Stable spray dried protein formulation and implementation in vaccine development

Doctoral thesis
Submitted in fulfillment of the requirements
for the degree of
Doctor in Natural Sciences
at the
Christian Albrecht University
Kiel, Germany
by
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Kiel 2014
Referee: Prof. Dr. Hartwig Steckel
Co-Referee: Prof. Dr. Natalie Medlicott
Date of exam: 29.07.2014
Accepted for publication: 29.07.2014

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“All our dreams can come true, if we have the courage to pursue them”

-Walt Disney
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Chapter 1 Introduction and Objective

1.1 Introduction

The market of protein-based therapeutics is rapidly escalating due to unprecedented recognition of their success in clinics (Dimitrov 2012). The development of stable vaccine or protein formulations is problematic due to their fragile three-dimensional structures which are prone to chemical and physical degradations (Capelle et al. 2007). Hence, long-term stability and insufficient stability during manufacturing is the bottleneck in the successful development and commercialisation of this macromolecules (Klibanov, Schefiliti 2004).

Vaccines usually need to be stored between 2-8°C (Chen, Kristensen 2009) and maintenance of cold chains is also a challenging task especially over long distance transport and reaching remote populations (PATH 2012). Poor cold chain compromises vaccine safety and efficacy and are vulnerable to spoilage especially in developing countries where refrigerators are available at the periphery of the health care system. The major reason for polio outbreak in South Africa in the mid-1990s was due to an incomplete cold chain of the polio vaccine. This problem is not only confined to developing countries, it was found that about 38% of vaccines sampled in Hungary were damaged by cold weather (Riviere, Buckley 2012). It is estimated that by 2019 another dozen vaccines may be introduced in developing countries, but due to unavailability of cold chains, these vaccines will not be effective (Kaufmann et al. 2011; Riviere, Buckley 2012).
Solid protein formulations show a great potential to overcome this instability barrier and assure storage stability along with easier distribution (Arakawa et al. 2001). They can improve thermostability and will prevent temperature damage promoting reduced wastage, decreased logistical and equipment requirements as well as lower costs of transportation and storage (Maa, Prestrelski 2000; PATH 2012). Vaccines not requiring any refrigeration which can be stored safely at room temperature are an important goal envisioned by WHO’s Global Immunisation Vision and Strategy (WHO 2005). Dried formulation will increase the safety, reduce risk of recalls (following breaks in the cold chain) and decrease the cost of temperature-controlled storage and transport of thermo labile products (Kristensen et al. 2011).

The most common drying method used for biomolecules is lyophilisation (freeze drying), which involves freezing a protein solution; drying it at reduced pressure to remove the majority of bulk water by sublimation and a secondary drying step by thermal desorption to produce a dry cake (Matejtschuk 2007). However, freeze drying has some limitations such as it does not lead to a dispersible fine powder; it is a time- and energy-consuming process and can hence be very expensive (Roy, Gupta 2004). Freeze drying can be harmful to proteins due to stress caused by freezing and also during drying which can induce conformational instability. During freezing the molecule needs to be protected by cryoprotectants from harmful effects of the forming ice crystals and a shift in pH, which may easily occur due to the formation of saturated solutions differing in salt composition from the original buffer during freezing. Afterwards, the molecule needs to be
stabilised by lyoprotectants from dehydration during the removal of water. This can be achieved by an exchange of water with other hydrophilic molecules which may replace it as hydrogen bond forming partner.

Another effective and versatile method for converting proteins into a dry powder is by spray drying (Schüle et al. 2008). This process involves atomisation of the protein solution via a nozzle into a drying chamber containing hot air forcing the protein solution to instantly dry into solid particles (Lee 2002). Generally, spray drying is a gentle process due to short process time (10^{-4} sec from droplet formation to the dried particle being collected in a laboratory equipment) (Elversson, Millqvist-Fureby 2005a) and low thermal stress due to the cooling of evaporation. Nonetheless, proteins may come across several stress factors during spray drying such as shear stress when the protein is atomised into fine droplets, exposure to the air/liquid interface at the droplet surface possibly causing denaturation and increased temperature when the protein comes in contact with the drying air and loses its hydration shell which may induce protein denaturation and aggregation resulting in loss of protein activity (Lee 2002). Therefore, the protein needs effective protection and stabilisation.

Another area of concern for production of biological macromolecules is aseptic processing. Great care must be taken to assure control over each process step to maintain sterility of the products which is generally very expensive and any contamination during aseptic fill and finish may lead to significant economic loss (Lambert et al. 2011). An alternative method approved by pharmacopoeias is use of terminal sterilisation (European
Pharmacopoeia 5.0, chapter 5.1). However most protein and peptide drug products do not tolerate the high energy of ionising radiation which may result in unacceptable levels of physical and chemical degradation as well as destabilisation/unfolding. Although the extent of degradation might be lower than that associated with the high temperature treatment during dry-heat sterilisation. Therefore, a need exists for new formulation approaches that can protect from sterilisation mediated damage of biological macromolecules.
1.2 Objective of this thesis

The present research aims at developing a stable dried formulation of vaccine using spray drying. The number of excipients that are commonly used in spray-dried protein-structured drug products is relatively small and protein degradation and aggregation still remains a significant problem. Therefore effective protein protecting formulations urgently needs to be identified. In this thesis the potential of different amino acids as stabilising excipients for biomolecules were systematically evaluated. Stabilising and Protecting Solution (SPS; LEUKOCARE, Munich, Germany) formulation technology was also evaluated as potential spray drying excipient and was used to develop a terminally sterilised Influenza A (H1N1) vaccine formulation.

More specifically, the objectives of the present study were:

- To evaluate potential of different amino acids in protecting catalase, lysozyme and haemagglutinin from degradation during spray drying and subsequent storage at elevated temperature
- To evaluate SPS as protein-protecting excipient during spray drying of proteins and the subsequent storage
- To develop a spray-dried, stable, terminally sterilised powder vaccine of influenza A (H1N1) and evaluate the efficacy of spray-dried influenza A vaccine in-vivo in non-human primates
The work for this thesis was performed within the ZIM (Zentrales Innovationsprogramm Mittelstand) funded project “Production of biologically active and stable vaccine formulations by spray drying” (PROVAC, KF2802101FR0) in collaboration with Leukocare biotechnology (Munich, Germany).
Chapter 2 Theoretical Background

2.1 Proteins

Proteins are biological polymers made up of one or more polypeptide chains derived from the serial condensation of various amino acid monomers (Alberts 2002). Proteins are build up from a set of twenty amino acids each having a different unique side chain. Amino acids are covalently bonded together in chains by peptide bonds which are formed between the carbonyl group of one amino acid and the amino group of next amino acid (Figure 2.1). This polypeptide chain consist of a free amino (NH₂) group at one end and a free carboxyl (COOH) group at the other end (Hartl, Ruvolo 2012) (Figure 2.1). The chemistry of this amino acid side chains are vital for protein structure as they can form bonds with each other to hold a length of protein in a certain shape or conformation (O’Connor, Adams 2010). Polar amino acids side chains are capable of forming hydrogen bonds, charged amino acids side chains can form ionic bonds while the nonpolar (hydrophobic) chains can interact with each other to form weak van der Waals interaction (O’Connor, Adams 2010; Alberts 2002). Peptides, polypeptides and proteins are distinguished from each other based on the number of amino acids they contain. If the chain length contains fewer than 20 amino acids it is called a peptide; longer chains containing about 20-50 amino acids are called polypeptides and if they contain more than 50 amino acids they are called proteins (Lodish et al. 2000).
Proteins possess four levels of structural complexity namely primary, secondary, tertiary and quaternary (Figure 2.2). The primary structure of a protein is the linear amino acids sequence and determines the structural and functional characteristics of the protein. The secondary structure includes the folding of polypeptide chain in space (Lodish et al. 2000). The backbone folds periodically into one of two geometric arrangements when stabilising hydrogen bonds is formed between certain residues. The most recognised secondary structure element is a regular spiral, or helical, conformation, called as α-helix which is formed when single polypeptide chain twists around on itself to form a rigid cylinder (Alberts 2002). The defining feature of α helix is that the hydrogen bond is formed between every
fourth peptide bond, linking carbonyl oxygen of amino acid and the amide hydrogen of another amino acid (Lodish et al. 2000). Therefore, α-helix contains 3.6 amino acids per turn as illustrated in figure 2.3. Another regular secondary structure element is a β-sheet, a planar structure formed when the backbone of the polypeptide chain is extended into a zigzag form rather than a helical structure (Figure 2.3). β-sheet can be either parallel or antiparallel depending on amino-to-carboxyl orientations (Cox, Nelson 2008). The hydrogen bonding in β-sheet occurs between neighbouring polypeptide chains rather than within. Finally, without any stabilising interactions a polypeptide adopts a random-coil structure. About 60% of the polypeptide chain in an average protein exists as α-helices and β-sheets and the remainder of the molecule is in random coils and turns (Lodish et al. 2000).

*Figure 2.2: Levels of Protein structure* (Cox, Nelson 2008)
Figure 2.3: Model of the α helix (left); two-stranded β sheet with antiparallel β strands (right) (Lodish et al. 2000)

Tertiary structure of protein is its three-dimensional structure (Figure 2.2). The interaction between the “side chains” on the amino acids determines its tertiary structure. In contrast to secondary structure which is mainly stabilised by hydrogen bonding, the tertiary structure is stabilised by hydrophobic interactions between the nonpolar side chains and also by disulphide bonds in some proteins (Lu et al. 2007). This bonding interactions between "side chains" may cause a number of folds, bends and loops in the protein chain (Lodish et al. 2000). Quaternary structure is the specific association of two or more macromolecules with tertiary structure to form a complex (Figure 2.2).
Proteins viewed from the therapeutic perspective represent a tremendous opportunity to cure diseases (Leader et al. 2008). Protein therapeutics can be classified as antibody-based drugs, vaccines, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, growth factors, hormones, interferons, interleukins and thrombolytics (Dimitrov 2012). At present, the number of approved protein and peptide products on the market numbers just over 100 and many more are in development (Leader et al. 2008; Walsh 2010). However, formulation and delivery of therapeutic peptides is of great concern due to lack of physical and chemical stability which is leading to significant degradation during processing and storage of the aqueous formulations.

### 2.2 Instability of proteins in solution state

Proteins are inherently unstable molecules (Frokjaer, Otzen 2005; Manning et al. 1989) and very sensitive even to slight changes in solution chemistry. In aqueous solution, proper folding of a protein in its native state is not infinitely stable (Shortle, 1996) and is compositionally and conformationally stable only within a relatively narrow range of temperature, pH and osmolarity (Wang 1999). The instability in solution can arise due to breaking or formation of covalent bond, resulting in the formation of new chemical entities (chemical instability), as well as due to non-covalent changes that result in alterations in secondary, tertiary and/or quaternary protein structures (physical instability) (Manning et al. 1989). Protein-degradation
pathways are mostly concomitant and synergistic (i.e. chemical events usually trigger physical events and vice versa) (Vasco Filipe et al. 2013).

2.2.1 Chemical instability

Chemical instability generally refers to the formation or cleavage of covalent bonds within a protein structure and includes processes, such as oxidation, reduction and hydrolysis reactions (Vasco Filipe et al. 2013; Patel et al. 2011). Chemical instability can result in alteration of primary structure of the protein and may also have an influence on its higher levels of structure. The chemical instability includes fragmentation, oxidation (mostly Methionine, Cysteine, Histidine, Tryptophan and Tyrosine residues), deamidation (mostly Asparagine residues) and disulfide scrambling (Wang et al. 2007; Vasco Filipe et al. 2013).

2.2.2 Physical instability

Physical instability includes denaturation, surface adsorption, unfolding, aggregation and precipitation (Manning et al. 1989) and can result in modification of secondary structure. Protein aggregation is the most common physical instability and the most problematic. Aggregation can happen at any stage of manufacturing, processing, storage and shipping of the protein drug resulting in partial or complete loss of activity, reduced solubility, and altered immunogenicity (Serno et al. 2011).
2.3 Requirements and benefits of dried formulation of proteins

Liquid protein formulation suffers from inferior stability during long-term storage (Vonhoff 2010). As described above proteins in aqueous solutions can undergo a variety of reactions, such as hydrolysis, cross-linking, oxidation, aggregation and disulfide rearrangements, which are quite unacceptable in terms of product performance and safety (Franks 1998). The aqueous protein formulations must always be stored in refrigerated conditions as an overexposure to extreme heat or cold can cause damages to the protein in the solution. Furthermore, shaking of aqueous protein solution might provoke aggregate formation during shipping due to an increase of the air/water interface upon bubble formation (Heljo 2013). The presence of any aggregates in an injected solution possess a potential to elicit adverse reactions which can be life-threatening for patients because it might provoke side effects and unappreciated immune response (Diwakar et al. 2011). A possible alternative for increasing the storage stability of a protein formulation is to dry it. By removal of water protein stability usually greatly increases which is related to the reduced mobility of the protein and to the absence of certain degradation pathways facilitated by water (Maltesen, van de Weert 2008; Vonhoff 2009).

Therefore, drying proteins is the most feasible option to produce a stable product that can be marketed. This is especially interesting for the field of vaccination, where poor vaccination rates can be a result of improper vaccine stability (Karen Miskinis 2013). Dried formulations of vaccines can
mitigate the problems of cold chain breaks. This can improve the effectiveness and efficiency of immunisations by preventing temperature damage to vaccines, reducing vaccine wastage, and decreasing logistical and equipment requirements as well as the costs of vaccine transportation and storage (PATH 2012).

2.4 Drying techniques

2.4.1 Lyophilisation

Lyophilisation or ‘freeze drying’ is the most common dehydration method used to manufacture protein products that are unstable in aqueous solution (Franks 1998). In freeze drying the water is removed from a frozen solution by sublimation accompanied by desorption (Roy, Gupta 2004). The freeze drying process can be divided into three steps: freezing, primary drying and secondary drying (Nail et al. 2002). An illustration of a conventional freeze drying cycle is shown in figure 2.4. The shift from one step to the next can usually be identified as the point when the phase transition in water occurs (e.g. from crystallisation to sublimation). The first step ‘freezing’ involves freezing of protein samples by either slowly cooling the samples by a gradual reduction in shelf temperature or by cooling them rapidly on a prechilled shelf (Chang B.S., Patro S.Y. 2004). After the product has been frozen, the chamber pressure is reduced, and the temperature of the shelves is increased to provide energy for primary drying. The water is removed by sublimation under vacuum in this step. The shelf temperature is further
increased and the chamber pressure is further decreased for secondary drying which facilitates the removal of unfrozen bound water (Banga 2006).

