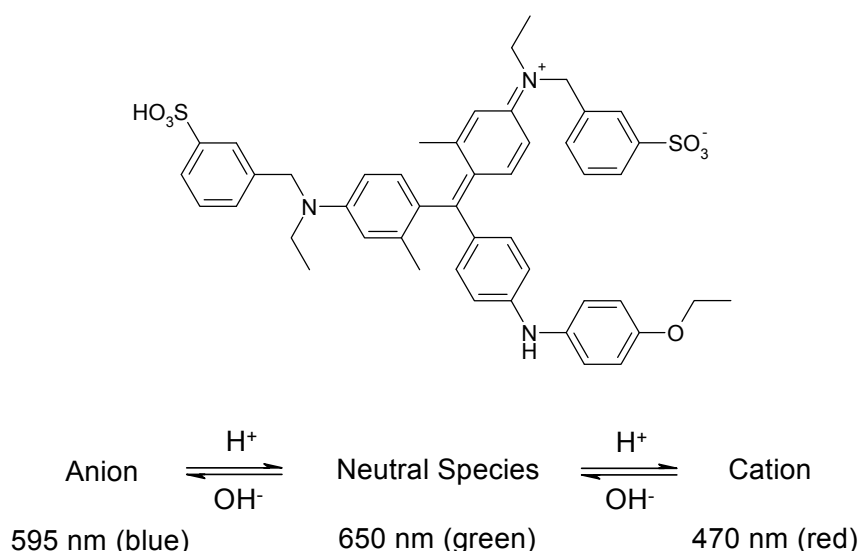
Figure 10. Structure of Coomassie blue dye and equilibrium of the different states with corresponding absorption maxima according to [107].

In its cationic form, i.e. under acidic conditions, CB is able to bind to protein which results in a spectral shift from a reddish/brown form to a blue-coloured form. This increasing UV/Vis absorption can be measured at 595 nm and the obtained absorption can be related to the protein concentration using a recorded standard curve [106, 107]. However, based on recently reported perturbations by polyethylene glycol [108] one has to consider that polymers in the sample can disturb the analysis, by disturbing both the reaction and the absorption measurement. For the use of the assay in the present work an investigation of possible disturbances by the pharmaceutical excipients that were co-released

with the protein was necessary (Paper II). Possible disturbances caused by polymers are light scattering or an interaction with either the dye or the protein. For the examination of possible interferences of the excipients with the Bradford method UV/VIS spectra were recorded instead of measuring the absorption at the fixed wavelength of 595 nm as specified in the Assay Kit. Pure excipient samples as well as protein samples in the presence of the excipients were investigated and compared with each other.

4.4 Drying of antigenic material

Antigenic material is often available or produced in the liquid state and needs to be converted into the dry state if it is to be used in a solid dosage form. In many cases it might be a challenge to find an appropriate drying process which does not affect the immunogenicity of the antigen. Different drying techniques are conceivable such as conventional oven drying, spray-drying, freeze-drying or spray-freeze drying. However, the choice of the drying method as well as the physico-chemical parameters during the drying process will influence the product properties. Spray-drying and freeze-drying are the most commonly used techniques for protein drying in the pharmaceutical industry [109], whereas other methods are more common on a research scale. Different parameters are important for the choice of a suitable method, such as cost efficacy and the desired route of administration.

In this work freeze-drying was chosen as the drying tool for the production of a dry bacterial whole-cell vaccine using *V. cholerae* as model vaccine where different conditions were evaluated (Paper IV). This drying technique is especially advantageous for heat-labile materials and was chosen due to several advantages discussed below that outweigh the disadvantageous long and cost consuming freeze-drying process.

4.4.1 Freeze-drying of killed whole-cell *Vibrio cholerae*

The principle of the freeze-drying process is based on the direct sublimation of frozen water, i.e. a phase transition from the solid ice state to the gas state [110, 111]. Prior to drying, the sample is therefore frozen so that ice crystals start to grow. This can be done in an external freezer or directly in the freeze-dryer where it is possible to control the freezing rate. In the present work the initial freezing-step of *V. cholerae* formulations was performed in an external freezer at − 70 °C overnight and the samples were subsequently put into the freeze-

dryer. To start the freeze-drying process the pressure in the drying chamber was lowered below the triple point of water to initiate sublimation. Since sublimation is an endothermic process, the shelf temperature was raised in order to provide additional energy and the drying was performed at a shelf temperature of -5 °C and a pressure of 0.78 mbar. After the primary drying it is possible to apply a second drying step where the temperature is further raised so that unfrozen water that is adsorbed to the solid ice-free matrix is desorbed. This is mostly done to lower the residual moisture content in the dried material and was not performed in the present study.

4.4.2 Advantages and challenges in freeze-drying of antigenic material

As mentioned above, the liquid is removed at low temperatures and in the freeze-dried state chemical or physical degradation is often inhibited or at least decelerated, thus resulting in a better stability of the product. The obtained porous cake has a large surface area that provides a fast dissolution, which might be advantageous if the product should be rapidly re-suspended, for example for immediate oral intake or injection. An additional advantage for parenteral formulations is the fact that sterile products are easy to obtain [109]. Furthermore, freeze-drying can also be used for the manufacturing of fast dissolving tablets [112].

The freeze-drying process includes however a variety of stresses that can affect the stability of proteins. At high freezing rates, smaller ice crystals and thus larger ice-water interfaces are formed, which can lead to surface-induced protein destabilization. Some buffer components tend to crystallize during freezing which can lead to a pH change and thus to conditions that might not be suitable for the material to be dried [111]. The dehydration process also removes hydrogen-bonded water and thus parts of the protein hydration shell [113]. When bacteria are dried this can lead to membrane damage and loss of function [114, 115]. Furthermore, as the sample becomes more concentrated during the water removal the possibility for interactions increases, and thus the risk for protein or bacterial aggregation. Such interaction can become a problem in the production of a dried antigen for a solid state vaccine. Regarding long-term stability of freeze-dried protein formulations, storage temperature, glass transition temperature and residual moisture content are important factors. Generally, one can say that the higher the storage temperature, the lower is the stability of a freeze-dried protein. The glass transition temperature, T_g, is defined as the temperature where a transition between the glassy (solid-like) and the rubbery (liquid-like) state occurs [113].

Storage above T_g of the dried preparation might result in cake collapse and can lead to a rapid crystallization of the amorphous components resulting in an accelerated instability of the protein [113]. The residual moisture content after freeze-drying is also of importance for long term stability. Water can either provide a reaction medium or react directly as a reactant with the dried material. The residual water can also act as a plasticizer and thus reduce the T_g of the formulation. The higher the residual moisture content, the more T_g decreases which in turn often lowers the stability of the product [116, 117].

4.4.3 Excipients for stabilization of bacterial antigens during freeze-drying

To meet the mentioned challenges associated with the freeze-drying process most freeze-dried protein products typically contain a buffer besides the active component, bulking agents such as mannitol or lactose to provide good cake properties and in the case of protein formulations stabilizing excipients. The stabilization capability of excipients needs to be examined in two stages: during the freeze-drying process and during long-term storage. The main stabilization mechanisms by excipients involve the formation of an amorphous glassy state, replacement of the removed water and hydrogen bonding with the protein. Non-reducing sugars are commonly used as stabilizing excipients for dried protein formulations since they can form hydrogen bonds with proteins. Different sugars can be differently efficient depending on the product to be dried and often a concentration-dependent stabilization efficacy is observed [113].

