Protein Drugs for Inhalation:

Investigating the Stability of Dry Powder Formulations during Production and Application

Dissertation

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vorgelegt von

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Für meine Tante Machti,

die mir zeigte wie wichtig es ist kreative Lösungen für Probleme zu finden…

Die Suche nach Wissen ist ein unendliches Abenteuer und jede Entdeckung eröffnet neue Horizonte der Möglichkeiten.

Rita Levi-Montalcini

Parts of this work were already published:

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1 Introduction

Over the past few years, protein-based drugs have become more and more important. Of the 37 novel drugs approved by the FDA for the year 2022, 15 were biologics: one mixture of proteolytic enzymes, ten monoclonal antibodies, one peptide hormone, one botulinum toxin A formulation, one enzyme and one fusion protein [1]. These numbers demonstrate the increasing importance of protein drugs especially concerning novel drugs. These protein drugs provide new or improved therapeutical options for several severe diseases for which there was previously no or inadequate medication. Nevertheless, proteins are delicate molecules with complex structures prone to degradation, which even nowadays remains a significant challenge for their therapeutic use. Due to their low stability in the gastrointestinal tract and their poor ability to penetrate through the gastrointestinal epithelium, protein formulations are commonly delivered parenterally. This comes with several disadvantages for the patient, the formulation and the environment, like the use of needles that cause pain or local reactions at the injection site, the need for sterility and the frequently needed cold storage. Therefore, alternative application routes are of increasing research interest. This thesis focuses on the pulmonary application of protein drugs as one of the most promising alternative routes.

For proteins a dry powder formulation for inhalation (DPI) seems to be the most suitable. Being a dry powder, protein DPIs are assumed to show higher storage stability than aqueous liquid protein formulations. Furthermore, the DPIs show several advantages in the development. Excipient concentrations and combinations can be chosen more freely since DPIs do not need to be isotonic or isohydric. Sterilisation is also not necessary, which is advantageous since many sterilisation methods are highly protein-degrading.

Although protein dry powder formulations are commonly produced by lyophilisation, this drying technique is not the best for protein DPI production. In this process, particle size and shape are not easy to manage and usually do not meet the inhalation criteria. Furthermore, the process is highly time- and energy-consuming. The best drying method for a protein DPI would be spray drying. The process produces homogenous, round particles whose size can be easily managed by the choice of spray drying parameters. Production of particles with an aerodynamic diameter from 1 µm to 5 µm meeting the inhalable size range is possible. Spray drying, however, exposes the protein to higher thermal and air-liquid interface stress and several other stress factors in another way than lyophilisation and is not as well-established for protein drying.

2 Objectives

This thesis focuses on DPI formulations of protein drugs. The aim is to identify and characterise destabilising factors that occur during the production and application of inhalable protein drugs. Additionally, the work approaches the challenges and opportunities of developing a stabilising formulation for a protein in the presence of these stress factors. Therefore, a deeper look into the stress factors and their influence on the protein's molecular integrity as well as stabilising parameters is part of this work. Furthermore, investigations of the stabilising influence of excipients, their needed concentration and possible combination, and their suitability for inhalation are performed. Last, different application for the DPI formulation opportunities are investigated.

The first part of this thesis aims to identify model proteins suitable for the investigation of the protein destabilising effect of spray drying. A suitable model protein would require a molecular structure that is influenced by the process but not completely degraded. Furthermore, a sensitive analytical method to investigate the protein´s stability needs to be found.

The second part evaluates and differentiates the influence of the specific stress factors during the different phases of the spray drying process. Additionally, it will answer the question of whether different spray drying systems and spray drying nozzles affect protein stability in different ways.

The following part focuses on the identification of suitable protein stabilising excipients for spray drying. The hypothesis is that excipients or excipient combinations selected based on specifically identified stress factors stabilise the protein the best.