![Graph showing process variables during freeze drying cycle](image)

*Figure 2.4: Process variables during freeze drying cycle (Banga 2006)*

The lyophilisation can induce conformational instability in proteins and is subject to degradation also in the solid state (Heller et al. 1999). Protein denaturation can be caused mainly due to two different types of stress, first stress is caused by the freezing itself and the second stress is caused by the drying process. Aggregation of protein drugs is the common mechanism of denaturation following lyophilisation (Banga 2006). Aggregates can be formed during freezing/drying step or also during reconstitution of structurally distressed protein molecules which may result in loss of biological activity of the protein drugs (Carpenter et al. 2002).
2.4.2 Spray drying

Spray drying is a well-established process for particle formation and drying widely used in industry as well as in laboratory (Vehring 2008). It is a continuous particle processing drying operation to obtain solids from the liquid feed stock (Sollohub, Cal 2010; GEA Niro). Spray drying involves several process stages to complete the drying operation. Firstly the aqueous feed is pumped through an atomiser device that produces fine mist or spray into the main drying chamber where the droplets are mixed with hot air (Mujumdar 2000). On contact with the hot air a rapid drying takes place due to evaporation of moisture from the droplets, converting the liquid droplets into powder particles which are finally separated from the air flow by cyclones (Brennan 2003). This transformation of liquid to solid only requires few milliseconds (Elversson, Millqvist-Fureby 2005b).

Spray drying is the method of choice when it comes to producing protein powders as it is a fast process, easy to perform, scalable and involves low operation cost compared to freeze drying. It also makes particle engineering more feasible and particle properties such as size distribution, dispersibility and surface can be manipulated easily (Chow, Albert H L et al. 2007). Lee et al. have demonstrated that spray drying is an effective process for producing dried formulation of peptide/protein suitable for parenteral or pulmonary administration with extended storage stability (Lee 2002). Spray drying of an aqueous protein formulation into a solid form will improve not only stability but also its handling and drug application.
2.5 Challenges in spray drying proteins

Protein native structure is a result of fine balance between various interactions including covalent linkages, hydrophobic interactions, hydrogen bonding and van der Waals forces (Diwakar et al. 2011). This intra-protein interaction together with protein-solvent interactions maintains the structure of protein and any change in surrounding environment can cause damage to these interactions and may triggers denaturation or inactivation. Protein may come across several stress factors during spray drying as illustrated in figure 2.5. Proteins being amphiphilic are prone to adsorption at interfaces which are readily formed during sample preparation. Protein may change conformation exposing the hydrophobic residues towards the air-water interface to avoid contact with water, which is accompanied by protein unfolding which leads to aggregation and possibly inactivation (Lee 2002). Proteins also come across shear forces in the pump and during atomisation in the nozzle. Shear forces alone are not so harmful for the protein but in combination with large air/liquid interfaces it may lead to enhanced aggregation and inactivation of protein (Banga 2006). After atomisation the protein is exposed to higher temperature which can cause reversible or irreversible thermal denaturation of protein due to weakening of hydrogen bonds and simultaneous strengthening of hydrophobic interactions. However, self-cooling effect of droplets due to solvent evaporation, prevents the temperature increase of the droplet surface above the wet bulb temperature (Schmid 2011). In summary, the removal of water itself as well as other stress factors can damage the protein during spray-
drying. Nevertheless, these instabilities can be ameliorated using appropriate formulation parameters by finding suitable formulation compositions and process conditions (Maury 2005).

Figure 2.5: Schematic representation of the possible stresses experienced by a protein during spray-drying (Adapted from Lee 2002)

2.6 Stabilisation during spray drying

Spray drying introduces dehydration stress to the protein, which can partly be overcome by the addition of excipients. These excipients usually replace the water molecules and decrease the structural perturbation of proteins upon dehydration (Maltesen, van de Weert 2008). Various mechanisms have been put forward that can (at least in parts) explain the lyoprotecting effects of different excipients (Elversson, Millqvist-Fureby 2005a):
2.6.1 Water replacement hypothesis

Proteins usually bind to many water molecules in solution and such hydrogen bonding is critical for thermodynamic stability of proteins (Liao et al. 2002). As water is removed during drying, the conformation of proteins can be altered leading to chemical degradation and aggregation of protein (Carpenter et al. 1994). The stabilisers form hydrogen bonds with the protein and substitute for the function of water. They create a water-like environment around the protein and strengthen the existence of its native tertiary structure preventing the unfolding of protein (Allison et al. 1999). The degree of stabilisation is greatly influenced by the extent and intimacy of hydrogen bond formation. Figure 2.6 demonstrates the mechanism of stabilisation of protein in dried state. Upon drying the excipient substitutes for water molecules at the protein surface by forming hydrogen bonds with the protein and thus, prevent denaturation.

![Figure 2.6: Protein in aqueous solution (left); Stabilisation of protein in dried state by hydrogen bonding to excipient molecules (Right) (Adapted from Tscheliessnig et al. 2012)]
2.6.2 Glassy state stabilisation

According to this hypothesis, stabilisers form a glassy matrix on drying in which the protein is molecularly dispersed and its molecular mobility is limited. Unfolding and degradation reactions that require mobility are difficult to take place in such a rigid matrix (Chang 2003). Hagen et al. showed that proteins conformational relaxation is decelerated and molecular mobility is restricted as amorphous glasses increase the viscosity of the formulation to as much as $10^{15}$ cP (Hagen et al. 1995). Due to the amorphous character of the glass it also enables the formation of hydrogen bonds with proteins allowing the stabilisers to act as a water substitute (Green, Angell 1989; Chang, Pikal 2009).

2.6.3 Reducing surface adsorption/concentration

Surfactants can be used to circumvent denaturation at the air–water interface by preventing protein adsorption at the interface (Adler, Lee 1999). According to this hypothesis surfactants bind to the hydrophobic surfaces of proteins that are potential unfolding sites and also compete with proteins to occupy the interfaces. As a result protein concentration at the surface of the drying phase decreases thereby inhibiting interfacial-induced denaturation (Maa et al. 1998b).
2.7 Excipients used in spray drying of proteins

The hydration of the protein is partly lost during spray drying and can lead to severe perturbations of higher structures resulting in protein denaturation. A variety of excipients have been studied that stabilise proteins during drying, examples include sugars, polyols, surfactants and amino acids (Labrude et al. 1989; Adler et al. 2000; Arakawa et al. 2007; Schüle et al. 2007). Different classes and different individual excipients have different protecting mechanism.

2.7.1 Sugars and polyols

The use of sugars to preserve the structure and activity of proteins during drying and rehydration has been thoroughly studied (Carpenter et al. 1994). Non reducing sugars such as sucrose and trehalose are most commonly used. They are believed to stabilise proteins during drying mostly by hydrogen bonding to the surface of the dried protein in place of the removed water layer and hence inhibiting unfolding. Apart from this water substitution action, the disaccharide forms an amorphous glassy solid during the drying process, which immobilises the protein molecules and provides enough kinetic stabilisation against protein unfolding (Abdul-Fattah et al. 2007). The stability of spray dried product IgG1 was found to be improved by addition of trehalose before spray drying (Schüle et al. 2008). Polyols such as mannitol are also effective hydrogen bond formers and may thus protect proteins from dehydration induced stress; however, their tendency to crystallize on drying reduces suitability as a stabilising
excipient in the formulation. Costantino et al. found that the ability of mannitol to provide the desired level of protein stabilisation for the spray-dried powder of recombinant humanised anti-IgE monoclonal antibody was limited by its tendency toward crystallisation (Costantino et al. 1998).

### 2.7.2 Surfactants

Surfactants are incorporated into several marketed protein solutions and help to protect the protein from surface induced (e.g., shaking or agitation) aggregation. They can also be used during drying, as these agents can displace protein molecules at the air/liquid-interface and thereby prohibits the exposure of the protein to denaturing surfaces. Polysorbates are commonly used in order to prevent protein surface adsorption and aggregation. Mumenthaler et al. showed that the addition of 0.1% (w/v) polysorbate 20 into the recombinant human growth hormone formulation reduced the formation of soluble and insoluble aggregates by approximately 90% on spray drying (Mumenthaler et al. 1994). However, the use of surfactants, especially polysorbates, is limited due to reduced long-term protein stability due to enhanced protein oxidation by residual peroxides (Schmid 2011).

### 2.7.3 Amino acids

Amino acids are well known stabilisers for biomolecule formulations (Hasija et al. 2013). Amino acids are incorporated in formulation as a buffing agent, antioxidant, to reduce surface adsorption and to inhibit aggregation. In solid state, amino acids are reported to stabilise proteins by direct binding to
protein molecules via hydrogen bonding or ion-dipole interactions (Hasija et al. 2013). A handful of amino acids are also reported to form amorphous solids upon drying (M. Mattern et al. 1999).
Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Model Proteins

3.1.1.1 Ovalbumin

Ovalbumin (OVA, Figure 3.1), a glycoprotein, is the main constituent of egg white, where it accounts for 60% to 65% of total protein content. It has a sequence homology with a group of proteinase inhibitors called serpins but it differs from this group as it does not undergo a conformational change upon proteolytic cleavage. The function of ovalbumin is not clearly understood but it is believed to be a storage protein. Ovalbumin is a single polypeptide chain of 385 amino acids and has a molecular weight of about 42.9 kDa (Nisbet et al. 1981) and an isoelectric point of 4.5 (Allen et al. 1984). Secondary structure of ovalbumin is composed of 33% α-helix, 5% of β-structure and 62% random coil (Batra 1991). The degradation temperature of ovalbumin was found to be 84°C as measured by differential scanning calorimetry (Donovan, Mapes 1976). In the present work, lyophilised ovalbumin from chicken egg white (Sigma-Aldrich, St. Louis, MO, USA) was used.
3.1.1.2 Catalase

Catalase (Figure 3.2) is a protective enzyme present in peroxisomes of nearly all aerobic cells and serves to protect the cell from the toxic effects of hydrogen peroxide by catalysing its decomposition into molecular oxygen and water (Deisseroth, Dounce 1970). Catalase is tetramer of 4 polypeptide chains each over 500 amino acid long (Vainshtein et al. 1981). Each polypeptide chain has a molecular weight of 60 kDa containing in its active centre a haeme group and NADPH (Kirkman, Gaetani 1984). The decomposition reaction of hydrogen peroxide by catalase occurs in two steps. Firstly, a molecule of hydrogen peroxide oxidises the haeme to an oxyferryl species resulting in generation of a porphyrin species when one oxidation equivalent is removed from iron and one from the porphyrin ring, a second hydrogen peroxide molecule then reduces and regenerates the resting state enzyme, generating a molecule of oxygen and water (Switala,
Loewen 2002). Secondary structure of catalase is composed of α-helix which account for 29% of its structure and β-structure for 14% (Lee et al. 2006). In the present study, catalase from bovine liver (Sigma Aldrich, St. Louis, MO, USA) as a lyophilised powder with an activity of 2,000-5,000 units/mg protein was used.

![Structure of bovine liver Catalase (RCSB Protein Data Bank)](image)

**Figure 3.2: Structure of bovine liver Catalase (RCSB Protein Data Bank)**

### 3.1.1.3 Lysozyme

Lysozyme (Figure 3.3) is an antimicrobial enzyme found in egg white, tears, and other secretions and is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus providing protection against infection (Newman et al. 1974). Lysozyme hydrolyses the β-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls. Gram-positive cells are quite susceptible to this hydrolysis as their cell walls have a high
proportion of peptidoglycan. Micrococcus lysodeikticus, a bacteria is especially susceptible to lysozyme, which is used for lysozyme activity assays (Imoto et al. 1968). Lysozyme is a single chain polypeptide of 129 amino acids cross-linked with four disulfide bridges and has a molecular weight of 14.3 kDa (Palmer et al. 1948). In the present study a crystalline powder of lysozyme (Sigma-Aldrich, St. Louis, MO, USA) with an activity of 70,000 units/mg was used.

![Figure 3.3: Structure of lysozyme (RCSB Protein Data Bank)](image)

**3.1.2 Influenza antigen**

Influenza is a contagious respiratory pathogen belonging to the family Orthomyxoviridae (Wilschut 2006). There are three types of influenza (A, B, C) which differ in pathogenicity and genome organisation and are mainly differentiated by the antigenic differences in their nucleoprotein and matrix protein. In humans, Influenza A and B viruses are the types that cause disease (Amorij et al. 2008). Influenza A virus (Figure 3.4) is composed of mainly two surface membrane glycoproteins Haemagglutinin (H) and
Neuraminidase (N). Other protein constituent of the virus includes matrix protein (MP1, MW 28 kDa; MP2, MW 12 kDa) and nucleoprotein (NP, MW ≈ 55 kDa) and several minor components from the membrane lipid matrix (Wilson, Cox 1990). Influenza A viruses are subdivided further into subtypes based on the surface antigens, H and N. In influenza A viruses 17 subtypes of H (H1-H17) and 10 subtypes of N (N1-N10) have been found (Centers for Disease Control and Prevention).

Figure 3.4: Structure of influenza virus (Adapted from Amorij et al. 2008)

Haemagglutinin (Figure 3.5) is responsible for both attachment of the virus to sialic acid containing receptors on the host cell surface and fusion of the viral and cellular membranes. Haemagglutinin ability to bind to sialic acids found on the surface of human erythrocytes and on the cell membranes of the upper respiratory tract which serves as the basis of haemagglutination, when viruses are mixed with blood cells, and enters into cells of the upper
respiratory tract. The name "haemagglutinin" comes from its ability to cause erythrocytes to clump together ("agglutinate"). Haemagglutinin is a trimeric glycoprotein (MW ≈ 230 kDa) of three identical monomers each having a MW of about 75 kDa. Each monomer consists of the polypeptides H1 (Mw ≈ 50 kDa) and H2 (MW ≈ 25 kDa) which are linked together by two intra-monomer disulfide bridges (Amorij et al. 2008). The Neuraminidase (N) is a tetrameric glycoprotein of MW of about 240 kDa and is responsible for transport of the virus particles through the mucin layer lining the respiratory tract (virus entry) and also mediates the release of newly assembled virus particles (virus release).