In this work focus was put on process stabilization, where the effect of three different excipients (Figure 11) on bacterial aggregation and antigen structure preservation was investigated (paper IV). Two common stabilizers, sucrose and trehalose, were investigated, but even the less effective substance mannitol was included in the study since mannitol is known to be a good bulking agent in lyophilized materials [99]. Sucrose, which is composed of the monosaccharides glucose and fructose, is otherwise frequently used as syrup in oral pharmaceutical formulations, as tablet or capsule diluent, in sugar coatings, as a granulating agent or to increase viscosity [99]. Trehalose, which is a disaccharide consisting of two glucose units, is less common in these contexts, maybe due to higher costs. The fact that both sucrose and trehalose are non-reducing sugars, since neither of the rings in the molecules can be opened to form aldehyde groups in solution, is one reason for their use as stabilizers in the freeze-drying of biological materials [114]. In the literature trehalose is often seen as the first choice stabilizer, but for each material the best one has to be

investigated individually. Since sucrose is the more common excipient in pharmaceutical formulations and it is much cheaper than trehalose, we wanted to examine and compare the capability of both sugars to provide stabilization of killed whole-cell bacteria during freeze-drying. Since both the type of excipient and the concentration play a role for the stabilizing capacity, all three excipients were examined in three different concentrations (0.25 mg/ml, 2.5 mg/ml and 25 mg/ml).
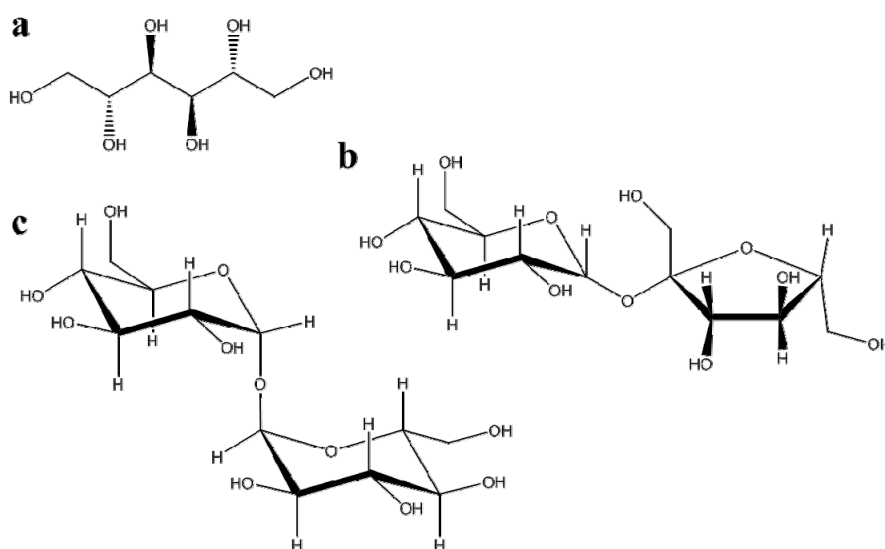

Figure 11. Structures of the excipients that were investigated in this thesis as stabilizers during freeze-drying of killed whole-cell bacteria. (a) mannitol, (b) sucrose, (c) trehalose.

4.5 Characterization of freeze-dried material

Freeze-dried samples can be characterized by different techniques both in the dry state and after dissolution or re-suspension. In this work the aim was to find stabilizing excipients that can avoid bacterial aggregation and maintain the functionality of the antigenic structures, the lipopolysaccharides (LPS) on the surface of *V. cholerae*. The latter could easily be investigated with re-suspended samples by ELISA. The extent of aggregation of the bacteria could successfully be visualized by confocal microscopy.

4.5.1 Confocal microscopy for evaluation of bacterial aggregation

Confocal microscopy (CM) is an optical imaging technique used to capture images of fluorescent samples by excitation of the sample and detection of the emitted signals. In contrast to a traditional wide-field fluorescence microscope where fluorophores through the whole sample are excited at once and signals

are collected from the sample and areas outside the plane of focus, the CM technique uses point illumination and collects fluorescent signals emitted from the sample via a pinhole aperture to eliminate background signals [118, 119]. This provides an increased optical resolution and contrast so that the images are not so blurry. A further advantage of the confocal microscope is that a series of optical sections at different depths can be used to reconstruct 3D images of the sample. The depth to which samples can be visualized depends on the transparency of the sample, the excitation wavelength and the objective [118]. The most commonly used type of confocal microscope, which was also used in this work, is a laser scanning confocal microscope (LSCM), i.e. a focused laser beam scans the sample and the emitted fluorescent signals are detected by a photo detector. Different immunofluorescence reagents or staining dyes can be used to study biological structures or functions. In order to make a visualization of the freeze-dried *V. cholerae* bacteria in Paper IV possible, the re-suspended samples were mixed with the biological stain safranin. This coloring agent has positive charges which can bind to negatively charged molecules in a biological sample, as for example the nuclei or the surface of bacteria. The samples to be investigated were excited at 488 nm and the emission was detected in the wavelength range 500 – 700 nm.

4.5.2 ELISA for functionality test of LPS antigen

An "inhibition" ELISA, or in the literature also called "competitive" ELISA, was used for the detection of intact surface LPS. This ELISA variant is used if two matching antibodies are not available [89]. Excess antibody is incubated with an antigen-containing sample, in this case with the re-suspended freeze-dried samples, and the mixture is then added to an antigen-coated plate to which the remaining free antibody binds. The better LPS is preserved in the sample, the less free antibody is available for binding on the plate and by comparing with a reference the maintenance of the LPS structures can be estimated.

Chapter 5

OUTCOME OF RESEARCH

In order to design solid dosage forms that are suitable for mucosal vaccination knowledge of crucial formulation properties and an understanding of how to obtain them are required. Figure 12 shows an overview of the key issues investigated in this thesis which will be discussed in the following chapters:
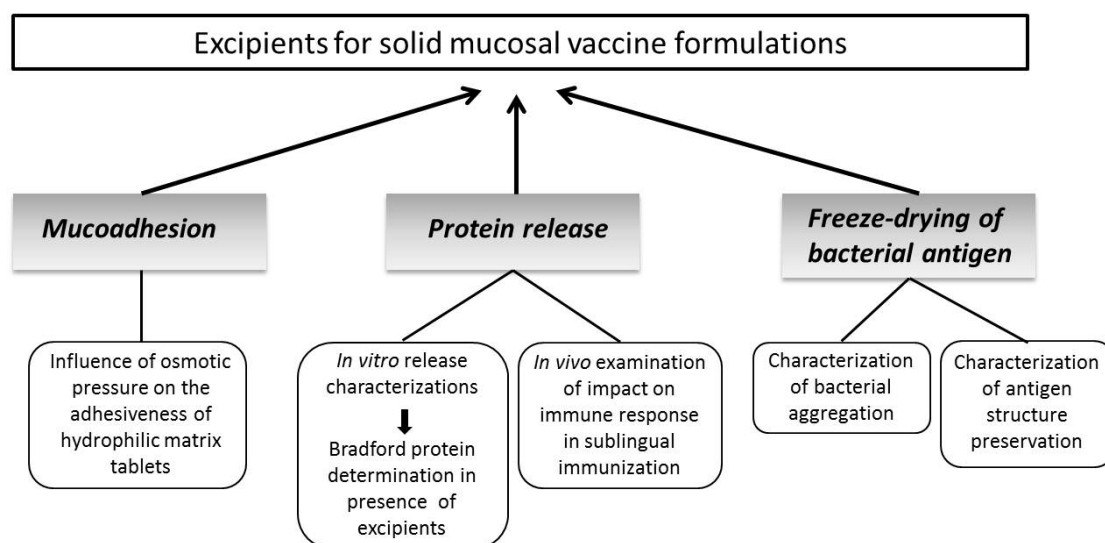

Figure 12. Overview of the investigations in this thesis concerning the influence of excipients in the design of solid dosage forms for mucosal immunization.

5.1 Investigations on mucoadhesion in relation to previous work

The first thoughts of this work concerned the development of a sticky solid formulation for vaccine delivery to the sublingual mucosa and the question arose whether an ER tablet could be a benefit for the efficacy of such a vaccine. Hydrophilic polymers can have mucoadhesive properties and are commonly used as a matrix for ER tablets. The well-known mucoadhesive and hydrophilic polymer Carbopol® was chosen as a starting point for the development of a sublingual mucoadhesive vaccine tablet.