The final part explores the application as an inhalable dry powder formulation and alternative application opportunities. The idea is to first formulate the protein as DPI formulation for patients that can use DPI devices and establish the possibility of redispersing the formulation and applying the liquid via nebulisation. The last aims to investigate formulations that can be used for patients with e. g. a very poor lung function.

3 Theoretical Background

3.1 Therapeutic Proteins

The first biopharmaceuticals were purified animal substances, like pig insulin. These naturally occurring proteins showed a remarkable affinity and specificity towards their clinical target [2]. However, they were not optimal for the use as a drug substance since the availability of these proteins was low and their immunogenicity high [3]. Nowadays, due to the enormous development in the field of biotechnology, protein drugs are mostly recombinant and lab-designed, like modern insulin products. Modifications in the molecular structure can have an impact on the mechanism of action, side effects, immunogenicity and efficacy [2]. They can also prolong the protein's half-life and thereby lower the dosing frequency. Furthermore, reducing the production cost and prolonging the shelf-life is possible [2]. To achieve the optimal drug molecule, it is, for example, possible to tailor the molecular structures of the protein to meet the precise need [2]. Furthermore, PEGylation or glycosylation of the protein can increase the protein´s in vivo stability [2].

In the last decade (2010-2019), 89 biological license applications (BLA), and 27 biosimilars were approved by the FDA [4]. For a long time, only new biological products were approved. In 2015, Zarxio (Sandoz Inc. Princeton, NJ USA) became to be the first biosimilar on the market. The API in Zarxio is filgrastim a recombinant methionyl human granulocyte colony-stimulating factor used to treat low neutrophil count in HIV/AIDS, chemotherapy or radiation poisoning patients. After Zarxio, numerous biosimilars followed [4].

The most important classes of drug proteins are recombinant proteins (including enzymes), monoclonal antibodies and antibody-drug-conjugates. They can have several therapeutic purposes. Therapeutic proteins can replace missing or abnormal proteins, enhance existing pathways, provide novel functions, interfere with molecules, and be used as a vaccination or diagnostic or for the delivery of other compounds like cytotoxic drugs [5]. Several severe diseases such as various kinds of cancer, diabetes or autoimmune diseases, which were previously unable to be treated effectively, can nowadays be treated using these drugs [5].

Therapeutic proteins are sensitive drugs with a highly complex molecular structure. Degradation of this structure can easily be caused by several chemical and physical stress factors. This degradation can occur during production, storage or application. Due to the high structural complexity already the synthesis of the protein active ingredient requires an expensive biotechnological production. This poses an increasing challenge for medical proteins that

require complex post-translational modifications for optimal secretion, drug efficacy and stability [6]. The integrity of the molecular structure, however, is crucial to secure the drug's effect. Low molecular stability generally leads to a short shelf-life. In vivo, proteins have a comparably short half-life due to several degradation pathways [7]. Immunogenicity presents a significant challenge during protein therapy, as many recombinant proteins can trigger immune reactions. Furthermore, misfolding and aggregation resulting from stress before or during application carry a high risk of causing immunogenic responses. These problems still limit or complicate the use of therapeutic proteins.

3.2 Protein Stability

Proteins have a high molecular weight (>5 kDa) and a primary, secondary, tertiary and sometimes even a quaternary molecular structure (Figure 1).