![Structure of Haemagglutinin (RCSB Protein Data Bank)](image)

In the present work, Pandemrix influenza vaccine (GlaxoSmithKline) was used which were formulated from an (H1N1)v (split virion, inactivated) A/California/7/2009 (H1N1)pdm09v like strain (x-179a)and is normally adjuvanted with AS03 adjuvant, provided in a separate flask and composed
of squalene (10.69 mg), DL-α-tocopherol (11.86 mg) and polysorbate 80 (4.86 mg) in phosphate buffer saline, being mixed with the antigen dispersion prior to injection. Only the antigen component (15 µg HA/mL) was used during formulation development. The Pandemrix influenza vaccine was kindly provided by the government of Brandenburg, Germany to Leukocare biotechnology (Munich, Germany) to be used within the ZIM project.

### 3.1.3 Stabilising and protecting solution (SPS)

The Stabilising and Protecting Solution (SPS; LEUKOCARE, Munich, Germany) is an aqueous solution (80 mg/mL) consisting of small molecule excipients, mainly amino acids and a glycosidic excipient glycyrrhizic acid (GA). SPS is a patented formulation for stabilisation and protection of biopharmaceuticals and combination devices (Margraf et al. 2010). SPS has shown to have excellent stabilising properties for biomolecules immobilised and dried on surfaces (Tscheliessnig et al. 2012). In the present study, several compositions of SPS were tested for formulation development of spray dried Pandemrix vaccine.

### 3.1.4 Amino Acids

#### 3.1.4.1 Arginine

L-Arginine (Arg, figure 3.6) is a positively charged polar amino acid with basic side chains and is very hydrophilic. It contains a guanidino group in the side chain which can be protonated at physiological pH. L-Arginine is used in several formulations containing therapeutic proteins as an excipient
highly effective in suppressing protein-protein interaction and therefore minimising protein aggregation (Hasija et al. 2013).

![Figure 3.6: Structure of L-Arginine](image)

**3.1.4.2 Glycine**

Glycine (Gly, figure 3.7) is the simplest of the amino acids. It does not have an asymmetric centre, and thus is not chiral. Glycine is commonly used in buffer solutions, in electrophoresis, and preparative chromatography. Glycine has also been used as a buffering agent in protein formulations and also as a bulking agent during lyophilisation (Pikal-Cleland et al. 2002).

![Figure 3.7: Structure of Glycine](image)

**3.1.4.3 Histidine**

L-Histidine (His, figure 3.8) has a basic side chain and is very hydrophilic. It contains an imidazole group in the side chain and this imidazole moiety can be either uncharged or positively charged, depending on the local environment (Sigma-Aldrich 2006). Histidine is often used as a buffering agent and also as an antioxidant in several protein formulations (Hasija et al. 2013).
3.1.4.4 Lysine

L-Lysine (Lys, figure 3.9) has basic side chains and is hydrophilic. It contains an N-butyl amino group in the side chain which gets protonated at physiological pH.

3.1.4.5 Leucine

L-Leucine (Leu, Figure 3.10) is a branched-chain aliphatic amino acid and is hydrophobic. It is important in haemoglobin synthesis and regulation of blood sugar and energy levels. Leucine is also used in pharmaceutical formulations as a “taste-influencing” agent (Ash, Ash 2007)
3.1.4.6 Methionine

L-Methionine (Met, figure 3.11) is one of the common sulfur-containing amino acid and is very hydrophobic. Methionine is used in oral pharmaceutical formulations as a flavouring agent and also in parenteral formulations as a pH controlling agent (Ash, Ash 2007).

![Structure of L-Methionine](image)

*Figure 3.11: Structure of L-Methionine*

3.1.4.7 Phenylalanine

L-Phenylalanine (Phe, figure 3.12) is a non-polar amino acid. It contains a benzyl side chain and is hydrophobic. The aromatic structure gives this amino acid useful absorption spectra, which can be monitored through UV spectrophotometry.

![Structure of L-Phenylalanine](image)

*Figure 3.12: Structure of L-Phenylalanine*

3.1.4.8 Tryptophan

L-Tryptophan (Trp, Figure 3.13) is a non-polar amino acid. It is used in the body to make vitamin-B (niacin) and also the neurotransmitters serotonin and melatonin. The aromatic structure gives this amino acid useful
absorption spectra, which can be monitored through UV spectrophotometry (280 nm) and can be used for detection and quantification of proteins.

![Structure of L-tryptophan](image)

*Figure 3.13: Structure of L-tryptophan*

**3.1.4.9 Tyrosine**

L-tyrosine (Tyr, figure 3.14) contains a bulky, uncharged, aromatic side group, a phenol group. The hydroxyl group present in the side chain gives it some slight polar characteristics. In addition, the aromatic structure gives this amino acid useful absorption spectra, which can be monitored through UV spectrophotometry (280 nm).

![Structure of L-tyrosine](image)

*Figure 3.14: Structure of L-tyrosine*
Table 1 summarises some important characteristics of amino acids used in this study.

**Table 1: Characteristics of amino acids used**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MW (Da)</th>
<th>Isoelectric point</th>
<th>Solubility (g/L)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>174.20</td>
<td>10.76</td>
<td>150</td>
<td>basic polar</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>5.97</td>
<td>250</td>
<td>neutral</td>
</tr>
<tr>
<td>Histidine</td>
<td>155.16</td>
<td>7.59</td>
<td>38</td>
<td>basic polar</td>
</tr>
<tr>
<td>Lysine</td>
<td>146.19</td>
<td>9.74</td>
<td>1500</td>
<td>basic polar</td>
</tr>
<tr>
<td>Leucine</td>
<td>131.18</td>
<td>5.98</td>
<td>24.26</td>
<td>non polar</td>
</tr>
<tr>
<td>Methionine</td>
<td>149.21</td>
<td>5.74</td>
<td>33.81</td>
<td>non polar</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>165.19</td>
<td>5.48</td>
<td>29.65</td>
<td>non polar</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>204.23</td>
<td>5.89</td>
<td>11.36</td>
<td>non polar</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>181.19</td>
<td>5.66</td>
<td>0.453</td>
<td>polar</td>
</tr>
</tbody>
</table>

### 3.1.5 Mannitol

Mannitol (Figure 3.15) is a hexahydric alcohol related to mannose and is isomeric with sorbitol (Ash, Ash 2007). Mannitol is widely used as filler in tablet formulations. Mannitol has also been used in some lyophilised preparations, as a carrier to produce a stiff, homogeneous cake and to improve the appearance of the lyophilised cake in a vial (Liao et al. 2007).
3.1.6 Trehalose

Trehalose (Figure 3.16), a disaccharide, is composed of two glucose molecules bound by an alpha, alpha-1,1 linkage. This α,α, glycosidic linkage makes it a non-reducing sugar and therefore it does not undergo the Maillard reactions. Trehalose is one of the most stable saccharides which has high thermostability and a wide pH-stability range (Simperler et al. 2006). Trehalose has been widely used for stabilising proteins during lyophilisation (Kaushik 2003).
Chapter 3: Materials and Methods

3.2 Methods

3.2.1 Spray drying

Spray drying was performed using a Büchi mini spray dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland). Figure 3.17 shows the schematic of a Büchi spray dryer. A B-290 mini spray dryer consists of a heating system, nozzle, drying chamber, sensors for measuring inlet temperature and outlet temperature, cyclone, collecting vessel, filter and aspirator (Buchi Laboratory). The aspirator is located behind the filter and aspirates the drying air by means of vacuum. For product separation Büchi spray dryer consists of a high performance cyclone having an electrically conductive layer of zirconium oxide on the inner wall of the cyclone to prevent the product from electrostatic binding to the cyclone wall.

Figure 3.17: Schematic of a Büchi mini spray dryer B-290
3.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to determine the molecular weight of proteins. In SDS-PAGE separations and migration of polypeptides is mainly governed by molecular weight. Sodium dodecyl sulphate (SDS), an anionic detergent, is added to the protein solution and then heated to denature the proteins so as proteins do not have any secondary, tertiary or quaternary structure (i.e. they retain only their primary amino acid structure). Apart from unfolding, SDS also gives a uniform negative charge to the protein (Hjemel, Chrambach 1981). Reducing agents, like 2-mercaptethanol are also used to break intra molecular disulphide bridges. The denatured protein is then loaded onto polyacrylamide gels and a constant current is applied onto the system which results in separation of protein according to their molecular weight (Sambrook, Russell 2006). In the present study, SDS-PAGE was used to determine the molecular weight integrity of model proteins after spray drying and subsequent storage. SDS-PAGE was carried out using a Bio-Rad Mini-Protean 3 gel electrophoresis system (Bio-Rad Laboratories, Hercules, USA). 10 µL of protein samples (0.5 mg/mL) was loaded into each well after incubating (95°C, 5 min) with 10 µL of sample buffer and subjected to electrophoresis at voltage of 120 V for 5 minutes and then 200 V for about 50 min. Coomassie blue (Carl Roth GmbH, Karlsruhe, Germany) staining was used to visualise the protein. A PageRuler Prestained Protein Ladder (Thermo Scientific, Waltham, MA, USA) was used as molecular weight marker.
For Pandemrix, non-reducing and reducing denaturating SDS-PAGE was performed from the cooperation partner (Leukocare Biotechnology, Munich, Germany) to monitor possible changes in the banding patterns of the split vaccine formulations upon the exposure to different kind of stresses, leading probably to aggregation and/or fragmentation of the viral antigens. Analysis was performed using a Novex X Cell II Mini cell system and Novex NuPAGE Bis-Tris Gels (4-12%); 10 or 12 wells at 1 mm thickness (Invitrogen, Darmstadt, Germany) with NuPAGE MOPS running buffer. Reconstituted spray dried products were reconstituted with an appropriate volume of water to adjust haemagglutinin concentration to 15 µg/mL. For denaturating conditions, the samples were prepared by mixing 12 µl of the vaccine formulation with 12 µL of the LDS-sample buffer 2 x concentrate (Invitrogen). The samples were denatured at 95°C for 10 min and then 20 µL of the samples were loaded to the gel wells. The applied protein amount was 0.1125 µg/well. Separation was done at a constant voltage of 200 V and running time was approximately 90 min. Gels were then stained with a silver staining kit (SilverXpress silver staining kit, Invitrogen). An unstained molecular weight standard (Novex Mark 12™ Unstained Standard, Invitrogen) was analysed on each gel to determine the molecular weight of the detected bands.

3.2.3 Circular Dichroism (CD)

Circular Dichroism (CD) is a powerful technique to determine the secondary structure of proteins (Herron et al. 1995). Polypeptides possess optical properties due to the asymmetric centres of their constituent amino acids
and thus interact differently with right- and left-circularly polarised light. The differences in the absorption of left-handed polarized light versus right-handed polarized light is measured in circular dichroism (Hagnauer 1982). In the far-UV region (200-250 nm) CD can be used for the prediction of secondary structure of a protein (Deutscher, Glasel 1995). Chromophore at these wavelengths is the peptide bond, and the signal arises when it is located in a regular, folded environment (Ranjbar, Gill 2009). α-helix, β-sheets and random coil structure give rise to characteristics (Figure 3.18) in shape and magnitude of CD spectrum, thus their approximate fraction present in any protein can be determined by analysing far UV CD spectrum (Banga 2006). In the present study, a Jasco J-720 spectropolarimeter (JASCO International Co. Ltd, Hachioji city, Japan) was used to assess the secondary structure of proteins before and after spray drying. CD spectra were recorded in far UV range (200 to 250 nm) region with a sampling interval of 1.0 nm in a 0.05 mm path length cuvette.

Figure 3.18: Characteristic CD spectra (Mathews, van Holde 1996)
3.2.4 Karl Fischer titration

Karl Fischer titration was used to determine the water content in the spray dried samples. The determination of water content in the samples by Karl Fischer is based on the quantitative reaction of water with iodine and sulphur dioxide in the presence of methanol and an organic base such as pyridine (Scholz, Boschke 1984). Methanol, sulphur dioxide (SO₂) and a base (RN) react to form an alkylsulfite intermediate (Reaction 1), this intermediate reacts with iodine (I₂) and the water from the sample (Reaction 2). Since water and I₂ are consumed in equimolar amounts and knowing the amount of I₂ consumed, the amount of water that was present in the sample can be calculated (Aldrich).

\[ \text{CH}_3\text{OH} + \text{SO}_2 + \text{RN} = [\text{RNH}]\text{SO}_3\text{CH}_3 \quad \text{(Reaction 1)} \]

\[ [\text{RNH}]\text{SO}_3\text{CH}_3 + \text{I}_2 + \text{H}_2\text{O} + 2\text{RN} = [\text{RNH}]\text{SO}_4\text{CH}_3 + 2[\text{RNH}]\text{I} \quad \text{(Reaction 2)} \]

In the present study, a Karl Fischer titrator (V20, Mettler Toledo) was used to determine the water content of the spray dried samples. About 50 mg of the sample was dissolved in 1 mL of dimethylsulfoxide and was added to the titration flask. Titration was then carried out using Karl Fischer reagent (HYDRANAL, Sigma-Aldrich, St. Louis, MO, USA) of previously determined titre (mg H₂O/mL). Water content was determined based on the titration volume (mL) and was subtracted for solvent.
3.2.5 Dynamic vapour sorption

Dynamic vapour sorption (DVS) is a gravimetric technique used to measure the water sorption isotherms. In DVS experiment the relative humidity is varied by mixing of dry gas with a water-saturated gas stream and the mass change of sample is constantly monitored as a function of time. The equilibrium mass values at each relative humidity step are then used to generate the isotherms (Hogan, Buckton 2001). In the present study, DVS experiments were carried out with a DVS 1 (Surface Measurement system Ltd., London, UK). Approximately 15 mg of samples was weighed onto a Cahn microbalance. The sample was then exposed to stepwise increasing rH with 10% increment until equilibrium moisture uptake was achieved up to rH 90%. Then, again the sample was stepwise dried in 10% steps to an rH of 0%, followed by a second cycle using the same protocol (Figure 3.19).