As described in Chapter 4 one mechanism that is discussed in the literature as the responsible one for the mucoadhesive behavior of hydrophilic polymer tablets is their ability to swell, i.e. to take up huge amounts of water upon

contact with a wet surface [96, 120]. The water uptake by the tablet results in a local lack of water in the mucosa generating an under pressure so that a suction force is developed [97]. A difference in the osmotic pressure is one main driving force for the movement of water and therefore the hypothesis was raised that osmotic pressure might have a direct impact on the adhesive force.

To study this relationship between osmotic pressure and adhesive force a model system was used where the osmotic pressure could be tuned. The weight increase of the tablet after the adhesion measurement was used as a parameter for the amount of water transported into the tablet. The first investigations dealt with the impact of the time of contact between the tablet and the agarose model surface on the water uptake by the tablets in correlation with the developed adhesive force. In Figure 13 it can be seen that forces of up to 0.7 N could be detected which is comparable to the gravity force of a tablet with a 200-fold higher weight than the dry tablet. Both the adhesive force and the tablet weight increased with a prolonged contact time. An interesting observation was that the initial distinct increase of the adhesive force leveled out while the water gain by the tablet continuously increased, which indicates that the tablet swells at a relatively constant rate and that a water transport from the agarose gel into the tablet occurs at all times. An explanation for this could be that the adhesive force develops mostly in the initial time phase by a negative pressure due to an initial local lack of water and when water starts to flow within the agarose gel to equal out this imbalance, the negative pressure does not increase as much as before so that the effect of the water flow into the tablet levels out. However, these are only speculative interpretations and more mechanistic investigations would be required in order to explain this phenomenon in more detail.
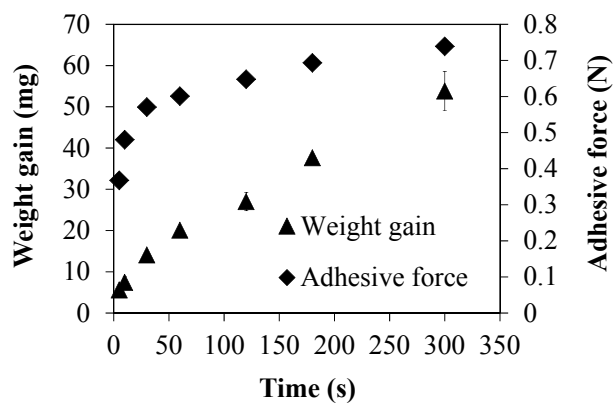
Figure 13. Impact of contact time on the water gain and the adhesive force of Carbopol® tablets. Each data point represents the mean (± SD) of five replicate measurements.

For the following investigations on the influence of osmotic pressure differences on the adhesive force a contact time of 180 s was chosen since the effect of the adhesion time had leveled out at that point in time. The osmotic pressure of the agarose gel was increased by the addition of different amounts of solute (0 – 1.0 M), which in turn decreased the osmotic pressure difference

between the agarose gel and the tablet. Thus, a decrease in the water flow into the tablet and hence a lowered adhesive force was expected according to the proposed hypothesis. Indeed, this could be confirmed both when NaCl or mannitol was added to the agarose gel as can be seen in Figure 14, where increasing additive concentrations result in decreasing adhesive forces.
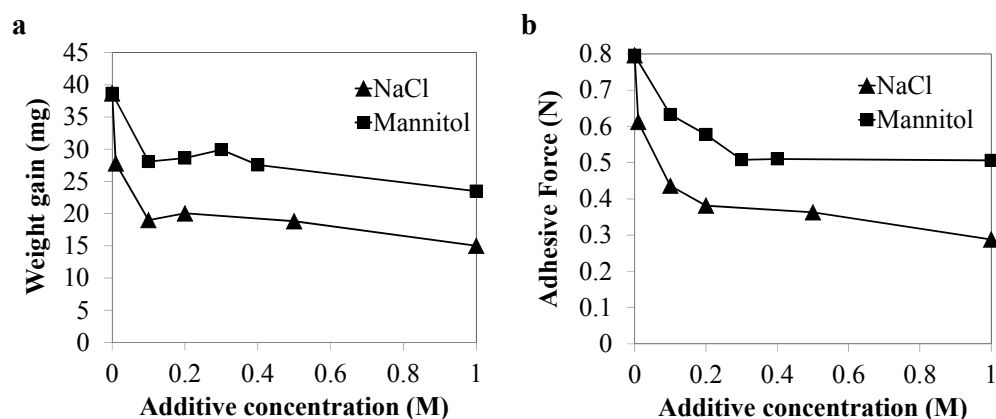

Figure 14. Effect of NaCl and mannitol addition (a) on the water uptake by Carbopol® tablets measured as weight gain and (b) on the adhesive force measured as the maximum force required for the detachment of the tablet from an agarose surface. Each data point represents the mean (± SD) of five replicate measurements.

Similar observations can be found in other published studies. Lejoyeux et al. [121] found for example that and an addition of NaCl and calcium chloride (CaCl₂) to a buffer solution surrounding Carbopol® tablets that were attached to sublingual and vaginal tissue samples resulted in lower adhesive forces of the tablets to the tissues, where $CaCl_2$ had a stronger effect than NaCl. The authors of that study discuss these results in terms of ionic shielding effects on the polyacrylic acid molecules by the divalent ions. However, this explanation does not hold in the case of mannitol since it has a very low shielding effect. In contrast, the theory evaluated in this work can be applied to the results by Lejoyeux et al; $CaCl_2$ has a higher van't Hoff factor than NaCl and thus decreases the osmotic pressure difference more which leads to a stronger decrease in the adhesive forces. Another study by Mortazavi and Smart showed that anionic polymers dehydrate a mucus gel more than neutral polymers [96], which can also be explained by the different osmotic pressures caused by the polymers. Similar observations were made when pure Carbopol® and HPMC tablets were compared by us with the mucoadhesion test method used in this work. The weight gain by neutral HPMC tablets was lower than by Carbopol® tablets. The measured adhesive forces showed also slight differences, where the HPMC tablets were less adhesive than the Carbopol® tablets, so that even here a correlation between osmotic pressure and adhesive force can be speculated

(Table 1). The same results were obtained with the method used in the study by Lejoyeux et al. where the positively charged Carbopol® showed a higher adhesiveness than HPMC [121].

Table 1. Influence of the polymer type on water uptake (measured as tablet weight gain) and adhesive force (measured as the force required to detach a 12 mm-tablet from an agarose surface).

Polymer	Weight gain (mg) (± SD)	Adhesive force (N) (± SD)
Carbopol® 974-P NF	38.7 (1.1)	0.8 (0.1)
HPMC 90 SH 100 000	28.2 (1.1)	0.7 (0.1)

Taken together, the results from this study as well as data from the literature show that osmotic pressure is an important parameter for the adhesiveness of dry tablets based on hydrophilic polymers. Both the composition of the mucosal tissue and the excipients in a dry formulation can alter the osmotic pressure. Thus, the influence of excipients on the mucoadhesiveness of a tablet should be considered in the design of such formulations. Since Carbopol® is known to be a good mucoadhesive polymer and the high charge density creates a high osmotic pressure in the formulation, Carbopol® was chosen as the mucoadhesive component to adhere tablets sublingually in mice.