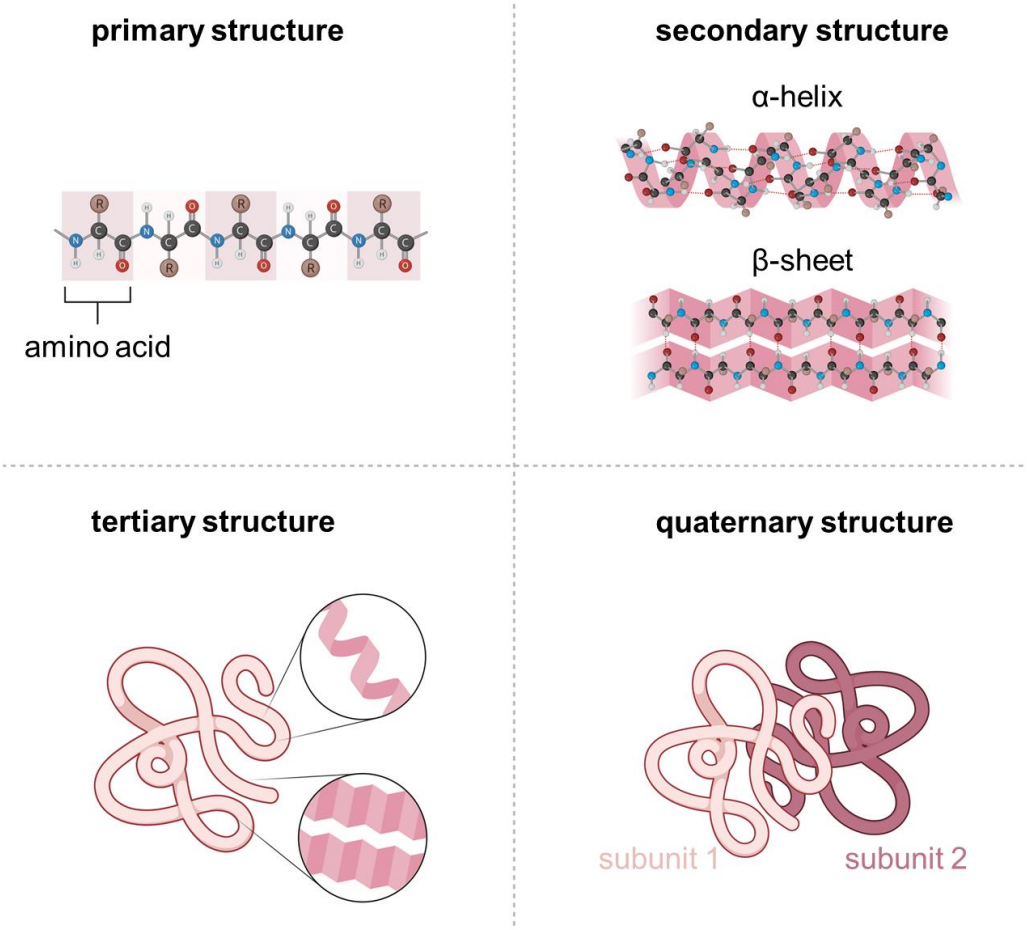

Figure 1: Molecular protein structures; created with BioRender

The primary structure is the peptide backbone formed by the amino acid sequence consisting of the twenty proteinogenic amino acids present in all living organisms. These amino acids have different characteristics depending on their side chains. The arrangement of the primary structure defines the higher protein structures. The amino acid sequences can either form α-helixes, β-sheets or β-turns which define the secondary structure. The three-dimensional arrangement of the peptide chain is called the tertiary structure. It usually defines the protein's characteristics and functionality. In some larger proteins, however, different protein subunits form one protein molecule. This structure is called the quaternary structure and is also essential for the protein´s functionality.

The free energy required to unfold the protein is minimal since the native structure is only marginally more stable than the unfolded structure [8]. Therefore, even minor changes in

the protein's environment can lead to denaturation. To minimise thermodynamically unfavourable interactions at protein-solvent interfaces proteins tend to aggregate [9]. This can be caused by unfolding but might also happen in the native state [10]. Maintaining the protein structure requires considering several chemical and physical degradation mechanisms. Chemical degradation like oxidation, hydrolysis or deamidation changes the chemical composition of the peptide chain and therefore the primary structure [11]. This usually leads to changes or destruction of higher protein structures. In physical degradation, by contrast, the chemical composition of the primary structure stays intact but the higher protein structures change. Denaturation, aggregation, precipitation and surface or interface adsorption are the most important ones (Figure 2) [11].