![DVS Relative Humidity Plot](image)

Figure 3.19: DVS plot showing step-wise increase in rH (Surface Measurement Systems)
3.2.6 Catalase activity assay

Enzymatic activity of catalase was determined using modified Beers and Sizer method (Beers, Sizer 1952) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit of enzyme decomposes one micromole of \( \text{H}_2\text{O}_2 \) per minute at 25°C and pH 7.0 under the specified conditions. 100 µL of diluted solution of catalase was added to 2.9 mL of substrate (1.9 mL of 0.059 M \( \text{H}_2\text{O}_2 \) + 1 mL of distilled water) contained in a cuvette of a Helios omega UV/Vis spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The decrease in absorbance at 240 nm was recorded and \( \Delta A_{240}/\text{min} \) was calculated; enzymatic activity of catalase was calculated using equation (1) (Worthington, Worthington V. 2011):

\[
\text{Units/mg} = \frac{((\Delta A_{240}/\text{min} \times 1000 \times \text{reaction vol.} \times \text{df}))}{(43.6 \times \text{sample vol.})} \quad \text{(Equation 1)}
\]

reaction vol. = Volume (in mL) of reaction mixture used  
\( \text{df} \) = Dilution factor  
Sample vol. = Volume (in mL) of enzyme solution used

3.2.7 Lysozyme activity assay

The activity of lysozyme was measured by monitoring the rate of lysis of a micrococcus lysodeikticus cell suspension in 0.066 M phosphate buffer, pH 6.24 at 25°C. Solutions (20 µg/mL) of pure and spray dried samples were
also prepared in the same buffer. The decrease in absorbance at 450 nm on addition of 0.1 mL of each sample solution to 2.5 mL of the bacterial suspension was monitored using a UV-Vis spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). A unit of activity decreases the absorbance at 450 nm at 0.001 min\(^{-1}\) under the above conditions (Shugar David 1952). The activity was determined using equation (2):

\[
\text{Units/mg} = \frac{((\Delta A450/\text{min Test} - \Delta A450/\text{min blank}) \times \text{df})}{(0.1 \times 0.001)}
\]  
(Equation 2)

\(\text{df} = \text{Dilution factor}\)

0.001 = Change in absorbance at 450 nm as per the unit definition

0.1 = Volume (in mL) of enzyme solution used

### 3.2.8 Haemagglutination assay (HA)

The Haemagglutination (HA) assay is a quantification of haemagglutinin protein of viruses. Influenza virus has surface or envelope proteins, which are able to agglutinate (stick to) human or animal red blood cells (RBC) and bind to its N-acetyleneuraminic acid (Sialic acid) (Chattopadhyay et al. 2009). If viruses are present, the red cells become bound to the virus particles in a lattice. This lattice then coats the well to form a uniform reddish colour (positive result). In the absence of virus the RBC’s will fall to the bottom of a culture well, forming a sharp dot (negative result). The titres were determined as the endpoint dilutions inhibiting the precipitation of red blood cells and are given as the inverse of the dilution factor. Figure 3.20 summarises the procedure of haemagglutination assay. In the present
study, Haemagglutination assay was performed by reconstituting the dried samples to adjust the haemagglutinin concentration to 15 µg/mL in each sample. 50 µL of the diluted split vaccine formulations were two-fold serially diluted in 50 µL PBS mixed with an equal volume of fresh 0.5% v/v suspension of red blood cells (Institute of Immunology, UKSH, Kiel, Germany) or 0.5% (w/v) chicken red blood cell suspension (Harlan Laboratories, Belton, Leicestershire, UK) in a U-bottom 96 well plate. After 45 minutes incubation at room temperature, the plates were scored for agglutination.

*Figure 3.20: Haemagglutination assay procedure*
3.2.9 Dynamic Light Scattering (DLS)

Dynamic light scattering also known as "photon correlation spectroscopy" (PCS) or "quasi-elastic light scattering" (QELS) measures the size of the particles by measuring the ‘Brownian motion’ of molecules in solution (Goldburg 1999). Brownian motion is the movement of particles due to the random collision with the molecules of the liquid surrounding the particle as a result of which small particles move quickly and large particles move more slowly. Because of this, light scattered from particles fluctuates with time. A correlation curve is produced from these fluctuations across very short time intervals, which is further deconvoluted using algorithms to obtain size distribution information (Mattison 2003). In the present study, Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) was used to determine any aggregates after spray drying of Pandemrix. A sample amount equivalent to 15 μg of protein (HA) was redispersed in double distilled water and was measured.

3.2.10 Particle size analysis by laser diffraction

Laser diffraction was used to determine the particle size distribution after spray drying. In laser diffraction particle size is measured by passing a laser beam onto a dispersed particulate sample and measuring the angular variation in intensity of scattered light (Malvern). The large particles usually scatters light at narrow angles with high intensity while the smaller particles scatter will light at wider angles but with low intensity (Chu 1991). Mie theory of light scattering is then used to calculate the size of the particles.
responsible for creating the scattering pattern (Johnson, Gabriel 1994). Another approach, the Fraunhofer approximation, can also be used as an alternative where the optical properties of samples are not known (Malvern). In the present study, particle size was measured by a HELOS diffractometer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) using a 50 mm focal length lens and a 6 mL cuvette. Few milligrams of spray dried formulations were dispersed in about 6 mL of Miglyol 812 (Sasol Olefins & Surfactants GmbH, Hamburg, Germany). The suspension was sonicated in a water bath (Transsonic T460/H, Elma GmbH, Singen, Germany) for 45 s to disperse any possible agglomerates before measurement. The particle size distributions were expressed in terms of x10, x50 and x90 values based on a volume distribution.

3.2.11 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) also known as gel filtration chromatography is a common method for separating proteins based on their size or hydrodynamic volume (Barth et al. 1994) and can be used for studying protein aggregation or for determination of low molecular weight degradation products (Watson, Kenney 1988). SEC uses a gel filtration column packed with particles of defined size. Proteins and its variants passes through this packed column and reaches the end of the column (Striegel 2009). Small variants have access to greater number of paths through the porous material that results in a larger accessible volume for these variants and therefore results in a longer retention time (Ozturk, Hu 2006; Mary E.M. Cromwell 2006). Larger variants have fewer paths and
therefore elutes first from the SEC column (Figure 3.21). SEC mobile phase used is usually aqueous salt solution and stationary phase consists of a column porous microparticles (Hagnauer 1982). In the present study, size exclusion chromatography (SEC) was performed using an Merck-Hitachi D 7000 system (Merck-Hitachi, Darmstadt, Germany) with UV detector at 214 nm and a 13 µm TSKgel GMPWXL SEC column (7.8 × 300 mm) (TOSOH Bioscience GmbH, Stuttgart, Germany). A sample amount equivalent to 15 µg of protein (HA) was redispersed in PBS and were injected into SEC. Samples were run in a mobile phase of PBS pH 7.4 at a flow rate of 0.7 mL/min.

*Figure 3.21: Separation by SEC (Adapted from Striegel 2009)*
3.2.12 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy is an important tool for structural characterisation of proteins. FTIR spectroscopy is based on a measurement of wavelength and intensity of the absorption of IR radiation by a protein sample (Jilie, Shaoning 2007). Characteristic bands found in the infrared spectra of proteins and polypeptides include the Amide I and Amide II which arise from the amide bonds (Figure 3.22). Amide I band is mostly used to perform the structural analysis of proteins. Amide I band absorption occurs in 1600-1700 cm\(^{-1}\) region and is directly related to the backbone confirmation with a major contribution from C=O stretching vibrations and some contribution from C-N stretching vibrations (Marco Van De Weert 2005).

![Fourier transform infrared spectroscopy diagram](image)

**Figure 3.22:** A typical protein FTIR spectra, indication various region in which vibrational motions of the amide backbone occurs; Amide I (1700–1600cm\(^{-1}\)), amide II (1600–1500cm\(^{-1}\)) and amide III (1330–1220cm\(^{-1}\)) (Vonhoff et al. 2010)
Chapter 3: Materials and Methods

In the present study, Fourier transform infrared measurements were conducted using a Spectrum 100 (Perkin Elmer, Rodgau, Germany) spectrometer. Spectra were recorded for spray dried samples in amide I region (1600-1700 cm\(^{-1}\)), they were corrected for background and were area-normalised in the amide I region. For each spectrum, 128 scans were collected in absorbance mode with a 2 cm\(^{-1}\) resolution using a MIRacle attenuated total reflection unit (Pike technologies, WI, USA). Second derivative spectra were also obtained in amide I region using the spectrum software version 6.3.4.0164 (Perkin Elmer, Rodgau, Germany).

3.2.13 X-ray powder diffraction (XRPD)

X-ray powder diffraction was used to determine the crystallinity of the unprocessed and spray dried products. In the present study, Stadi P X-ray diffractometer (Stoe & Cie GmbH, Darmstadt, Germany) in transmission mode using Cu-K\(\alpha\) radiation (40 kV, 30 mA) was used. The samples were scanned from 5 to 50° 2-Theta, with steps of 2° 2-Theta and duration of 80 s per step.

3.2.14 Fluorescence Spectroscopy

Fluorescence spectroscopy is a sensitive method for characterising any conformational changes in structure of protein. Tryptophan’s and tyrosine’s intrinsic fluorescence can be used to determine conformational changes of proteins (Herron et al. 1995). Apart from the intrinsic fluorescence measurements some extrinsic fluorescent dyes also offer great opportunities for characterising structural perturbations in proteins. An extrinsic
fluorescent dye interacts non-covalently with proteins and protein degradation products via hydrophobic or electrostatic interactions. These interactions between dye molecules and proteins can lead to changes of fluorescence emission by a change of the preferred relaxation pathway (Hawe et al. 2008). In this work, fluorescence spectra were recorded using a fluorescence spectrometer (LS55 from PerkinElmer, Waltham, USA) using a 1 cm path length cuvette. Intrinsic fluorescence of 1-Anilino-8-Naphthalene Sulfonate (ANS; Sigma-Aldrich, St. Louis, MO, USA) was measured by exciting it at 360 nm and the emission spectrum was recorded in 390-550 nm range. Fluorescence spectra were corrected for the background spectrum of solvent.
Chapter 4: Results and Discussion

4.1 Stabilising proteins with amino acids

Despite their use as stabilising excipients in biopharmaceuticals, there are only limited studies on the use of amino acids as a potential stabilising excipient and subsequent investigation of their mechanism of stabilisation during spray drying (Chang, Pikal 2009). The Stabilising and Protecting Solution (SPS; Leukocare, Munich, Germany) is also a mixture of various amino acids and therefore it is of great interest to evaluate the stabilising effect of single amino acids. Catalase has a complex structure, is intrinsically unstable (Lee et al. 2006) and was found to be quite unstable on drying as studied by Liao et al. (Liao et al. 2002). Therefore it was chosen as an ideal candidate to examine the stabilisation ability of amino acids in this study.

4.1.1 Spray drying catalase with single amino acids


Before conducting spray drying together with catalase, single amino acids were investigated with Dynamic Vapour Sorption (DVS) to estimate their impact on final powder properties. DVS isotherms of amino acids are shown in figure 4.1. L-Arginine and L-Lysine showed strongest water vapour interaction and hence are hygroscopic at rH of 40% rH and above, while the
other amino acids have an almost negligible interaction with water vapour. Arginine and lysine, despite being hygroscopic were studied as they have shown to be remarkably good stabilisers to prevent protein aggregation in solution state (Falconer et al. 2011; Baynes et al. 2005).

An aqueous solution of catalase (1.5%, w/v) was spray-dried using a Büchi B-290 laboratory spray dryer (Büchi, Flawil, Switzerland). The feed solution was pumped at a speed of 5-7.5 mL/min through a two fluid nozzle (1.5 mm inner diameter) into the drying chamber. The catalase solution was dried at an inlet air temperature of 180°C, the atomising air was 470 L/h and the outlet air temperature was between 90–95°C. On spray drying at low outlet temperature of 50°C catalase did not show considerable loss of activity and therefore high outlet temperature (90°C) was used to stress catalase further and to get substantial loss of the activity. The aspirator flow rate was 35 m³/h (=100%). The dried product was collected in the product container using a high performance cyclone and was stored in closed glass vials at -20°C until assayed. Catalase was spray dried with different amino acids as summarised in table 2. Addition of different single amino acids to the catalase solution prior to spray drying resulted in more or less stable products compared to spray dried catalase without any amino acids (Figure 4.2). Especially the addition of arginine, glycine or histidine to the spray drying solution appeared to have a good stabilising effect and resulted in an activity ranging from 60 to 80% compared to only 50% of pure catalase spray dried. Activity maintenance was higher when these amino acids were used in a 1+2 ratio (protein + amino acid) than if used in a 1+1 ration. These batches were then stored for 1 month at 25°C/60% rH and 40°C/75% rH to get further insight on stabilising capability. Change in enzymatic activity after 1 month storage is shown in figure 4.3. In general, activity was less upon storage at 40°C/75% rH compared to storage at milder conditions.
Other studies had already shown that increased temperature leads to increase in intermolecular β-sheet in catalase resulting in reduced enzymatic activity (Lee et al. 2006). Formulation with arginine, glycine or histidine may partially inhibit this effect.