5.2 Modified protein release from tablet formulations

All samples from the protein release studies were analyzed using the Bradford method. When the fractions of released protein were calculated, it was observed that the total amount of protein released from the Carbopol® matrix tablets after complete dissolution of the tablets was not in accordance with the actual amount incorporated in the tablets. Thus, the analysis method was put into question and standard curves of OVA in the absence and the presence of Carbopol® were detected. Remarkable differences in the absorption at 595 nm, the monitoring wavelength of the Bradford Assay, were found where all OVA samples had a higher absorbance as compared to the equivalent samples without Carbopol® (Figure 15).
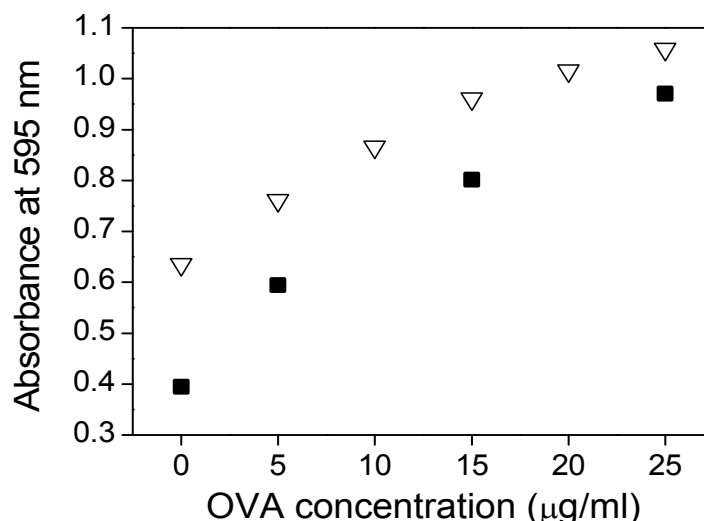
Figure 15. Absorbance of OVA-Coomassie Blue samples at the monitoring wavelength of the Bradford Assay (595 nm) as a function of the protein concentration in the absence of polymer (filled squares) and in the presence of 100 µg/ml Carbopol® (open triangles).

5.3 Development of an analysis method for protein quantification in the presence of pharmaceutical excipients

As a consequence of the detected disturbances by Carbopol® in the analysis of OVA samples by the Bradford method, a general evaluation of the method in the presence of excipients was performed. Three different hydrophilic polymers that might be of interest for mucoadhesive formulations were investigated: positively charged Carbopol®, neutral HPMC and negatively charged Chitosan. In addition, all other excipients used in the formulations of the release study were also tested for their influence on the detection of OVA with the Bradford assay in order to be sure that all protein determinations were performed correctly. In addition to measurements at the monitoring wavelength of the assay wavelength scans in the range of 350 – 850 nm were recorded in order to make the finding of disturbances easier.

No disturbances were found by HPMC, mannitol, lactose or MCC. In contrast, the presence of Chitosan or Carbopol® changed the absorption spectra of the CB dye as it can be seen in Figure 16.
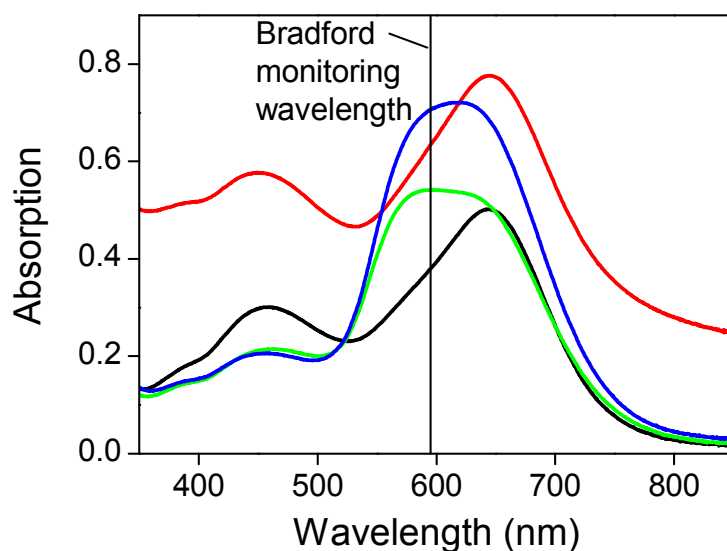
Figure 16. Absorption spectra of the Bradford reagent solution: in absence of protein and polymer (continuous black), in presence of 10 µg/ml OVA (blue line), in presence of 100 µg/ml Carbopol® (red line) and in presence of 5 µg/ml Chitosan (green line).

The presence of Chitosan resulted in a shift of the main peak at 645 nm to lower wavelengths similar to that obtained when OVA is present, which indicated an interaction of Chitosan with the CB dye. The presence of Carbopol® did not lead to a spectral shift of the main peak but instead to an increase in the absorbance. This increase was detected at all wavelengths including those where CB does not absorb, i.e. the spectrum of the CB dye was shifted upwards without any changes in the shape. Since this effect was also observed in a solution of the same pH in the absence of CB dye (Paper II) the disturbance by Carbopol® was interpreted as a turbidity effect caused by small Carbopol® aggregates due to the low pH of the reagent solution.

In the case of Chitosan further investigations showed a concentration-dependent effect on the absorbance where higher concentrations of the polymer resulted in an increased absorbance. This observation further indicated that chitosan interacts with the dye. Since the interaction of Chitosan and the CB dye disturbs the CB spectrum at the monitoring wavelength of the Bradford Assay in such a concentration-dependent manner, the monitored absorbance of protein samples will differ with different Chitosan concentrations and it is difficult to make corrections for this perturbation. In the case of Carbopol® however only scattering perturbations and no indications for an interaction with the dye were found. This scattering effect was also found to be concentration-dependent where the absorbance caused by scattering increased with increasing polymer concentrations. Since the scattering contribution of Carbopol® could be related to the amplitude at 850 nm where CB itself has no absorbance, the

absorbance at 850 nm could be used to make corrections for the disturbance by subtracting the value from the value measured at 595 nm. Since light scattering increases with decreasing wavelengths, the absorption at 850 nm had to be multiplied with a constant that takes this effect into account and a value of 1.5 was found to be appropriate under Bradford conditions:

$$A_{corr} (595\ nm) = A\ (595\ nm) - 1.5 \cdot A\ (850\ nm).$$

Figure 17 shows OVA standard curves recorded in the presence of different Carbopol® concentrations after correction by this method. The absorbance increased with increasing OVA concentration and the same absorbance was measured in samples with equal OVA concentrations regardless of the Carbopol® concentration, showing that the correction method works.
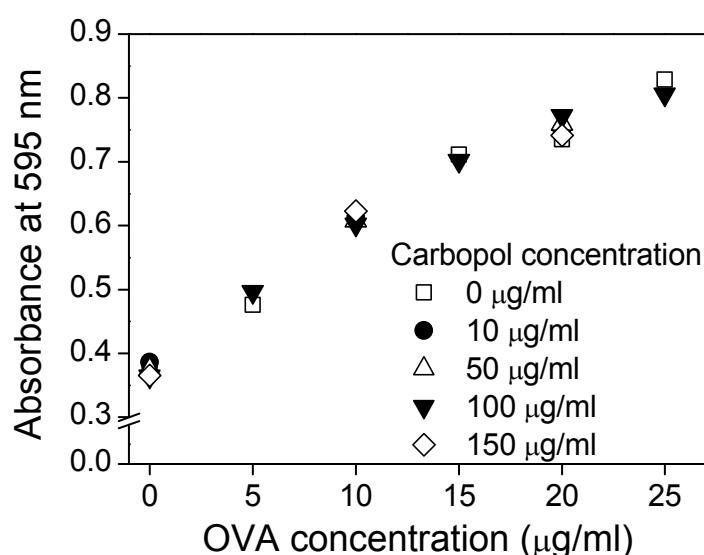
Figure 17. Absorbance at the monitoring wavelength of the Bradford assay (595 nm) of OVA-CB dye samples containing different concentrations of Carbopol® after correction for the scattering effect by Carbopol® according to the described method.