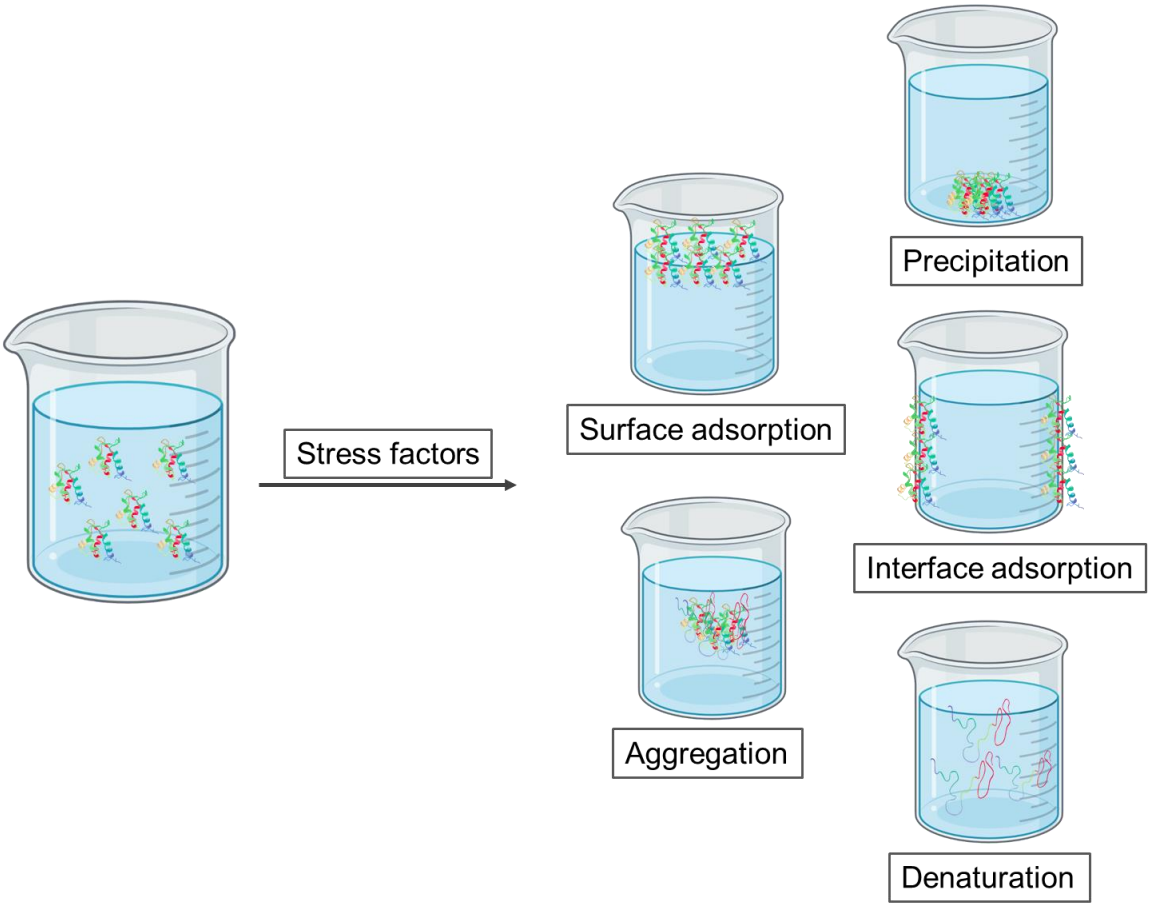

Figure 2: Mechanisms of physical protein degradation; created with BioRender

Physical degradation can also lead to chemical degradation and vice versa. Many of these degradation pathways tend to be irreversible [11].