Table 2: Formulation composition of spray dried batches of catalase with amino acids (* indicates reduced concentrations due to saturation solubility)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Catalase (% w/v)</th>
<th>Amino acids</th>
<th>Concentration (% w/v)</th>
<th>Ratio AA+protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.5</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1.5</td>
<td>arginine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>III</td>
<td>1.5</td>
<td>arginine</td>
<td>3</td>
<td>2+1</td>
</tr>
<tr>
<td>IV</td>
<td>1.5</td>
<td>glycine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>V</td>
<td>1.5</td>
<td>glycine</td>
<td>3</td>
<td>2+1</td>
</tr>
<tr>
<td>VI</td>
<td>1.5</td>
<td>histidine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>VII</td>
<td>1.5</td>
<td>histidine</td>
<td>3</td>
<td>2+1</td>
</tr>
<tr>
<td>VIII</td>
<td>1.5</td>
<td>leucine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>IX</td>
<td>1.5</td>
<td>lysine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>X</td>
<td>1.5</td>
<td>lysine</td>
<td>3</td>
<td>2+1</td>
</tr>
<tr>
<td>XI</td>
<td>1.5</td>
<td>methionine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>XII</td>
<td>1.5</td>
<td>methionine</td>
<td>3</td>
<td>2+1</td>
</tr>
<tr>
<td>XIII</td>
<td>1.5</td>
<td>phenylalanine</td>
<td>1.5</td>
<td>1+1</td>
</tr>
<tr>
<td>XIV</td>
<td>1.5</td>
<td>tryptophan</td>
<td>1*</td>
<td>0.7+1</td>
</tr>
<tr>
<td>XV</td>
<td>1.5</td>
<td>tyrosine</td>
<td>0.04*</td>
<td>0.03+1</td>
</tr>
</tbody>
</table>
Chapter 4: Results and Discussion

Figure 4.2: Enzymatic activity of catalase after spray drying with amino acids (n=3, untreated =100%, error bars indicate sd)

Figure 4.3: Enzymatic activity of spray dried catalase with amino acids after storage for 1 month (n=3, untreated =100%, error bars indicate sd)
Spray drying involves atomising the feed solution into fine droplets followed by thermal dehydration (Lee, 2002). For labile biologicals the stress imposed during drying can result in destabilisation and degradation as shown for spray drying of alkaline phosphatase without any added excipient leading to a significant decrease in enzymatic activity (Li et al., 2010). Catalase appears to follow this trend upon spray drying. The main cause of a decrease in activity could be the destruction of hydrophobic interactions on dehydration and the breakdown of intermolecular hydrogen bonds by removal of the hydration shell which in turn causes conformational changes of catalase as visible in FTIR. The FTIR spectra of pure catalase (untreated) and catalase spray dried are shown in figure 4.4. A shift in the amide I spectra in the region between 1600 and 1700 cm\(^{-1}\) can be seen upon spray drying. FTIR spectra in this region are a good measure for changes in secondary structure, especially due to changes in backbone structure with a major contribution of the amide stretching vibration (Marco Van De Weert 2005). It has been shown for lysozyme and catalase that a similar shift occurs when the protein is dried without stabilising excipients (Jain, Roy 2009; Mönkedieck 2013). In contrast, when stabilising hydrogen bonds are present as it is the case in solution or in a stabilised version of the dried protein, this shift fails to appear (Jain, Roy 2009). The mechanism by which these amino acids can stabilise catalase on drying is by forming hydrogen bonds with catalase molecule. They substitute water molecules which are removed during drying and help catalase to maintain its tertiary structure (water replacement hypothesis). FTIR second derivative spectra of spray
dried catalase also showed an alteration in the amide I band especially at 1651 cm$^{-1}$ (Figure 4.8) indicating conformational changes in secondary structure of catalase. This band is influenced by hydrogen bonds and any variation corresponds to possible changes in secondary structure. Charged hydrophilic amino acids can form good hydrogen bonds with catalase due to presence of protonated nitrogen. This probably is a reason for arginine and histidine to be especially effective in stabilising catalase. Tian et al. reported that during freeze drying of antibodies with arginine and histidine the amino acids become partially amorphous which allows them to interact intimately with the protein via hydrogen bonds and with this they stabilise the antibody in the dried state (glassy state stabilisation) (Tian et al. 2006).

![Figure 4.4: FTIR spectra (amide I band) of Catalase pure and Catalase SD, mean of 128 scans](image)

Figure 4.4: FTIR spectra (amide I band) of Catalase pure and Catalase SD, mean of 128 scans
XRPD measurements revealed that catalase was crystalline when spray dried without additives, whereas the product with histidine was completely amorphous and the combination with arginine turned to be semi-amorphous (Figure 4.5). Combination with methionine resulted in a crystalline product, which has been shown to have no stabilising effect. In contrast, glycine, which has also shown to stabilise catalase, is crystalline upon drying. Nonetheless, as it is the smallest amino acid, it allows very close contact to the protein, similar to water molecules. Hence, it may replace water hydrogen bonds perfectly.

![Figure 4.5: XRPD spectra of Catalase pure, catalase spray dried, catalase spray dried with histidine, catalase spray dried with arginine, catalase spray dried with methionine (1+2 ratio of AA to protein)](image)

*Figure 4.5: XRPD spectra of Catalase pure, catalase spray dried, catalase spray dried with histidine, catalase spray dried with arginine, catalase spray dried with methionine (1+2 ratio of AA to protein)*
Chapter 4: Results and Discussion

4.1.2 Spray drying catalase with combination of amino acids

Arginine, glycine and histidine showed good potential of stabilising catalase during spray drying. These amino acids were then combined in a 1+1(+1) ratio and spray dried in 1+1 or 1+2 ratios in combination with catalase either as it has been described, that a mixture of amino acids may have synergistic stabilising effects (Shukla, Trout 2011; Markus HZ 1982). Formulation composition of spray dried batches of catalase with combination of amino acids is presented in table 3.

Table 3: Formulation composition of spray dried batches of catalase with combination of amino acids (1+1(+1) mass ratio)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Catalase (% w/v)</th>
<th>Amino acids</th>
<th>Total amino acid concentration (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>arginine + glycine</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>glycine + histidine</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>arginine + histidine</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>arginine + glycine + histidine</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>arginine + glycine</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>glycine + histidine</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>arginine + histidine</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
<td>arginine + glycine + histidine</td>
<td>3</td>
</tr>
</tbody>
</table>

The enzymatic activity of these batches after spray drying is shown in figure 4.6. Some formulations resulted in a surprising maintenance of activity almost up to 100%. Especially, the combination of arginine and glycine
showed an excellent stabilising effect with respect to enzymatic activity of catalase which still was almost 80% after storage for 1 month at 40°C/75% rH (Figure 4.7).

**Figure 4.6: Enzymatic activity of catalase after spray drying with amino acids (n=3, untreated =100%, error bars indicate sd)**

**Figure 4.7: Enzymatic activity of spray dried catalase with amino acids after spray drying and storage for 1 month at high temperature/humidity (n=3, untreated =100%, error bars indicate sd)**
In FTIR data, the aforementioned change in pattern of second derivative spectra was not observed, when catalase was spray dried together with Arg + Gly indicating their stabilising effect (figure 4.8). Shukla et al. showed in a previous study, that arginine interacts with oppositely charged residues on the protein surface and covers the adjacently exposed hydrophobic residues, thereby inhibiting the protein aggregation via reduced hydrophobic interactions (Shukla, Trout 2011). As discussed before, it also results in an amorphous matrix for the protein. In contrast to polymers, which may also be used to stabilise proteins during spray drying and often form a glassy matrix, the low MW amino acids do not influence solution viscosity of the spray drying solution significantly. Hence, shear stress of the protein during nozzle passage is not enhanced. In combination with the small molecule glycine, stabilisation seems to be superior. In contrast, a combination of both amorphous amino acids (Arg + His) as well as a triple combination with histidine does not show similar results, especially if storage stability at stress conditions is looked at. This may be due to the fact that arginine and glycine are regarded as chemically stable amino acids, whereas histidine has an increased chemical instability (S.Li et al. 1995). Furthermore, due to the surface activity of arginine and its smaller size compared to catalase, arginine has a higher molecular mobility in the spray droplet, which allows it to diffuse quickly to the droplet surface with this preventing the protein from concentration at the air-liquid interface and with this preventing interfacial stress. In the dried state the formation of hydrogen bonds between amino acids and catalase is mainly responsible for preservation of
native structure of catalase together with the formation of an amorphous matrix which also plays an important role in restricting the formation of aggregates on drying.

Figure 4.8: FTIR second derivative amide I spectra of catalase pure, catalase + (Arg + Gly) SD and catalase SD, mean of 128 scans
4.1.3 Stabilising influenza vaccine with amino acids

As the combination of arginine and glycine showed extraordinary capability for stabilising catalase activity during spray drying, it was examined whether this concept can be transferred to other protein structures. As a model for vaccine antigens, the antigenic component of the Pandemrix influenza split vaccine was chosen. The antigenic component of Pandemrix was spray dried in combination with Arg and Gly (1+1 in a AA:protein ratio of 2:1) along with trehalose (160 mg/mL) as a bulking excipient. Trehalose was selected, as protein content in the original liquid was too less (15 µg/mL) to obtain a product upon spray drying without the use of a bulking agent. Trehalose has been shown to have a certain stabilising effect itself in spray drying as it may incorporate the protein in a glassy matrix (Amorij et al. 2007). After spray drying with bulking agent only as well as with addition of the amino acid mixture, a one log step loss of haemagglutination titre was observed for the Pandemrix batch (Figure 4.9). These formulations were then further stored at 2-8°C, 25°C/60% rH and 40°C/75% rH and haemagglutination titre was determined after 1, 3 and 6 months. The liquid Pandemrix was stored at the same conditions for comparison. Pandemrix dried with trehalose and Arg + Gly showed a better HA titre when stored for 1 month at 25°C/60% rH than the original liquid vaccine (Figure 4.9). Liquid Pandemrix vaccine was completely inactivated and did not show any HA titre when stored at 40°C/75% rH, whereas loss of haemagglutination titre was much lower when stored in spray dried form (Figure 4.9). The mechanism of stabilisation is likely to be similar to that of
catalase i.e. by formation of hydrogen bonds with the protein and also immobilising of protein into glassy matrix which helps in protecting the protein during spray drying and storage.

![Figure 4.9](image.png)

*Figure 4.9: HA titre of original Pandemrix vaccine and spray dried vaccine after production and after storage for 1, 3 and 6 months (n=2)*

### 4.1.4 Stabilising lysozyme with amino acids

Since both proteins studied here are of similar high molecular weight (240 kDa) and may have similar ratios of surface area to volume (usually high molecular weight proteins have smaller ratio of surface area to volume) which mainly dominates the number of hydrogen bonding sites on the protein surface. It would hence be expected that even at lower ratio of stabiliser to protein, the hydrogen binding site for high molecular weight protein can easily be saturated compared to that for a low molecular weight protein (Chang et al. 2005). To exclude that the stabilisation effect of the
selected amino acid combination is due to coincidental similarities in MW and supramolecular structure of catalase and haemagglutinin, further studies with a low molecular weight protein (lysozyme) have been performed to evaluate, whether the stabilising effect of the combination of Arg and Gly is transferrable to a different type of protein. In contrast to catalase and haemagglutinin, lysozyme is a single chain molecule which does not require any supramolecular structure for functionality (Strynadka, James 1991; Nagendra et al. 1996).

An aqueous solution of lysozyme (1.5%, w/v) and also together with arginine and glycine (1+1) in mass ratio of 1:2 (protein to amino acid blend) was spray-dried using a Büchi B-290 laboratory spray dryer (Büchi, Flawil, Switzerland) using the same process parameters as used for catalase (chapter 4.1.1). The activity results after spray drying lysozyme are shown in figure 4.10. The activity loss of spray dried lysozyme in absence of any stabiliser was about 14%, while in the presence of the amino acid mixture only 6% loss was observed. These formulations were further stored at 40°C/75% rH for 1 month and it could be shown that with the presence of the stabilising amino acid mixture no further loss of activity was obtained for lysozyme indicating that the stabilising concept of Arg and Gly can also be transferred to a smaller protein.
Figure 4.10: Enzymatic activity of spray dried lysozyme with amino acids after spray drying and storage for 1 month at high temperature/humidity (n=3, untreated =100%, error bars indicate sd)

As spray drying is a process leading to a fine powder in a single step, which can be used without further processing such as milling, powder particle size is an interesting characteristic to look at. Particle size has implications for flowability and bulk powder handling as well as for later use (dissolution, powder inhalation etc.). The particle size of the catalase and lysozyme products after spray drying alone or in combination with arginine and glycine are shown in table 4.
**Table 4: Particle size of spray dried catalase and lysozyme formulations**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X10</td>
</tr>
<tr>
<td>Catalase spray dried</td>
<td>1.98</td>
</tr>
<tr>
<td>Catalase SD with Arg + Gly (1+2)</td>
<td>1.75</td>
</tr>
<tr>
<td>Lysozyme spray dried</td>
<td>1.37</td>
</tr>
<tr>
<td>Lysozyme SD with Arg + Gly (1+2)</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Mean particle size (x50) of all spray-dried particles was found to be between 3.3 µm and 4.8 µm. It can be seen that addition of amino acids does not alter mean particle size or size distribution. Such low micron particles will be capable for rapid dissolution as it would be needed for redispersion of therapeutic proteins prior to injection. They might also be capable for inhalation to the lung, although dry powder dispersion and aerodynamic behaviour was not the main scope of this study and hence was not evaluated.
4.1.5 Conclusion

The effects of amino acids on catalase stability as measured by activity assay showed that there are appreciable differences in the stabilising efficacies of these amino acids with respect to protection against the stress during spray drying and storage at elevated temperatures. From the results presented it can be concluded that arginine and glycine have a profound effect on the preservation of the enzyme during spray drying. Catalase can be effectively stabilised by a combination of arginine and glycine. This combination can also be used for stabilising an influenza antigen preparation (Pandemrix) in spray drying and subsequent storage as dry powder. Pandemrix in spray dried form was found to be more stable after storage for 3 months under harsh conditions (40°C/75% rH) compared to classical liquid vaccine which completely loses its activity under stress conditions. Finally, it could be shown that also smaller proteins such as lysozyme may also be stabilised by this combination. The result obtained thus demonstrates that amino acids can be effectively used for stabilising proteins during spray drying and SPS being a mixture of various amino acids might prove to be an effective spray drying excipient which will be further investigated.
4.2 Spray drying of a model protein (ovalbumin)

4.2.1 Development of a suitable spray drying process for ovalbumin

Ovalbumin is a well-studied protein and therefore was used as model antigen for spray drying experiments. The selection of suitable spray-drying conditions ('process variables') has a major impact on extent of protein aggregation and/or inactivation (Carpenter, Manning 2002). Hence, a number of spray drying runs of an aqueous solution of 3% (w/v) ovalbumin were conducted to optimise the spray drying process parameters (mainly inlet air temperature and feed flow rate). Outlet air temperature is the crucial variable in spray drying, especially in the case of temperature sensitive proteins. Therefore process parameters were optimised with the aim of not harming the protein and to minimise process time. Inlet air temperature was selected to have a quick evaporation of solvent and feed rate was adjusted to get an outlet air temperature of 50-55°C. High aspirator rate of 100% was used to get a higher separation in the cyclone and nozzle diameter of 1.5 mm was selected to facilitate smooth spraying of protein solution. The optimised process parameters used for spray drying are summarised in table 5.
Table 5: Optimised parameters used for spray drying of Ovalbumin

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet air temperature</td>
<td>120°C</td>
</tr>
<tr>
<td>Outlet air temperature</td>
<td>50-55°C</td>
</tr>
<tr>
<td>Spray flow rate</td>
<td>470 L/h</td>
</tr>
<tr>
<td>Aspirator air flow</td>
<td>35 (m³/h) = 100%</td>
</tr>
<tr>
<td>Flow rate</td>
<td>5-7.5 mL/min</td>
</tr>
</tbody>
</table>

4.2.2 Stabilising and Protecting Solution (SPS) as a spray drying stabilising excipient

Stabilising and Protecting Solution (SPS) was evaluated as a potential stabilising excipient. In DVS measurements (chapter 4.1.1), L-Arginine and L-Lysine showed strongest water vapour interaction and hence are hygroscopic at rH of 40% and above. These hygroscopic components could increase water ingestion in the dried powder and limit stability. Therefore, arginine and lysine were deleted from the SPS and three different compositions of SPS were evaluated as summarised below:

- SPS (original): Patented composition (Margraf et al. 2010)
- SPS 3.0.3: SPS without Arginine and Lysine
- SPS 3.0.3.1: SPS 3.0.3 + Phenylalanine + Leucine
3% (w/v) of ovalbumin along with 6% (w/v) SPS were spray dried using a Büchi B-290 laboratory spray dryer. Table 6 summarises the formulation composition of spray dried batches. The parameters used for the spray drying were the same that were previously optimised and are shown in table 5. 25 mL of solution was spray dried and final product was collected and stored in dry and refrigerated conditions until analysed.