5.4 *In vitro* and *in vivo* evaluations of controlled release tablets for sublingual immunization

With the gained knowledge from the evaluation of the Bradford method and the possibility to correct for perturbations by co-released Carbopol® all samples from the OVA release study could be analyzed correctly. As desired, the three ER tablets released OVA during differently prolonged times, whereas all protein was immediately released from the MCC/lactose formulation. The

entire protein amount incorporated in the latter formulation could already be detected at the first sampling time point of 5 minutes (Figure 18).

The Carbopol® matrix tablet dissolved as expected very slowly and hence after one day just under 60 % of the total amount were released. Both HPMC formulations showed a prolonged release whereof the formulation containing 40 % mannitol had a steeper release profile and almost 90 % of the total amount of protein was already released after 10 hours; in contrast to the formulation consisting solely of HPMC which had released all protein after approximately one day. Thus, the tablets showed different protein release profiles and were subsequently tested in mice.
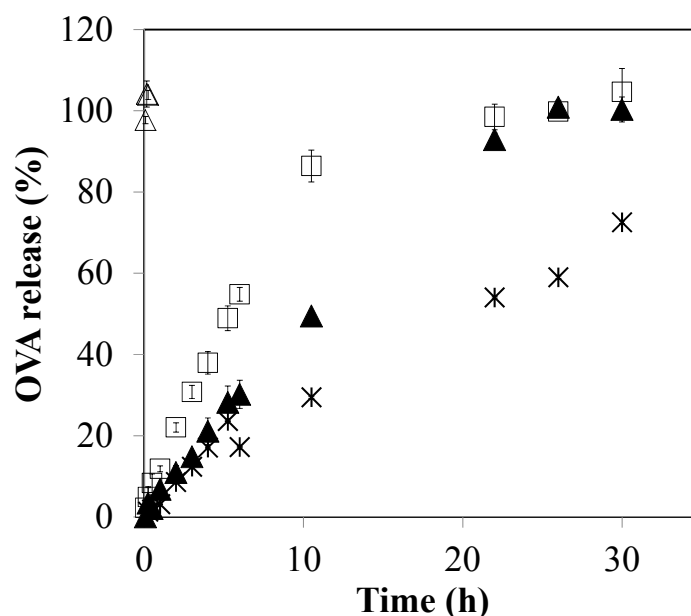
Figure 18. Release of OVA at 100 rpm paddle stirring rate from tablet formulations containing OVA in a matrix of lactose/MCC (open triangles), Carbopol® 974 P-NF (crosses), HPMC 90 SH 100000 (filled triangles) and HPMC 90 SH 100000/mannitol (open squares). Each data point represents the mean (± SD) of three different tablet preparations.

After the characterization of the release profiles it was examined if it is possible to evoke an immune response with a sublingually administered tablet formulation and how the release rate affects the elicited immune response. The analyzed serum and intestinal samples from the sublingual immunizations in mice showed that it was indeed possible to evoke immune responses with a tablet formulation (Figure 19).

Figure 19. Serum and intestinal anti-OVA antibody responses in mice immunized sublingually with ovalbumin (OVA) and CT as adjuvant. Doses of 250 μg OVA and 7.5 μg CT were given on days 0 + 1 and 16. Five groups of five mice each received tablet formulations. The reference group was immunized with a solution of OVA in PBS. (a) Serum IgG responses and (b) standardized small intestine (SI) IgA responses were measured against OVA by ELISA and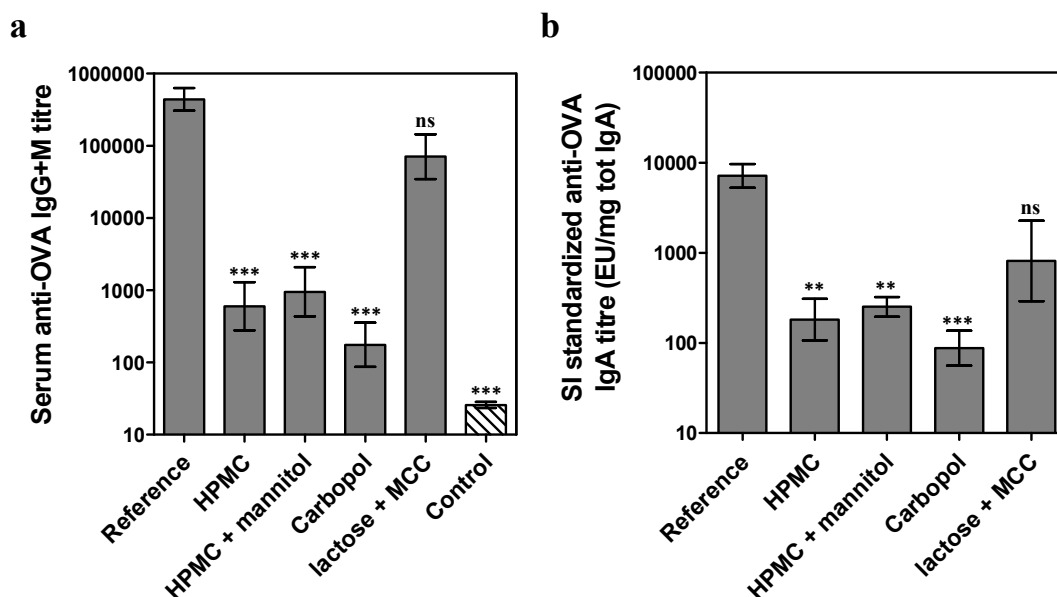 are expressed as $\log_{10}$ antibody titres (GM ± SEM). Asterisks denote significant differences (** $P < 0.01$, *** $P < 0.001$, ns = non-significant) compared to the reference group.

With the IR tablet systemic and mucosal antibody levels not significantly lower than those obtained with the OVA solution could be elicited. However, only poor responses were evoked with the three ER tablets; the antibody levels in all three groups were significantly lower, both in serum and intestinal samples. Interestingly, even if not significant, the differences in the antibody levels between the three groups that had received ER tablets had the same tendency as the differences in the release profiles: the lowest antibody levels were measured in mice that had been immunized with Carbopol® tablets (slowest ER tablet) whereas the highest levels were detected in mice that had received the HPMC/mannitol formulation (fastest releasing ER tablet).

These results gave rise to the two interpretations that a prolonged release might be unsuitable for sublingual immunization and that the slower the release of the antigen, the lower the evoked immune response. The latter is, however, more a speculation and more significant data are required for proving this interpretation. It is also important to consider that it is only valid for the model system used in this study that ER tablets are not resulting in any advantages for sublingual immunization since there are several factors that could have influenced the outcome of the present investigations. Since only small amounts of antigen are presented to the immune system at each point of time, the

immune response might depend on the total amount in the formulations and the type of antigen. In addition, the fact that CT was not co-released but given at once when the sublingual tablets were administered might have had an impact on the immune responses since the earlier observed time-dependent adjuvant effect of CT by Lycke et al. [78] was even observed in the investigations of Paper III. Furthermore, it is possible that the ER tablets were not kept at the administration site until erosion was complete. Even if the adhesion of test formulations could be confirmed for four hours in several mice, there is, of course, still a risk that some mice swallowed the gelled tablets or gel pieces.