Degradation can be caused or accelerated by various stress factors. Heat accelerates chemical reactions such as oxidation and deamidation of proteins. This directly affects the conformation of proteins' molecular structure and can lead to temperature-induced unfolding

and higher aggregation levels [10]. Many of these heat-induced conformational changes are irreversible [12]. The so-called melting temperature of a protein is protein-specific and can be used to evaluate the thermal stability of a protein. At the melting temperature, 50% of protein molecules are unfolded during a thermal unfolding transition [10]. The pH of the solution determines the charge of the protein and thereby its electrostatic interactions [13]. Proteins are usually only stable at a narrow pH range. Outside of this range, the free energy of the unfolded state is lower than that of the folded state and specific interactions like salt bridges are less stable [13]. Proteins are highly surface-active molecules and tend to adsorb at interfaces with more hydrophobic solids, gases or liquids when dissolved in aqueous solutions. Especially, air-liquid interfaces can destabilise proteins, but some studies also found negative effects of water-oil and solid interfaces [14]. Freezing and thawing introduce the protein to new ice-liquid interfaces [10]. In addition, there may be changes in the concentration of the protein and other solutes, such as buffer salts, and pH changes due to the crystallisation of buffer components which might occur [10]. Degradation caused by mechanical shear on its own is thermodynamically unlikely [14]. Nevertheless, the combination of interfacial stress and other shear-related effects, like molecular rotation caused by turbulence, appears to be able to destabilise proteins [14]. Several further factors like dehydration, storage in solution, organic solvents, UV light or oxygen can trigger protein degradation as well [10, 15].

During the production of protein drugs, the protein concentration needs to be considered. Since protein molecules tend to self-associate intermolecularly, the viscosity of protein solutions increases strongly with increasing protein concentration [16]. Depending on the protein, this can additionally lead to an increase in aggregation [16].

Considering the high molecular sensitivity, finding a stabilising formulation is essential for preserving a protein drug's effect. Several techniques exist to stabilise protein drugs. Storing the protein as a dry powder increases its long-term stability compared to storage in an aqueous solution [17]. In these solutions, proteins tend to be less stable and usually have to be stored under cool storage or even cold chain conditions at 2-8°C. Furthermore, the packing can protect the protein in liquid as well as in dry formulations against UV light or oxygen.

Several excipients can stabilise proteins during drug production or storage. To determine the appropriate excipient for the desired function in the formulation, conducting pre-formulation compatibility experiments is essential [18]. Basic knowledge of the physical and chemical properties of the specific protein must be taken into consideration [18]. Additionally, it is crucial to comprehend the specific mechanism of stabilisation between the excipient and

each drug-protein [18]. Of these excipients, only a few are suitable for dry powder inhalation (see Section 3.4.1.2).

3.2.1 Detection of Changes in Protein Structure

The complexity of proteins´ molecular structure and degradation pathways leads to several possible types of changes with different intensities [19]. Additionally, analysed samples often contain a mixture of native and degraded proteins. This highly complicates analysing these changes. Furthermore, many analytical methods require sample preparation steps like filtration, dilution or an increase in concentration, buffer changes or heating that can themselves interfere with the protein's structural integrity. Therefore, the detected structural changes in some cases might be caused by the conditions during sample preparation or the measurement. Various strategies exist to identify changes in the morphology and chemical and physical properties of proteins.

Aggregation is a frequently analysed physical property of proteins. It is usually linked to other forms of degradation and therefore suitable for an evaluation of the overall protein stability. One strategy relies on characterising the molecular size by measuring the hydrodynamic size, the molecular weight or a combination of both. Dynamic light scattering (DLS), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography with or without multi-angle light scattering detector (SEC (-MALLS)) are commonly used methods to measure these properties [19] (Table 1).

Table 1: Selection of commonly used analytical methods for the detection of protein aggregates [19]

Method	Detection of	Size range	Throughput
DLS	Hydrodynamic size	1 nm–5 µm	High
Light obscuration	Concentration/size	1–600 µm	Medium
Optical microscopy	Size/morphology	>1 µm–mm	Low
SDS-PAGE	Molecular weight	kDa–MDa	High
SEC (-MALLS)	Hydrodynamic size	1–100 nm	High
Turbidity	Optical density > 350 nm (wavelength)	Not defined	High
Visual inspection	Visible particles	>50 µm–mm	High