Table 6: Formulation composition of spray dried batches of ovalbumin

<table>
<thead>
<tr>
<th>Batch</th>
<th>Ovalbumin (mg/mL)</th>
<th>SPS (mg/mL)</th>
<th>SPS 3.0.3 (mg/mL)</th>
<th>SPS 3.0.3.1 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA SD</td>
<td>30</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>OVA + SPS SD</td>
<td>30</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA + SPS 3.0.3 SD</td>
<td>30</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA + SPS 3.0.3.1 SD</td>
<td>30</td>
<td></td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

After spray drying SDS-PAGE was performed to check the molecular weight integrity of ovalbumin. Stress during the spray drying process can degrade the protein into smaller fragments and this can be observed by presence of lower molecular bands on the polyacrylamide gel. SDS-PAGE of OVA control, spray dried OVA without and with SPS is shown in figure 4.11. All the samples present the same gel pattern and no lower molecular bands are visible on the gel, hence no relevant degradation of the OVA molecule has taken place upon spray drying.
Since no significant change in primary structure of ovalbumin was observed, circular dichroism (CD) was conducted to determine whether the secondary structure of ovalbumin was altered on spray drying. It has been shown that CD spectroscopy in far UV region is a fairly reliable technique to monitor the secondary structure of proteins (Kelly et al. 2005). All versions of SPS being composed of amino acids showed strong signal in CD (Figure 4.12) and therefore had to be removed to analyse the structure of ovalbumin alone. The separation of SPS from the ovalbumin was performed using an ultrafiltration device Vivaspin 6 (Sartorius Stedim biotech, Göttingen, Germany). Vivaspin 6 consists of twin vertical membranes of polyethersulfone with a MWCO of 5000 Daltons and has a capacity of 6 ml. Spray dried powder was dissolved in water and 5 ml was filled on the top of Vivaspin 6.
Centrifugation was performed at 4000 rcf for 30 min to obtain a purified ovalbumin solution and was then measured directly in the CD.

![Graph showing CD spectra of SPS, SPS 3.0.3 and SPS 3.0.3.1](image)

*Figure 4.12: CD spectra of SPS, SPS 3.0.3 and SPS 3.0.3.1*

When analysed for CD, the far UV CD spectra of OVA SD looks similar to that of the pure OVA signifying that there is no significant change in secondary structure (Figure 4.13). Nonetheless, the signal becomes fluctuating indicating some change in secondary structure. Ovalbumin is susceptible to surface denaturation (Galyean, Cotterill 1979) and therefore gets partially denatured by exposure to new surface areas on atomisation during spray drying which resulted in obtained changes in secondary structure. On addition of SPS to ovalbumin sample prior to spray drying, these fluctuations can be diminished signifying its stabilising effect (Figure 4.13). SPS is believed to work by replacing stabilising interactions between protein and water with the similar interactions of less reactive small molecules (Scholz, Lücking 2012). Upon removal of water, SPS thus
substitutes water molecules and forms a protecting shell by forming H-bonds at various sites within the ovalbumin during drying and thus protects ovalbumin’s native structure. The other tested variants of SPS did not show the same stabilising effect indicating that SPS (original) is superior in stabilising the ovalbumin secondary structure (Figure 4.14) and therefore was selected for further formulation development. Arginine was found to be very effective in stabilising protein during spray drying as studied in chapter 4.1; as other SPS variants (SPS 3.0.3, SPS 3.0.3.1) are deficient of arginine and hence may have less stabilising effect compared to SPS (original).

![Figure 4.13: CD spectra of OVA pure, OVA SD and (OVA+ SPS) SD](image)
Figure 4.14: CD spectra of OVA pure, (OVA+ SPS) SD; (OVA+ SPS 3.0.3) SD and (OVA+ SPS 3.0.3.1) SD

Fluorescence spectroscopy was further conducted to monitor conformational change and to analyse the integrity of ovalbumin after spray drying. An extrinsic fluorescent dye 1-(anilino)-naphthalene-8-sulfonate (ANS) dye was used to examine the conformational changes of ovalbumin. ANS is practically non-fluorescent in water, but shows fluorescence upon binding to hydrophobic sites that exists on proteins and which is greatly increased when the protein is denatured (Figure 4.15). This phenomenon was used to characterise the integrity of OVA after spray drying. As seen in Figure 4.16 the control and spray dried OVA with SPS possess the same fluorescence intensity indicating that no relevant denaturation event had taken place during spray drying.
Figure 4.15: Enhancement of fluorescence intensity of ANS on binding to pure OVA and denatured OVA, respectively.

Figure 4.16: Fluorescence intensity of OVA control, (OVA+SPS) SD (spectra of protein only) after binding with ANS.
4.2.3 Storage stability

To get more insight on the integrity of ovalbumin over time after spray drying, spray dried formulations (OVA SD, OVA+SPS SD) were placed for stability studies at 40°C/75% rH for 3 months. Samples were analysed using CD spectroscopy and were compared with the reference sample (pure OVA). CD spectra of the formulation stored for stability can be seen in figure 4.17. The OVA SD formulation stored at 40°C/75% rH for 3 months shows some fluctuations in CD spectra (Figure 4.17) indicating further changes in secondary structure and this fluctuation were fewer with formulation containing SPS (figure 4.18) signifying its stabilising effect. An important assessment during stability study is analysis by an activity assay. As ovalbumin does not have any functionality, evaluation using an activity assay is certainly deficient to further evaluate changes in its integrity.

![CD Spectra of OVA pure, OVA SD (t=0) and after storage at 40°C/75% rH for 3 months](image)

*Figure 4.17: CD Spectra of OVA pure, OVA SD (t=0) and after storage at 40°C/75% rH for 3 months*
Figure 4.18: CD Spectra of OVA pure, (OVA+SPS) SD and after storage at 40°C/75 % rH for 3 months

4.2.4 Conclusion

Ovalbumin was successfully spray dried and process parameters were optimised to obtain a dried product. The results obtained suggest that only slight alteration was obtained after spray drying and no large scale degradation of ovalbumin can be seen as measured by SDS-PAGE. Secondary structure of ovalbumin after spray drying as analysed by circular dichorism showed some fluctuations when compared to the pure OVA and this fluctuations were diminished by addition of SPS. The examination of the storage stability of the spray dried ovalbumin revealed that storage at elevated temperature and relative humidity (40°C/75% rH) leads to a further changes in secondary structure and addition of SPS before spray drying to ovalbumin can decrease such secondary structure changes. SPS is therefore
helpful in preserving the native structure of ovalbumin during spray drying and subsequent storage.
4.3 Development of Influenza A (H1N1) powder vaccine formulation

Influenza vaccines have been available since long time but still there high protection levels and widespread patient coverage is limited due to instability of liquid formulation and dependency on cold chain (Kim et al. 2010; Amorij et al. 2008). It has been shown that thermal stability of vaccines can be greatly increased when the antigen is in dried state (Ohtake et al. 2010). Therefore, the dry influenza powder formulations offer the potential of a more stable product making vaccine delivery easier and safer by decreasing dependency on the cold chain and facilitates stockpiling and vaccine targeting strategies (Amorij et al. 2007; Friede, Aguado 2005). As evaluated previously in chapter 4.2, the model protein ovalbumin could be effectively stabilised during spray drying by addition of SPS. This stabilising concept is further evaluated for an influenza antigen (H1N1) for development of a stable dry influenza vaccine formulation.

4.3.1 Formulation development of spray dried Influenza A (H1N1) vaccine formulation

Spray drying of influenza A (H1N1) vaccine (Pandemrix) was performed using previously evaluated conditions for ovalbumin (Table 5) and were set to mild conditions in order not to harm the antigen excessively. Spray drying was performed using Büchi mini spray dryer B-290 and the process parameters used are summarised in table 7. Pandemrix vaccine on spray drying did not result in any product yield; therefore spray drying was performed together with added bulking excipients mannitol/trehalose. The formulation variants

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81
of spray dried batches of Pandemrix is summarised in table 8. The goal was to determine the best formulation composition and bulking excipient for the formulation. Original Pandemrix was either used as supplied and mixed with the bulking component in the respective amount or in case of the SPS containing formulations it was dialysed overnight against SPS in the respective concentrations at pH 7, using Slide-A-Lyzer® dialysis cassettes (MWCO 3.5 kDa; volume 3–12 mL) (Thermo Scientific, Schwerte-Geisecke, Germany). Approximately 11.5 mL of solution was spray dried and final product was collected and stored in dry and refrigerated conditions until analysed.

Table 7: Parameters used for spray drying of Pandemrix

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet air temperature</td>
<td>120°C</td>
</tr>
<tr>
<td>Outlet air temperature</td>
<td>50-55°C</td>
</tr>
<tr>
<td>Spray flow rate</td>
<td>470 L/h</td>
</tr>
<tr>
<td>Aspirator air flow</td>
<td>35 (m³/h) = 100%</td>
</tr>
<tr>
<td>Flow rate</td>
<td>5-6 mL/min</td>
</tr>
</tbody>
</table>
### Table 8: Overview of spray dried formulations of Pandemrix

<table>
<thead>
<tr>
<th>Formulation</th>
<th>HA (µg/mL)</th>
<th>SPS (mg/mL)</th>
<th>Trehalose (mg/mL)</th>
<th>Mannitol (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD T80</td>
<td>15</td>
<td>--</td>
<td>80</td>
<td>--</td>
</tr>
<tr>
<td>SD M80</td>
<td>15</td>
<td>--</td>
<td>--</td>
<td>80</td>
</tr>
<tr>
<td>SD T</td>
<td>15</td>
<td>--</td>
<td>160</td>
<td>--</td>
</tr>
<tr>
<td>SD M</td>
<td>15</td>
<td>--</td>
<td>--</td>
<td>160</td>
</tr>
<tr>
<td>SD SPS40</td>
<td>15</td>
<td>40</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SD SPS80</td>
<td>15</td>
<td>80</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SD SPS40 T80</td>
<td>15</td>
<td>40</td>
<td>80</td>
<td>--</td>
</tr>
<tr>
<td>SD SPS40 M80</td>
<td>15</td>
<td>40</td>
<td>--</td>
<td>80</td>
</tr>
<tr>
<td>SD SPS80 T80</td>
<td>15</td>
<td>80</td>
<td>80</td>
<td>--</td>
</tr>
<tr>
<td>SD SPS80 M80</td>
<td>15</td>
<td>80</td>
<td>--</td>
<td>80</td>
</tr>
<tr>
<td>SD SPS80 T</td>
<td>15</td>
<td>80</td>
<td>160</td>
<td>--</td>
</tr>
<tr>
<td>SD SPS80 M</td>
<td>15</td>
<td>80</td>
<td>--</td>
<td>160</td>
</tr>
</tbody>
</table>

The functional activity of spray dried formulation of Pandemrix was determined using Haemagglutination (HA) assay. The HA-titres for the batches of Pandemrix spray dried with added bulking excipients mannitol/trehalose +/- SPS is presented in figure 4.19.
Figure 4.19: HA titre of original Pandemrix vaccine and spray dried vaccine after production

Trehalose at a concentration of 160 mg/mL when spray dried with Pandemrix (SD T) resulted in an HA-titre comparable to the corresponding HA-titre of the original Pandemrix vaccine. On the other hand, lowest HA titres were obtained for spray dried batches of Pandemrix containing mannitol as a bulking excipient (SD M). It is known for mannitol that it forms a polymeric crystalline phase on drying and this can have a profound negative influence on the stability of dried proteins (Costantino et al. 1998). In contrast, trehalose forms an amorphous phase and thereby stabilises the protein. The obtained result supports this theory. On spray drying Pandemrix with SPS (SD SPS40, SD SPS80) without any added bulking excipient no product was obtained. The SPS/trehalose combination batch SD SPS80 T showed no loss of activity in the HA assay. Further dilution of this formulation (SD SPS40 T80 and SD SPS80 T80) resulted in a sticky product with poor quality but still showed retention of activity. The
SPS/mannitol combination batch SD SPS80 M also resulted in a sticky poor quality product with only a small product yield. Nonetheless, the presence of SPS led to an increase of the HA-activity of the spray dried product to a HA-title of 128, compared to mannitol formulation without SPS (HA titre 32), suggesting a stabilising activity of SPS upon spray drying. In solution state, the stabilising and protecting effects of SPS assumed to be in accordance with the concepts of preferential exclusion and preferential binding (Tscheliessnig et al. 2012). On drying SPS is believed to form an amorphous matrix (Scholz, Lüking 2012) and this amorphous character in combination with trehalose but not with mannitol resulted in superior stabilisation during spray drying.

Spray dried batches of Pandemrix were also analysed for residual moisture content and obtained water content is shown in figure 4.20. The spray dried batches of Pandemrix with mannitol (SD M80, SD M) showed the lowest water content 0.7-1%, as determined by Karl Fischer titration. For all the other batches the water content was in the range of 7-9%. Generally chemical stability of protein is decreased with high moisture content in a solid formulation as water may act as a medium for mobilisation of reactants and increases the risk of physical or chemical instability (Maa et al. 1998a). A modified version of SPS without hygroscopic arginine and lysine (as seen in DVS, chapter 4.1.1) was also tested as these components being hygroscopic could increase water ingestion and limit stability. The effect of water content in spray dried batch (SD SPSv1 T) was found to be negligible but HA activity was further decreased (HA titre: 64). From all the
formulation variants tested combination of SPS at concentration of 80 mg/mL with 160 mg/mL trehalose showed the best retention of activity.