5.5 Freeze-drying of killed whole-cell bacteria

If one wants to produce a solid vaccine for mucosal administration, finding of a drying process for the manufacturing of dry antigen is crucial. In the work in Paper IV killed whole-cell *V. cholerae* strain JS 1569 and the commercially available vaccine Dukoral™ were used as model vaccines for the evaluation of stabilizing excipients during freeze-drying of a bacterial vaccine. The *in vitro* characterization of freeze-dried formalin-killed *V. cholerae* O1 strain JS 1569 showed clear differences between the samples depending on how the bacteria were freeze-dried. The presence of a stabilizing agent was required both to avoid aggregation and to preserve the LPS antigen functionality on the surface of the bacteria and the stabilizing capacity was dependent on the type of stabilizer and the concentration. The results of the performed LPS inhibition ELISA are summarized in Table 2 and indicate a remarkable loss of the functionality of the surface LPS, the main protective antigen of *V. cholera*, in the absence of any potential stabilizer (only 37 % LPS recovery). The presence of stabilizer concentrations of 0.25 mg/ml or 2.5 mg/ml resulted in a better LPS recovery, but still no sample was above a 70 % recovery rate. This was even the case in the presence of 25 mg/ml mannitol or trehalose. In contrast, when 25 mg/ml sucrose was added the LPS functionality could sufficiently be maintained, as can be seen in the LPS mean recovery rate of 89 %, which was not significantly lower as compared to the untreated bacteria suspension ($p >$ 0.05). Thus, these results indicated that only the latter condition could preserve the important LPS surface antigen of *V. cholerae* during freeze-drying to a satisfactory extent.

Table 2. Preservation of *V. cholerae* O1 surface LPS after freeze-drying (FD) with and without excipients of different concentrations. The samples were re-suspended in PBS and the LPS recovery was determined by LPS inhibition ELISA.

Formulation	mg/ml	LPS recovery (% ± SE)	ANOVA
Untreated	n.a.	100 ± 12	n.a.
FD	n.a.	37 ± 3	$P < 0.001$
FD + mannitol	0.25	52 ± 12	$P < 0.001$
	2.5	61 ± 3	$P < 0.05$
	25	60 ± 4	$P < 0.01$
FD + sucrose	0.25	36 ± 3	$P < 0.001$
	2.5	62 ± 1	$P < 0.01$
	25	89 ± 10	**$P > 0.05$**
FD + trehalose	0.25	28 ± 1	$P < 0.001$
	2.5	63 ± 5	$P < 0.01$
	25	70 ± 7	$P < 0.05$

In the following characterizations by confocal microscopy all samples containing excipient at the highest tested concentration (25 mg/ml) were analyzed and compared with an untreated suspension (Figure 20a) in order to study eventual changes in the morphology, size or aggregation. Sucrose even showed promising results here. An image of a re-suspended freeze-dried sample without any excipient for stabilization is shown in Figure 20b which clearly shows large and densely packed bacterial aggregates of partly over 10 µm diameter. Much smaller and less dense, but still numerous aggregates were found in the samples freeze-dried with mannitol (Figure 20c). In contrast, *V. cholerae* freeze-dried with sucrose and even those with trehalose showed no changes in the morphology and no aggregates could be found by confocal microscopy.
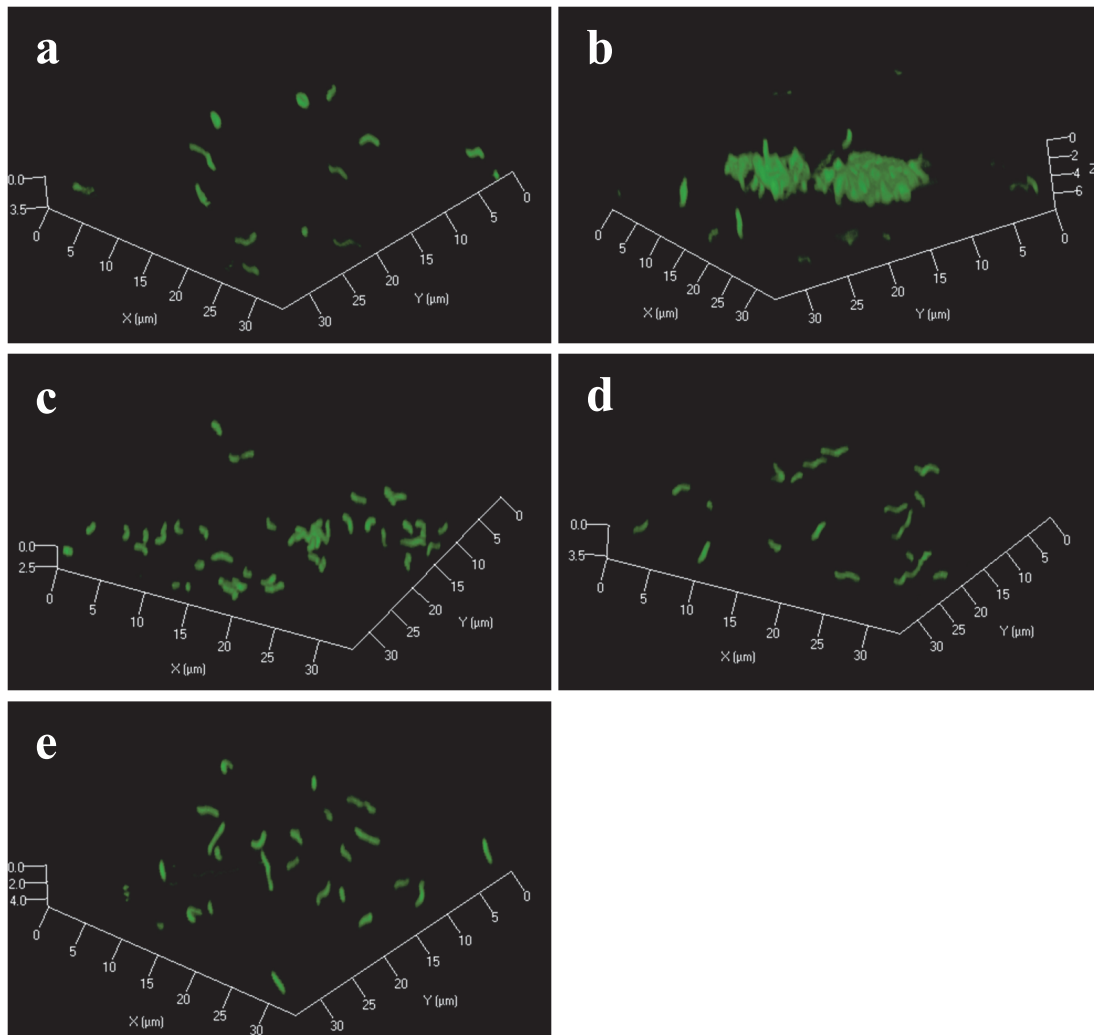
Figure 20. Images of formalin-killed JS 1569 *V. cholerae* (suspended in PBS) taken by confocal microscopy. (a) untreated, (b) freeze-dried, (c) freeze-dried with 25 mg/ml mannitol, (d) freeze-dried with 25 mg/ml sucrose and (e) freeze-dried with 25 mg/ml trehalose. The x- and y-axes of the z-stack images are scaled in 5 µm units and the dimensions are 33.81 µm x 33.81 µm in x- and y- direction and vary between 2.75 and 6.52 µm in z-direction depending on the properties of the sample.