DLS determines the hydrodynamic size of proteins. The measurements usually only require uncomplicated sample preparation, like filtration, and only take a few minutes [20]. The results are very accurate and reproducible for monodisperse samples. In polydisperse samples, however, the results are heavily influenced by a small number of larger particles that cover a large number of smaller particles [20]. SDS-PAGE discriminates protein species by their molecular weight. The set-up is inexpensive and easy to use, compared to other measurement techniques, and is therefore a traditional technique to analyse covalent aggregation as well as fragmentation [19]. SEC is a well-established method to separate protein species by their hydrodynamic size (measurement principle described in Section 6.5.2). It is very suitable for the quantification of dimers, trimers and oligomers [21]. The detection is, however, limited by the (molecular weight) cut-off of the column. Furthermore, the columns are highly sensitive and have a short life expectancy compared to other columns [21].

For the determination of particles in the sub-visible size range, regulatory agencies in the US, Europe and Japan describe light obscuration and optical microscopy [19]. These techniques can measure the geometric size of particles and are required in the production of parenteral (protein) drugs. For larger aggregates or higher concentrations of aggregates, a visual inspection as well as a turbidity measurement might be possible. Visual inspection under defined conditions by specially educated operators is also commonly used in industry to evaluate parenteral drug products [19]. On a laboratory scale, it is also a very simple method to detect very large aggregates in large amounts. Turbidity measurements in a UV/VIS-Spectrometer can detect aggregates that cause light scattering.

The analysis of protein aggregates can be challenging since they come in various forms and sizes [19]. They usually consist of degraded protein molecules, but some proteins also tend to form aggregates in the native state. Aggregates can vary in size from a few nanometers to a few millimeters and can therefore be visible or sub-visible. They can be water soluble as well as insoluble. Analytical methods, however, can usually only measure a certain size range, either soluble or insoluble particles and can usually not differ between native and degraded aggregation.

Chemical degradation products can be detected using different chromatographic methods, like ion exchange chromatography (IEXC) and reversed-phase high-performance liquid chromatography (RP-HPLC), capillary isoelectric focusing (cIEF) or (liquid chromatography-) mass spectroscopy (LC-MS) [19] (Table 2).

Table 2: Commonly used analytical methods for the detection of chemical changes in proteins [19]

Method	Detection of	Throughput
cIEF	Charge variants	High
IEXC	Charge variants	High
(LC-) MS	Molecule loss/gain	Low
RP-HPLC	Hydrophobicity	High

The main technique for the analysis of chemical changes in proteins is MS [19]. It detects molecule loss or gain. Several precise and specialised MS techniques exist [22]. Although, it is a very powerful analytical tool, the complex sample preparation and the highly cost-intensive equipment can be challenging. Other methods, like IEXC and cIEF detecting charge variants and RP-HPLC detecting hydrophobicity changes are also frequently used for the detection of chemical changes [19]. They are less accurate than MS-based methods but usually require less complicated sample preparation.

Conformational changes can cause e. g. changes in the absorption of different light wavelengths of the molecule, caused by alterations in the environment of amino acids or alterations in hydrophobicity. Commonly used analytical techniques are circular dichroism- (CD), ultraviolet/visible- (UV/VIS), fluorescence-, Fourier-transform-infrared- (FTIR) or Raman-spectroscopy and differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF) [19] (Table 3).

Table 3: Commonly used analytical methods for the detection of conformational changes in proteins [19]

Method	Detection of	Throughput
CD spectroscopy	Secondary/ tertiary/ quaternary structure	Medium
DSC	Thermal variables	Low
DSF	Melting temperature, aggregation onset temperature	Low
Fluorescence (extrinsic/ intrinsic) spectroscopy	Tertiary/ quaternary structure	High
Infrared spectroscopy	Secondary structure	Medium
Raman spectroscopy	Secondary structure	Low
UV spectroscopy	Tertiary structure	High

DSC and DSF are commonly used to analyse heat-induced protein-unfolding processes [19]. They can determine e. g., the onset of the thermal unfolding or the melting temperature (for NanoDSF see Section 6.5.6). The spectroscopic techniques to analyse conformational changes are all well-established [19]. FT-IR and Raman spectroscopy detect secondary structure changes, CD depending on the wavelength secondary, tertiary or quaternary structure changes, UV tertiary structure changes and fluorescence spectroscopy (see Section 6.5.4) tertiary and quaternary structure changes.