![Figure 4.20: Water content of the spray dried batches of Pandemrix](image)

**4.3.2 Effect of sterilisation**

The effect of irradiation sterilisation on the spray dried formulation of Pandemrix was investigated. Native proteins on sterilisation can undergo physicochemical modifications which might lead to complete loss of its biological function (Garrison et al. 1962; Zbikowska et al. 2006). Incorporation of vaccines in amorphous glassy sugar matrices to stabilise the antigen is well studied, however no study evaluating the effect of terminal sterilisation on dried vaccine formulation has been reported in the past. Sterilisation of the spray dried Pandemrix samples was performed in
sealed glass vials by Beta-Gamma Service (Bruchsal, Germany) using β-irradiation at 25 or 40 kGy. The results of HA-titre before and after sterilisation are shown in figure 4.21. Pandemrix spray dried with mannitol (SD M80, SD M) did not show any protection during sterilisation and the sample had lost all its activity after sterilisation. Trehalose when used at a concentration of 160 mg/mL (SD T) showed some protection against sterilisation especially at a dose of 25 kGy but with some loss of activity and at a 40 kGy dose all the activity was lost. Pandemrix when spray dried with combination of 80 mg/mL SPS and 160 mg/mL along with trehalose (SD SPS80 T) showed the best retention of activity after sterilisation (figure 4.21). On drying SPS is believed to form an amorphous layer and stabilise the three dimensional structure of haemagglutinin in de-hydrated conditions and increase its stress resistance and also protect against damages during sterilisation.
Figure 4.21: HA titre of formulation variants of spray dried Pandemrix before and after sterilisation

4.3.3 Incorporation of adjuvants into the spray dried formulation

It was investigated whether it is possible to incorporate an adjuvant directly during the drying process in the formulation without affecting the product properties and the activity of the vaccine adversely. Spray dried formulation of Pandemrix containing SPS at 80 mg/mL together with 160 mg/mL trehalose showed the best HA titre and also showed a good protection against sterilisation and therefore it was used for further formulation development. Various adjuvants (Glycyrrhizic acid (GA), Chitosan-HCl, Alum and Monophosphoryl Lipid A (MPL)) were added to this formulation before spray drying as summarised in table 9.
Table 9: Formulation variants of Pandemrix with different added adjuvants (each formulation contains 15 µg/mL HA together with 80 mg/mL SPS and 160 mg/mL trehalose)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>GA (mg/mL)</th>
<th>Chitosan-HCl (mg/mL)</th>
<th>Alum (µg/mL)</th>
<th>MPL (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPS80 T GA</td>
<td>4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SPS80 T GA+C</td>
<td>2</td>
<td>2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SPS80 T C</td>
<td>--</td>
<td>4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SPS80 T Alum</td>
<td>--</td>
<td>--</td>
<td>375</td>
<td>--</td>
</tr>
<tr>
<td>SPS80 T MPL</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>15</td>
</tr>
</tbody>
</table>

The result of HA-titre for the spray dried batches of Pandemrix containing adjuvant is presented in figure 4.22. Surprisingly, the addition of glycyrrhizic acid and chitosan-HCl to the SPS formulation (SPS80 T GA+C) and chitosan-HCl (SPS80 T C) alone led to a pronounced shift to higher HA titres. This finding was further investigated and was found that data was biased due to strong haemagglutination activity of chitosan-HCl which interferes with the functional readout system for antigenic activity of haemagglutinin in the analysed vaccines. Chitosan has been shown earlier to improve vaccination efficacy due to improved bioadhesion of the vaccine on the mucosa, to increase bioavailability and to boost mucosal immune
response (Illum et al. 1994; Illum et al. 2001). Chitosan was found to have a stabilising effect on proteins especially in combination with SPS as shown by a Dynamic Spectral Fluorometry (DSF) assay (personal communication with Dr. Kemter, Leukocare, Germany). In combination with amino acids, glycyrrhizic acid (GA) also has been shown earlier to stabilise biomolecules under stress conditions (Tscheliessnig et al. 2012). Therefore, the utilisation of glycyrrhizic acid and chitosan is of special interest for the production of vaccines since these excipients stabilise the antigen and elicit adjuvant activity in vivo (Scherliess et al. 2013; Sun et al. 2009) and were added to SPS formulation for animal study.

Figure 4.22: HA titre of formulation variants of spray dried Pandemrix containing adjuvants
4.3.4 In vivo study

To determine the efficacy and safety of spray dried formulation of Pandemrix an in vivo study was conducted in collaboration with Leukocare biotechnology (Munich, Germany) and Public Health England (Porton Down, UK). All experimental protocols were approved by the Ethical Review Process of Public Health England, Porton, Salisbury, UK and the Home Office via Project License PPL30/2993.

4.3.4.1 Study design

In-vivo potency of the dried powder formulation of Pandemrix was assessed in Macaca fascicularis. Twenty six animals of either sex were selected from a cohort of animals and were screened for absence of any influenza antibodies. Formulation variants that were studied in vivo are summarised in table 10. Spray drying of all the batches used for in vivo study was performed using an additional inlet air filter to avoid any contamination from the surrounding and all the components of spray dryer were disinfected with 70% (v/v) isopropanol. Spray dried product were filed in individual single doses in pre-sterilised vials (Type I, 2 R, Pro-Pac e.k. Eschweiler, Germany) as shown in figure 4.23. Sterilisation by β-irradiation of the spray dried batches was performed at 25 kGy which is recommended by the European Pharmacopoeia as the standard dose for terminal sterilisation (European Pharmacopoeia 5.0, chapter 5.1). All spray dried samples were reconstituted with sterile water and adjuvant immediately prior to vaccination. Original Pandemrix was injected after mixing with the
AS03 adjuvant provided in the separate flask. All vaccinations were given by intramuscular injection. Each animal was given 0.5 mL vaccine preparation containing 3.75 µg HA antigen (human adult dose). Table 11 summarises the in vivo study design and vaccination schedule. Control sera were taken eight days prior to vaccination. All five groups of animals were given prime vaccination on day 0 and booster vaccination was given on day 28 with an equal dose of the appropriately-treated vaccine. Sera samples were taken at day 21 after post immunisation and after day 34 and 48 following boost immunisation.

Table 10: Animal group and formulations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Formulations</th>
<th>Description</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Phosphate Buffer Saline (PBS)</td>
<td>Negative control</td>
<td>2</td>
</tr>
<tr>
<td>Group 2</td>
<td>Original Pandemrix</td>
<td>Positive control</td>
<td>6</td>
</tr>
<tr>
<td>Group 3</td>
<td>SD M 25 kGy</td>
<td>Pandemrix spray dried with Mannitol (160 mg/mL) and irradiated</td>
<td>6</td>
</tr>
<tr>
<td>Group 4</td>
<td>SD SPS T final</td>
<td>Pandemrix spray dried with SPS (160 mg/mL) + Trehalose 160 mg/mL + GA (2 mg/mL) + Chitosan-HCl (2 mg/mL)</td>
<td>6</td>
</tr>
<tr>
<td>Group 5</td>
<td>SD SPS T final 25 kGy</td>
<td>Pandemrix spray dried with SPS (160 mg/mL) + Trehalose 160 mg/mL + GA (2 mg/mL) + Chitosan-HCl (2 mg/mL) and irradiated</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 4.23: Formulations for in-vivo study (Right vial: Group 3 (Pandemrix SD M 25 kGy), Centre vial: Group 4 (Pandemrix SD SPS T final), Left vial: Group 5 (Pandemrix SD SPS T final 25 kGy)

Table 11: In-vivo study design

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Timeline (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-8</td>
</tr>
<tr>
<td>Prime immunisation</td>
<td>0</td>
</tr>
<tr>
<td>Sampling</td>
<td>21</td>
</tr>
<tr>
<td>Boost immunisation</td>
<td>28</td>
</tr>
<tr>
<td>Sampling</td>
<td>34, 48</td>
</tr>
</tbody>
</table>
### 4.3.4.2 Immune response to the vaccine

Haemagglutination inhibition (HAI) assay was used to analyse the systemic immune response after vaccination. The HAI assay detects serum antibodies to the viral haemagglutinin by measuring the inhibition of virus-mediated agglutination of erythrocytes (Noah et al. 2009). HAI assay is based on the fact that antibodies to influenza virus will prevent attachment of the virus to red blood cells and therefore haemagglutination is inhibited when antibodies are present. After vaccination the sera obtained from animals was treated with receptor-destroying enzyme (RDE, Denka Seiken Co., Japan), followed by heat-inactivation. Treated sera were then subjected to 2-fold serial dilutions in 96-well U-bottom plates, followed by the addition of 4 HA units of virus (influenza A (H1N1)/California/ 07/09). After incubating at room temperature, a 0.5% w/v suspension of chicken red blood cells was added in a U-bottom 96 well plate. After 45 minutes incubation at room temperature, the plates were scored for agglutination.

The results of HAI titres after serum sample analysis is presented in figure 4.24. The immune response obtained correlated well with the in-vitro HA assay. After vaccination negative control group (group 1) did not show any seroconversion. Group 2, 4 and 5 shows similar haemagglutination inhibition (HAI) titres. All animals in group 4 & 5 (SD SPS T final & SD SPS T final 25 kGy) were seroconverted (HAI titre ≥ 40) and 5 out of 6 animals in group 2 (original Pandemrix) were seroconverted after vaccination. All 6 animals in group 3 (SD M 25 kGy) remained sero-negative (HAI titres ≤ 20). After giving the booster dose the animals in group 3 also showed
seroconversion. However, the mean titre of group 3 remained significantly lower than the mean titres of groups 2, 4 and 5.

![Graph](image)

**Figure 4.24: Immune responses in macaques to Pandemrix vaccine (HAI titre)**

- **Group 1:** Negative control group (PBS); **Group 2:** Original Pandemrix; **Group 3:** SD M 25 kGy; **Group 4:** SD SPS T final; **Group 5:** SD SPS T final 25 kGy.

*Significant difference from group 2, 4 and 5 means, p < 0.01. Means for groups 2, 4 and 5 are not significantly different from one another at any time-point. (Bars represent the geometric mean titre for each group)*

Thus, the immunisation of Macaca fascicularis with all SPS-protected Pandemrix formulation induced strong systemic humoral responses and serum HAI titres obtained were similar to that of original liquid vaccine and in absence of such stabilisation it led to >10-fold reduction in mean HAI titre.
4.3.5 Long-term storage stability

To facilitate vaccine distribution, vaccines formulation must have sufficient thermal stability. To evaluate the storage stability Pandemrix spray dried formulation variants (SPS SD M 25 kGy, SPS SD T final, SPS SD T final 25 kGy) were stored at 2-8°C and 25°C/60% rH in sealed vials and were analysed using gel electrophoresis, dynamic light scattering and size exclusion chromatography after 1, 3 and 6 months.

4.3.5.1 Gel electrophoresis

Molecular stability of spray dried influenza vaccine formulations was determined by non-reducing and reducing SDS-PAGE. In non-reducing SDS-PAGE original Pandemrix showed multiple band patterns of about six separated bands (Figure 4.25A) which is typical for a split vaccine (Feshchenko et al. 2012). Original Pandemrix typically migrate as monomers and disulfide-linked oligomers under non-reducing conditions. It was found that Haemagglutinin monomer (HA0) band migrates at approximately 65 kDa, and dimer and trimer bands approximately at 120 and 200 kDa, respectively (Figure 4.25A). However, lack of protein band with molecular weight higher than that of HA0 under reducing conditions suggest that the oligomeric forms observed under non-reducing conditions were disulfide linked oligomers of HA0 particularly dimers and trimmers (Figure 4.25B). The band at approximately 55.4 kDa could correspond to smaller amounts of HA1 in complex with the nucleoprotein. A small band between 31 and
21.5 kDa may correspond to the protein complex of HA2 and the matrix protein (M) (Figure 4.25B).

Pandemrix with added mannitol (Liquid SD M) before spray drying shows the same migration pattern compared to the original pandemrix in the non-reducing as well as in the reducing SDS-PAGE (Figure 4.25A and B, lane 6). However, on spray drying and irradiation (SD M 25 kGy) this batch showed almost complete loss of the migration pattern of the split vaccine, suggesting substantial fragmentation of the protein constituents (Figure 4.25A and B, lane 7). SDS-PAGE of SPS formulated vaccines (SD SPS T final & SD SPS T final 25 kGy) are shown in Figure 4.25A and B. It is well known from literature that chitosan can bind to lipids (Wydro et al. 2007). Due to possible binding of chitosan to the residual fragments of the lipid membrane of the split vaccine, the chitosan containing vaccine preparation liquid SD SPS T final, SD SPS T final, SD SPS T final 25 kGy (Figure 4.25A and B, lanes 2-5) and also original Pandemrix in combination with chitosan-HCl resulted in a long smear overlaying individual bands (Figure 4.26; lane 8). There was no loss of any protein band particularly of haemagglutinin SPS protected vaccines after spray drying and irradiation.
Figure 4.25 A) Non-reducing SDS-PAGE and B) Reducing SDS-PAGE of the different formulations of the influenza vaccine before and after spray-drying and subsequent irradiation at 25 kGy at the time point \( t = 0 \). The samples are loaded for each treatment condition in the following order: Mark12 (lane 1); liquid SD SPS T final (lane 2); SD SPS T final (lane 3); SD SPS T final 25 kGy (lane 4); original Pandemrix (lane 5); liquid SD M (lane 6); SD M 25 kGy (lane 7); Mark12 (lane 8).

SD M 25 kGy batch after storage for 1 and 3 months at 25°C/60% rH resulted in further loss of the residual protein components of the split vaccine (Figure 4.26A, B and C, D; lane 7) compared to storage at 2-8°C (Figure 4.26 A, B and C, D; lane 6) and time point \( t = 0 \) (Figure 4.25A and B; lane 7). However after storage for 1 and 3 months at 2-8°C and 25°C/60% rH SPS containing Pandemrix batches (SD SPS T final and SD SPS T final 25 kGy) showed no changes in the migration pattern (Figure 4.26A, B and C, D; lanes 2-5) when compared with the corresponding migration pattern at the starting time point \( t = 0 \) (Figure 4.25A & B, lanes 3-4). This shows that the SPS-stabilised spray dried and particularly the
subsequent irradiated formulation remains stable not only upon refrigerated storage, but also at 25 °C/60% rH demonstrating increased thermo stability.