From the *in vitro* characterizations it could be concluded that sucrose and possibly trehalose at a concentration of 25 mg/ml seem to sufficiently stabilize *V. cholerae* during freeze-drying. Since all excipients at lower concentrations failed as stabilizing agents, the *in vivo* evaluations in BALB/c mice were only performed with freeze-dried samples of JS 1569 containing either 25 mg/ml of excipient or no excipient. The ability to elicit systemic and mucosal immune responses after peroral administration was investigated by comparing these formulations with untreated JS 1569 suspension. The results are shown below in Figure 21.
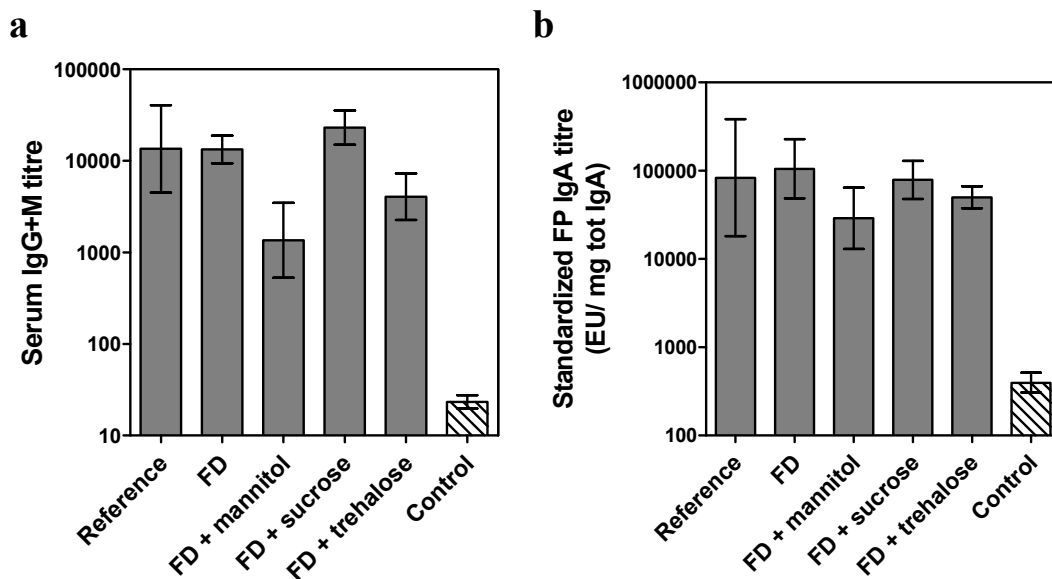
Figure 21. Serum and intestinal anti-O1 LPS antibody responses in mice immunized perorally with formalin-killed *V. cholerae* strain JS 1569. All groups of mice (four mice in each group) were immunized with doses containing 5×10^8 bacteria on days 0 and 2, 13 and 15, 41 and 43. Four groups received bacteria that had been freeze-dried (FD) in the absence or in the presence of an excipient (mannitol, sucrose or trehalose) and one group received untreated bacteria (reference). (a) Serum IgG+IgM responses and (b) standardized fecal pellet (FP) IgA responses were measured against O1 LPS by ELISA and are expressed as $\log_{10}$ antibody titres (GM±SE). The mean antibody level of sham-immunized mice (control group) was significantly lower than the reference group (*** $P < 0.001$) whereas no significant differences to the reference group were found in all other mice.

The antibody titres of the negative control group originate from previous experiments and are included to show that no responses can be evoked by sham-immunization with PBS alone. All groups of mice except for the control group developed both serum and intestinal anti-LPS antibodies. Variations between the effects of the excipients were found, as anticipated from the *in vitro* evaluations, however, the differences were not as distinct as *in vitro*. JS 1569 freeze-dried with mannitol elicited, as expected from the *in vitro* results, immune responses with lower systemic IgG+IgM antibody levels as compared to the untreated reference. The IgA levels detected in the small intestine were also lower in these mice. Freeze-dried JS 1569 stabilized with sucrose or trehalose had almost the same immunogenicity as can be seen in the similar systemic and mucosal responses. However, since slightly higher IgG+IgM and IgA antibody levels were measured in mice that had been immunized with freeze-dried JS 1569 stabilized by sucrose, the overall conclusion from the *in vitro* and *in vivo* data was that sucrose seems to provide the best stabilizing properties of all tested excipients during the drying process. Surprisingly, antibody levels comparable to the reference group were also found in mice that had received JS 1569 freeze-dried without any excipient. This formulation was

not expected to have a good immunogenicity according to all *in vitro* results. A speculative explanation could be that the recognition of bacterial surface LPS is not the only important property for an *in vivo* effect. It is also possible that an aggregation of the bacteria hampers the LPS recognition by the ELISA method, whereas the immune system is able to recognize the antigen, for example by processing and breaking up the aggregates. However, if the latter would be the case, even JS 1569 freeze-dried with mannitol should be as immunogenic as the freeze-dried preparation without excipient.

Summarizing both the *in vitro* and *in vivo* characterizations of freeze-dried JS 1569 formulations it could be concluded that sucrose was a promising stabilizer throughout all experiments. Trehalose showed a similar potential, however, this excipient is more expensive than sucrose and also less common in pharmaceutical products on the market, so that it provides no benefits over sucrose. According to the *in vitro* results, mannitol provided a poorer stabilizing capacity with regard to both bacterial aggregation and LPC maintenance and even the *in vivo* immunizations indicated the lowest ability to maintain the immunogenicity of JS 1569 during freeze-drying. Based on these considerations, the freeze-drying process was applied to the commercially available oral cholera vaccine Dukoral[TM], with either no stabilizer or with 25 mg/ml sucrose as the stabilizing excipient and the freeze-dried vaccine was compared with the original vaccine suspension in peroral immunizations in BALB/c mice. Since the efficacy of Dukoral[TM] is connected with both antibacterial and antitoxic IgA antibodies [70], sera and intestinal samples were analyzed for both anti-O1 LPS and anti-rCTB antibodies. In addition to FP estimates the intestinal antibody response was even measured in tissue extracts of the small intestine, since any possible impact of naturally excreted IgA from the liver via bile to the intestinal lumen [122] is avoided in those samples so that they provide direct evidence of the site-specific local production of IgA in the gut.

The antibody levels of all immunized mice are shown in Figure 22. The negative control data are as in Figure 21 taken from earlier experiments. All immunized mice elicited antibody titres significantly higher than this control. As already observed in the peroral immunizations with freeze-dried JS 1569, no significant differences were found between the groups. For the antitoxic responses similar antibody titres were expected both in sera and intestinal samples since it could be confirmed by a GM1 ELISA that CTB maintains its binding capacity during the freeze-drying process (data not shown). Compared to untreated Dukoral[TM], vaccine freeze-dried with sucrose evoked as observed

with the single JS 1569 strain, similar and not significantly lower antibacterial antibody levels. Only slightly lower antibacterial antibody levels were measured in mice that had been immunized with freeze-dried Dukoral[TM] without sucrose stabilization, so that the surprisingly good immunogenicity of freeze-dried killed whole-cell *V. cholerae* in the absence of any excipient was confirmed even with the mixture of the different *V. cholerae* strains present in the Dukoral[TM] vaccine.
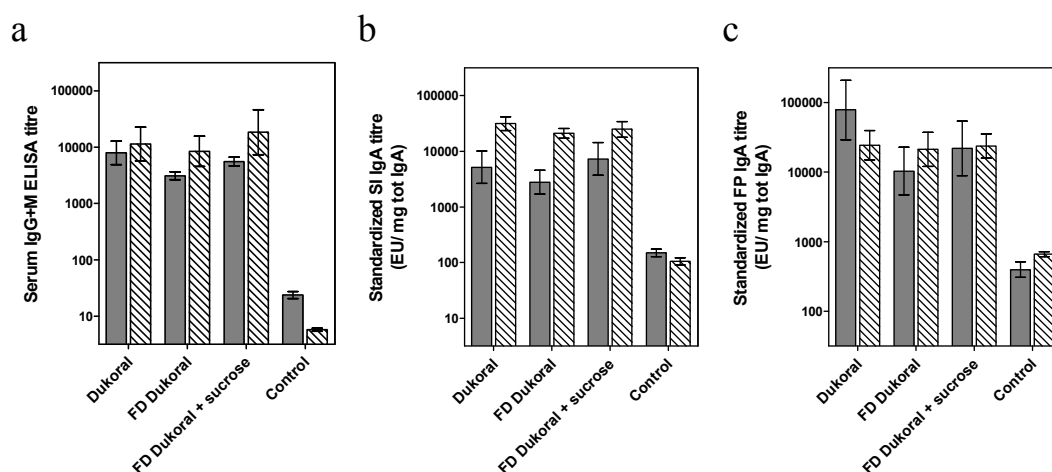

Figure 22. Antibacterial and antitoxic responses in mice immunized perorally with Dukoral[TM] vaccine. All groups of mice (seven animals per group) were immunized with doses containing 5×10^8 bacteria in three rounds on days 0 and 1, 14 and 15, 27 and 28. Two groups received Dukoral[TM] formulations that had been freeze-dried in the absence and in the presence of sucrose, respectively, and the reference group received untreated vaccine. Immune responses were measured against O1 LPS (filled bars) and rCTB (striped bars) by ELISA and are expressed as $\log_{10}$ antibody titres (GM ± SE). (a) Serum IgG+IgM responses, (b) standardized small intestine (SI) IgA responses and (c) standardized fecal pellet (FP) IgA responses.