For several enzymes specific activity assays exist. The enzyme usually processes a substrate which then changes its properties resulting in e. g. a change in UV absorption (for chymotrypsin activity assay see Section 6.5.5). Furthermore, specific potency assays that are based on cells, tissues, organs or animals exist [19]. Both kinds of assays are capable of analysing a protein´s functionality which is essential for therapeutic proteins. However, it has to be kept in mind that not all structural changes cause a change in functionality.

Due to the different principles, the methods rely on, they detect different kinds of protein degradation. In the production of protein drugs, it is, therefore, necessary to establish a set of analytical methods covering a broad range of measurement principles and addressing different aspects of protein integrity [19]. Depending on the number of samples, the throughput of the methods should also be considered when choosing suitable methods as it can vary greatly between different methods. However, due to time and resource limitations in

laboratory research, it is not always possible to implement several different analytical methods. It therefore has to keep in mind that a single method might detect the presence of structural changes, but cannot prove their absence.

3.3 Administration of Protein Drugs

In general, oral delivery is preferred for drug administration due to the patient-friendly administration and the easy and well-established formulation strategies. For protein drugs, however, the suitability of this application route is limited since proteins are highly sensitive active ingredients (API). The low pH in the stomach, the high proteolytic enzyme concentration, the hepatic-first-pass effect and poor epithelium permeability due to the protein´s high molecular weight lead to a very low bioavailability [23]. Nevertheless, clinical trials investigated the oral delivery of some peptide hormones and some other proteins including antibodies [24]. In 2019 the FDA approved the glucagon-like peptide-1 receptor agonist Semaglutid (Rybelsus®, Novo Nordisk, Bagsværd, Denmark) for oral peptide delivery against diabetes mellitus [25]. However, until today no protein or antibody has been approved for oral delivery [26]. Furthermore, a local treatment especially with missing enzymes as well as oral vaccination is reasonable and possible. The FDA approved some pancreatic enzymes, sacrosidase and a peanut protein for hypo sensibilisation for oral administration [26].

Due to the challenges of oral application, protein drugs are commonly given via parenteral application. The formulations are traditionally aqueous solutions which decrease their storage stability. Many of them have to be stored cool [15] and often a cold chain is necessary. This requires a lot of energy and effort. Especially, in warmer regions or low-income countries, this can be challenging. The parenteral application, also, comes with the need for sterility and depending on the application's site and the applicated volume isotonicity and an isohydric/euhydric pH. This complicates the formulation process. Most sterilisation methods expose the drug to harsh conditions, like high temperatures, pressure or radiation which can lead to protein degradation. The preferred method is, therefore, sterile filtration. During the production process, multiple filtration steps might be necessary [27]. At each step, the protein is exposed to shear-related stress as well as adsorption to the membrane material. Isotonic and isohydric conditions might not meet the criteria for the optimal environment of the specific protein as the stability and solubility of proteins can highly depend on the solvent's pH and salt content [28].

Furthermore, there are several disadvantages for the patients due to the invasive application of parenteral formulations. Often these drugs are injections or infusions that need to be administered by a healthcare professional usually in a practice or hospital. The injection frequently comes with pain and can cause local side effects like skin irritation or injuries. Subcutaneous formulations can usually be administered by the patients themselves or their families. These drugs come with a high incidence rate of longer-lasting (hours till days) injection

site reactions with symptoms like pain, itching, bruising and swelling [29]. An optimal injection technique can reduce the risk for local side effects but includes injecting also in more painful injection sites, like the upper arm [29]. The discomfort caused by invasive application might reduce the patient's quality of life and therapy adherence. Furthermore, the invasive application often comes with the production of hazardous and contagious waste e. g. contaminated needles.