Figure 4.26 Non-reducing SDS-PAGE (A & C) and Reducing SDS-PAGE (B & D) of the different formulations of the influenza vaccine before and after spray-drying and subsequent irradiation at 25 kGy at the time point t = 1 (A & B) and t = 3 months (C & D). The samples are loaded for each treatment condition in the following order: Mark12 (lane 1); SD SPS T final 2-8°C (lane 2); SD SPS T final 25°C/60% rH (lane 3); SD SPS T final 25 kGy 2-8°C (lane 4); SD SPS T final 25 kGy 25°C/60% rH (lane 5); SD M 25 kGy 2-8°C (lane 6); SD M 25 kGy 25°C/60% rH (lane 7); original Pandemrix + chitosan-HCl (lane 8); original Pandemrix (lane 9); Mark12 (lane 10).
4.3.5.2 Dynamic light scattering

Dynamic Light Scattering (DLS) data showed that the original Pandemrix consist of colloidal components of a certain size distribution without presence of any large aggregates (Figure 4.27a). On spray drying Pandemrix with mannitol (SD M), aggregates were visible as seen by the second peak at around 5000 nm (Figure 4.27a). On irradiation this formulation (SD M 25 kGy) shows a further increase in intensity of aggregation and intensity was further increased after storage especially at 25°C/60% rH for 6 months (Figure 4.27c). In contrast the SPS T final formulation (Figure 4.27a) did not show any aggregation and after irradiation only a slight increase in aggregation can be seen for the SD SPS T final 25 kGy formulation (Figure 4.27a). On storage this SPS stabilised spray dried formulation did not show significant increase in aggregation irrespective of the storage conditions (Figure 4.27d, e, f and g) indicating no destabilising processes take place over storage time which would increase aggregation upon redispersion.
Chapter 4: Results and Discussion

**Figure b**
Size Distribution by Intensity

**Figure c**
Size Distribution by Intensity

**Figure d**
Size Distribution by Intensity

**Figure e**
Size Distribution by Intensity
Figure 4.27: Particle size distribution by dynamic light scattering: a) Original Pandemrix; SD M, SD M 25 kGy; SD SPS T final; SD SPS T final 25 kGy; b) SD M 25 kGy, prepared freshly and after 1, 3 and 6 month storage at 2-8°C; c) SD M 25 kGy, prepared freshly and after 1, 3 and 6 month storage at 25°C/60% rH d) SD SPS T final, prepared freshly and after 1, 3 and 6 month storage at 2-8°C; e) SD SPS T final, prepared freshly and after 1, 3 and 6 month storage at 25°C/60% rH f) SD SPS T final 25 kGy, prepared freshly and after 1, 3 and 6 month storage at 2-8°C; g) SPS T final 25 kGy, prepared freshly and after 1, 3 and 6 month storage at 25°C/60% rH

4.3.5.3 Size exclusion chromatography (SEC)

Pandemrix spray dried formulations stored for stability testing were analysed using size exclusion chromatography. SEC chromatograms are shown in figure 4.28. In SEC the highly hydrophobic nature of the protein led to elution as protein complexes. The first peak at about 11 mL in the
original Pandemrix could be assigned to a high molecular weight complex of the protein constituents >500 kDa, second peak at approx. 16 mL may represent the HA trimer of 255 kDa and the third peak could represent the HA monomer (Figure 4.28a). It can be seen that when Pandemrix was spray dried with mannitol (SD M), the HA trimer peak became larger (Figure 4.28a). On irradiation this formulation (SD M 25 kGy) resulted in further increase of HA trimer peak and the HA monomer peak was completely lost (Figure 4.28a). As a result of the changes in the protein constituents of the original formulated split vaccine with mannitol upon stress exposure, such as spray drying and irradiation, the composition of the complexes formed in this formulation may have changed. The SD SPS T final and SD SPS T final 25 kGy exhibits the same peaks of the trimer and the monomer as original Pandemrix, but lost the high molecular weight complex (Figure 4.28a and c) indicating a stable formulation. On storage at 25°C/60% rH after 1, 3 and 6 months the SD M 25 kGy formulation showed changes in the trimer peak area (Figure 4.28b) indicating that the antigen was not stabilised properly leading to increased instability and aggregation upon redispersion which was also seen in the SDS-PAGE results. In contrast the SD SPS T final or the SD SPS T final 25 kGy formulation (Figure 4.28c and d) showed no change in the peak area over storage for 1 and 3 months at either storage condition compared to the freshly prepared formulation. Thus, SEC analysis showed that irradiation induces severe aggregation and degradation in spray dried Pandemrix formulation without containing SPS and the formulations
stabilised with SPS can withstand a long period of exposure without significant loss of potency, confirming the findings by SDS-PAGE.

Figure 4.28: SEC chromatograms of a) original Pandemrix; SD M 25 kGy; SD SPS T final; SD SPS T final 25 kGy  b) SD M 25 kGy, prepared freshly and after 1 and 3 month storage at 2-8°C and 25°C/60% rH  c) SD SPS T final; prepared freshly and after 1 and 3 month storage at 2-8°C and 25°C/60% rH  d) SD SPS T final 25 kGy, prepared freshly and after 1, 3 and 6 month storage at 2-8°C and 25°C/60% rH
4.3.6 Conclusion

Pandemrix vaccine could be effectively spray dried together with SPS and added bulking excipients (mannitol/trehalose). Several formulation compositions of bulking excipient +/- SPS were spray dried and evaluated using in vitro Haemagglutination assay. Pandemrix when spray dried with mannitol did not show any protection of activity after irradiation sterilisation and was hence used as a negative control in the in vivo study. SPS/trehalose combination resulted in superior stabilisation characteristics during spray drying. The combination of SPS at concentration of 80 mg/mL with 160 mg/mL trehalose showed the best protection of activity. The stabilising effect of SPS was even more pronounced after β-irradiation of the spray dried products at 25 or 40 kGy. Formation of an amorphous coat by SPS upon drying is believed to protect haemagglutinin against physical destabilisation and destructive impact of sterilisation. Glycyrrhizic acid and chitosan were further added to this formulation as they resulted in highly stable compositions and both substances might enhance the immune response in vivo due to their known adjuvanting effect. The in vivo study conducted in non-human primates (Macaca fascicularis) revealed that SPS protected Pandemrix formulation elicit a strong systemic humoral response comparable to original liquid vaccine, whereas in the absence of SPS, there was significant reduction in HAI titre. The storage stability determined at 1, 3 and 6 months using SEC analysis, DLS and SDS-PAGE revealed that potency of the SPS stabilised dried Pandemrix vaccine was fully maintained even after storage under stress conditions (25°C/60% rH).
Chapter 5 Summary

Proteins and peptides have escalated as an important class of therapeutic drugs for treating several chronic and acute illnesses. However, these macromolecules mostly have to be stored and transported under refrigeration as they are unstable at ambient temperatures. Limited storage stability also impedes their commercialisation. To increase their stability, biologicals are often formulated in dried state which generally requires stabilising excipients to protect proteins during drying. This thesis reports a successful development of biologically active and stable protein formulation using suitable excipients during spray drying.

In this work, the ability of amino acids (ranging from large hydrophobic to small hydrophilic) and their combinations to stabilise proteins during spray drying was evaluated. On spray drying with the combination of arginine and glycine it could be shown that model proteins catalase and lysozyme maintain their activity on drying and remain stable after storage. It was shown that an influenza A (H1N1) vaccine (Pandemrix) in spray dried form is more stable compared to classical liquid vaccine which completely loses its activity especially when stored for 1 month at stress conditions whereas the dried formulation is found to be stable for more than 3 months under such harsh conditions. Surface activity of these amino acids protects the proteins from interfacial stress during spray droplet formation combined with water replacement mechanisms and creation of an amorphous matrix to ensure stability in the dried state. This combination is tailored to the needs of
protein stabilisation during spray drying and may hence be utilised in dry powder formulation of biomolecules with superior stability characteristics.

Stabilising and Protecting Solution (SPS; Leukocare, Germany) formulation technology which makes use of the stabilisation effect of amino acids was also evaluated as a potential spray drying protecting excipient. The effectiveness of SPS as a stabilising excipient for proteins during spray drying was established first using the model protein (ovalbumin) and then the results were transferred to influenza A (H1N1) vaccine. Several characterisation methods such as gel-electrophoresis, size exclusion chromatography, dynamic light scattering and a functional assay were used to optimise the dried formulation and to evaluate the long term storage stability. It could be shown by haemagglutination assay that the Pandemrix vaccine spray dried with SPS is stable and as functional as the original liquid vaccine. Terminal sterilisation was also possible for this formulation without harming the vaccine. Storage data showed high stability of protected vaccines at 2-8°C and 25°C/60% rH, for at least six months. The preclinical in vivo study (done in cooperation with Public Health England, Proton Down, UK) showed that effectiveness of the dried influenza vaccine is comparable to that of the original liquid vaccine. Animals receiving original Pandemrix exhibited expected levels of seroconversion after 21 days (prime) and 48 days (boost) as assessed by haemagglutination inhibition assay. Boost immunisation with SPS-protected vaccine resulted in a strong increase in seroconversion but had only minor effects in animals treated with non SPS-protected vaccine. The results indicate the potential utility of spray-dried
and terminally sterilised vaccines stabilised by mixtures of amino acids which likely solve the problems associated with the long term instability and cold chain requirement of liquid vaccines.
Chapter 6 Zusammenfassung der Arbeit


In dieser Arbeit wurden einzelne Aminosäuren (von großen hydrophoben bis zu kleinen hydrophilen) sowie Kombinationen daraus hinsichtlich ihrer Fähigkeit untersucht, Proteine während des Sprühtrocknungsprozesses zu stabilisieren. So konnte nach der Sprühtrocknung mit einer Kombination von Arginin und Glycin gezeigt werden, dass die Aktivität der Modellproteine Catalase und Lysozym beim Trocknen erhalten und die Formulierung auch nach weiterer Lagerung stabil blieb. Es wurde weiterhin gezeigt, dass ein Influenza A (H1N1)-Impfstoff (Pandemrix) in sprühgetrockneter Form im Vergleich zur klassischen flüssigen Formulierung eine verbesserte Stabilität aufweist. Der flüssige Impfstoff verliert seine Aktivität vollständig, wenn er für einen Monat bei Stressbedingungen gelagert wird, während die

Zusätzlich wurde eine Formulierungstechnologie basierend auf Stabilising and Protecting Solution (SPS; Leukocare, Deutschland), die ebenfalls den stabilisierenden Effekt von Aminosäurekombinationen ausnutzt, als potenzieller (Schutz-) Hilfsstoff für die Sprühtrocknung untersucht. Die Effektivität der SPS als stabilisierender Hilfsstoff für Proteine während der Sprühtrocknung wurde zunächst mit dem Modellprotein Ovalbumin überprüft, um anschließend die Ergebnisse auf den Influenza A (H1N1)-Impfstoff zu übertragen. Dabei wurden mehrere Charakterisierungsmethoden wie Gel-Elektrophorese, Größenausschlusschromatographie, dynamische Lichtstreuung und ein funktionaler Assay verwendet, um die getrocknete Formulierung zu optimieren und deren Langzeitlagerstabilität zu beurteilen. Es konnte durch Hämagglutinations-Assay gezeigt werden, dass der mit SPS sprühgetrocknete Impfstoff stabil ist und die gleiche Aktivität wie der
flüssige Originalimpfstoff aufweist. Zudem war eine Endsterilisation dieser Formulierung möglich, ohne den Impfstoff zu schädigen.


# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ala</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>ANS</td>
<td>1-Anilino-8-Naphthalene Sulfonate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GA</td>
<td>Glycyrrhizic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>His</td>
<td>L-Histidine</td>
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<td>Leu</td>
<td>L-Leucine</td>
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<tr>
<td>Lys</td>
<td>L-Lysine</td>
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<tr>
<td>Met</td>
<td>L-Methionine</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MPL</td>
<td>Monophospholipid A</td>
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<tr>
<td>Mw</td>
<td>Molecular weight</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Phe</td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
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<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SD</td>
<td>Spray drying/spray dried</td>
</tr>
<tr>
<td>sd</td>
<td>Standard deviation</td>
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<tr>
<td>SPS</td>
<td>Stabilising and Protecting Solution</td>
</tr>
<tr>
<td>Trp</td>
<td>L-Tryptophan</td>
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<tr>
<td>Tyr</td>
<td>L-Tyrosine</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>XRPD</td>
<td>X-ray powder diffraction</td>
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RCSB Protein Data Bank: Ovalbumin. Available online at www.rcsb.org/pdb.


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Acknowledgements

I would like to take this opportunity to thank all those people who helped me through the course of the journey towards completion of my PhD.

Foremost, I am extremely grateful to Prof. Dr. Hartwig Steckel for giving me an opportunity to perform my PhD thesis in the Department of Pharmaceutics and Biopharmaceutics, CAU, Kiel.

I express my deep sense of gratitude for my supervisor Dr. Regina Scherließ for her continuous advice and guidance for this research project with her bright ideas and constructive feedback. I was fortunate to have such a supervisor, who was always available to answer my queries and to check my manuscripts in a short period of time. Her sincere suggestion helped me greatly in bringing out the best in this research.

I would like to acknowledge Zim ‘Zentrales Innovationsprogramm Mittelstand’ for providing the financial support of this thesis. I also would like to thank Prof. Dr. Martin Scholz and Dr. Kristina Kemter from Leukocare Biotechnology for their excellent collaboration for this project.

Moreover, I would like to thank all techno colleagues for their help during my research work and providing a friendly work environment. I owe my special and in-depth gratitude to my lab colleagues Andrea (aka ‘AED’) and Gereon (aka ‘Granimal’) for all the help, enjoyable lab conversations and German lessons. It was always nice to discuss and chit-chat with you guys on various topics.
I also would like to thank Eike, Friederike, Lars, Philip, Sven, Sabrina and Simon for their help and support whenever needed. I am also grateful to Mohammad for helping me out with numerous German forms and insurance related issues.

I sincerely acknowledge the courtesy and help of Rüdi for creating the best graphical drawings for this work. I also would like to thank Hanna, Regina K., Maren, Simone, Detlef, Volkmar and Dirk for the analytical and technical support.

My time in Kiel has been remarkably enjoyable in large part due to my friends and would like to thank all of them who made my stay memorable and pleasant. Special thanks to Faizan and Priya for the great time after work and all the delicious food which we cooked together. My time in Kiel was also enriched by the Kiel Cricket Club and would like to thank all players of my cricket team for all the fun and exciting matches we had played.

Finally, my heartfelt gratitude goes to my family for their love and support throughout my life. Many thanks to my wife Mahek for her love and ever support. She always has lots of things to share and listening to her always makes my day and all the tense moments to fade away.

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