Although no significant differences in the immunogenicity of the freeze-dried formulations were found regardless of the presence or absence of any excipient, one would not prefer a vaccine consisting of aggregated bacteria with obviously poor LPS antigen activity found in a LPS inhibition ELISA. The *in vivo* results of this study give raise to different questions to be solved, but it can anyhow be concluded that the use of excipients should be considered for a stabilization of killed whole-cell bacteria during freeze-drying and that sucrose might be a potential candidate.

Chapter 6

CONCLUDING REMARKS

The use of solid dosage forms for vaccines has so far been less common since most vaccines are injected as a liquid. However, there is a great need to develop delivery systems for non-invasive mucosal vaccines against infections starting at a mucosal surface, where injectable vaccines are not sufficiently protective. Solid dosage forms would provide a variety of advantages over conventional injectable vaccines and research is therefore needed to define important properties of suitable delivery systems for an efficient presentation of protective antigen to the mucosal immune system. Since the performance of a formulation is dependent on the right excipients in the formulation, the emphasis in the studies presented in this thesis was put on the influence of excipients associated with different aspects concerning the design of mucosal vaccine delivery systems.

Figure 23 summarizes the key findings of the different areas discussed in the previous chapters:
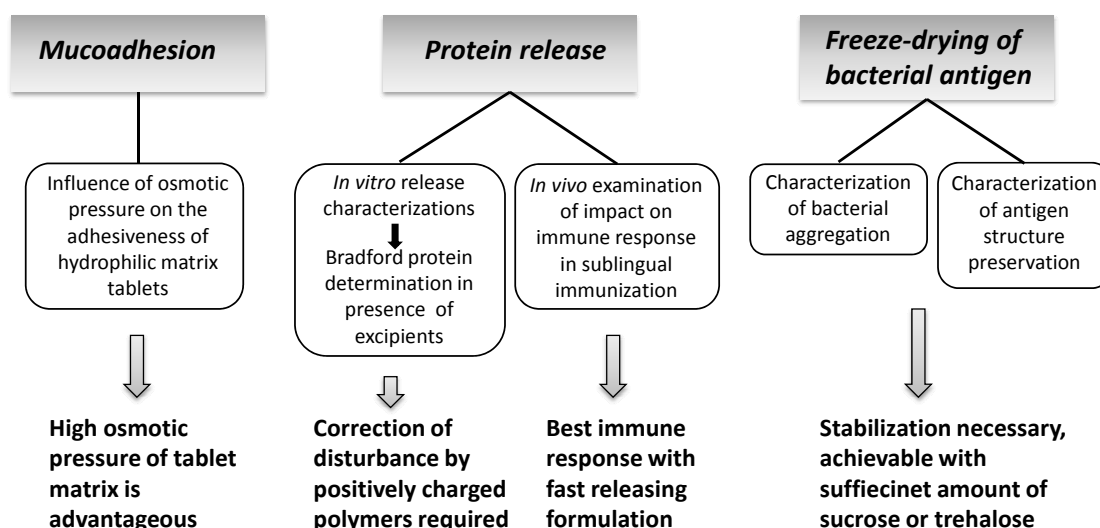

Figure 23. Overview of the outcome of the investigations in this thesis concerning the role of excipients.

It was found that osmotic pressure is an important factor for the mucoadhesive properties of tablets that swell upon contact with water. Since the nature of different excipients will reveal different osmotic pressures in a formulation, it

might be important to keep these findings in mind if a good mucoadhesiveness is desired. *In vivo* investigations in mice showed that tablets can be kept at the sublingual mucosa for several hours using the mucoadhesive polymer Carbopol® and that it is possible to efficiently deliver antigen formulated in a solid dosage form. The use of hydrophilic matrix tablets, which often also provide mucoadhesive properties, did not seem to be advantageous as sublingual vaccine tablets against enteric diseases since they release the antigen too slowly in order to be able to induce a sufficient immune response in the gut. Excipients that lead to an immediate release of the antigen should instead take preference. A fast dissolving sublingual tablet formulation is also most likely to be accepted by the patient than one that has to be kept under the tongue over a longer period of time. On the way to the findings of the release study a very commonly used analytical method for protein determination, the Bradford Assay, was evaluated and it could be concluded that the presence of charged excipients in protein samples can disturb the determination. For the disturbances caused by Carbopol® a correction method could be developed. The results of the assay evaluation show not only the effect of different pharmaceutically relevant polymers, but moreover also how important it is to question traditional analytic tools that are always mentioned as fast and simple in the literature but might carry a risk for false results if one is not observant. The freeze-drying study showed that it is possible to produce a freeze-dried cholera vaccine formulation based on killed whole-cell bacteria if the bacteria are stabilized by sucrose so that no loss in the antigen functionality and the immunogenicity of the vaccine occurs.

Given the huge number of aspects that are important in the design of an efficient mucosal vaccine formulation, a lot of work remains to be done. The fact that the studies in this thesis have shown that it is possible to evoke mucosal and systemic immune responses with a mucosal solid state formulation if the right excipients are used motivates a continued research within this interdisciplinary field. Especially the positive results with the IR tablet in the sublingual immunization study indicate great possibilities due to the ability of the sublingual mucosa to disseminate immune responses to various mucosal tissues. Clinical trials are ongoing on several mucosal vaccines including the improvement of both existing vaccines and new vaccines [33] and in my opinion the success of these vaccines will depend on a proper formulation work where the findings presented here can provide helpful knowledge.

Chapter 7

FUTURE OUTLOOK

The results presented in this thesis give rise to several ideas for related future studies that would be interesting to perform:

In spite of the efficacy of Dukoral^{TM} the vaccine is relatively cost intensive to produce. This is due to different *V. cholerae* strains and two different inactivation methods so that the development of new improved cholera vaccines is still ongoing. It would thus be interesting to apply the present findings on such future cholera vaccines, but even on other future vaccines against bacterial gastrointestinal infections as those caused by ETEC or *Helicobacter pylori*. Combining the findings in Paper III that an immediate release of antigen might be most effective in sublingual vaccination, the freeze-drying conditions of Paper IV and the fact that freeze-dried materials commonly are easy to dissolve with the knowledge that sublingual immunization can induce strong responses in the gut, the design of a sublingual vaccine tablet against enteric infections based on a freeze-dried formulation of killed whole-cell bacteria seems possible. For the development of such a formulation it would be required to investigate the influence of pressure during the compression of the tablet on the morphology and immunogenicity of the bacteria and how excipients can be used to provide stabilization during the processing if required. Regarding stabilization it is of course also of interest to perform long-term stability studies of these solid state vaccines and what excipients one should choose in order to achieve the desired stability properties.

Besides the application of the present findings on different "real" antigens it would also be interesting to investigate the use of other drying techniques. Since freeze-drying gives more dried cakes rather than powders, spray-freeze-drying is an interesting alternative. I would further like to study the effect of the release rate in mucosal immunization with other antigens and at mucosal administration sites other than the sublingual. It remains of course also to be seen to what extent the gained knowledge is applicable to humans where features such as the bacterial and enzymatic environment, the nutritional status or the physiological conditions might differ from the murine model.

Chapter 8

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Chapter 9

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