As alternatives, several non-invasive application routes have been studied. The most promising routes for systemic treatment are sublingual, buccal, transdermal and pulmonary application (see Section 3.4). Clinical trials for buccal delivery mainly investigated the application of insulin [24]. Clinical trials for the transdermal delivery of biologics investigated particular microneedles, transdermal patches and micro injectors for peptide hormones or vaccination [24]. Nasal delivery in general is a favourable alternative to invasive application. For protein drugs, however, it comes with some challenges since molecules with a molecular weight of over 1000 Da penetrate through the nasal epithelium very poorly [30]. Furthermore, most proteins and peptides are highly hydrophilic which also hinders penetration [30]. The nasal delivery of biologics is most suitable for smaller peptides as well as vaccination. A few peptides like oxytocin or desmopressin and an influenza vaccine are FDA-approved for nasal application [24]. For some targets like the skin or the lung also a local application is possible. Several clinical trials investigate protein drugs for pulmonary applications [23]. For alternative application routes, the FDA approved four sublingual allergen extracts and 43 protein drugs for topical administration mainly containing fibrin or thrombin as well as four pulmonary formulations (see Section 3.4.3) [26].

3.4 Pulmonary Delivery of Therapeutic Proteins

Pulmonary delivery has several advantages compared to the parenteral application. Inhaled drugs can be administered without health care professionals by the patients themselves or with the help of e. g. a family member at home. Furthermore, the application is painless and does not cause any tissue irritations. This can increase the patient's comfort with the therapy and therefore might increase the patient's adherence [31]. The needle-free application does not produce any hazardous or contagious waste. This is especially advantageous for hospitals or medical practices in low-income regions, where secure, but cost-intensive, disposal can be challenging, or for the application without healthcare professionals at home.

3.4.1 The Respiratory System

The respiratory system (Figure 3) is responsible for gas exchange between the human body and the atmosphere.

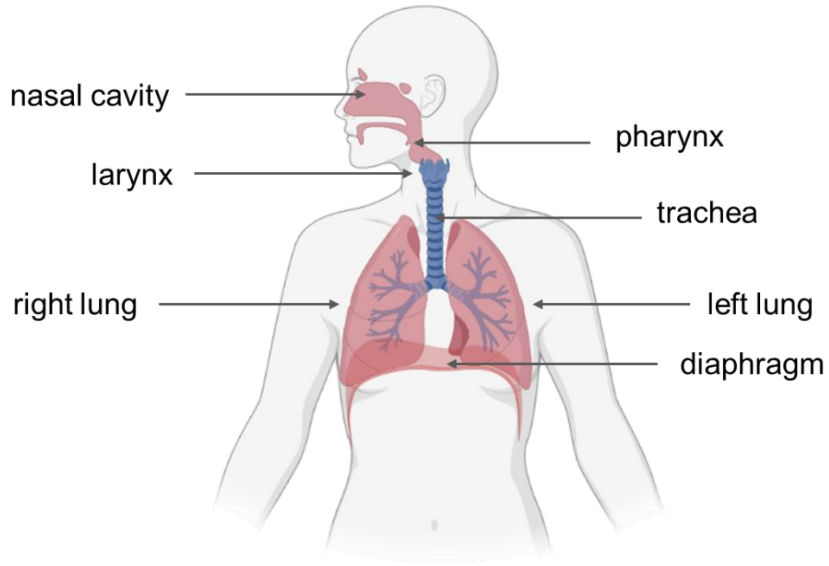

Figure 3: Respiratory system; created with BioRender

During inhalation, the air gets warmed, humidified and filtered in the upper parts of the respiratory system [32]. This air then flows through a complex pipe system splitting at bifurcations (trachea and bronchial tree, Figure 4) into the peripheral region of both lungs [32].

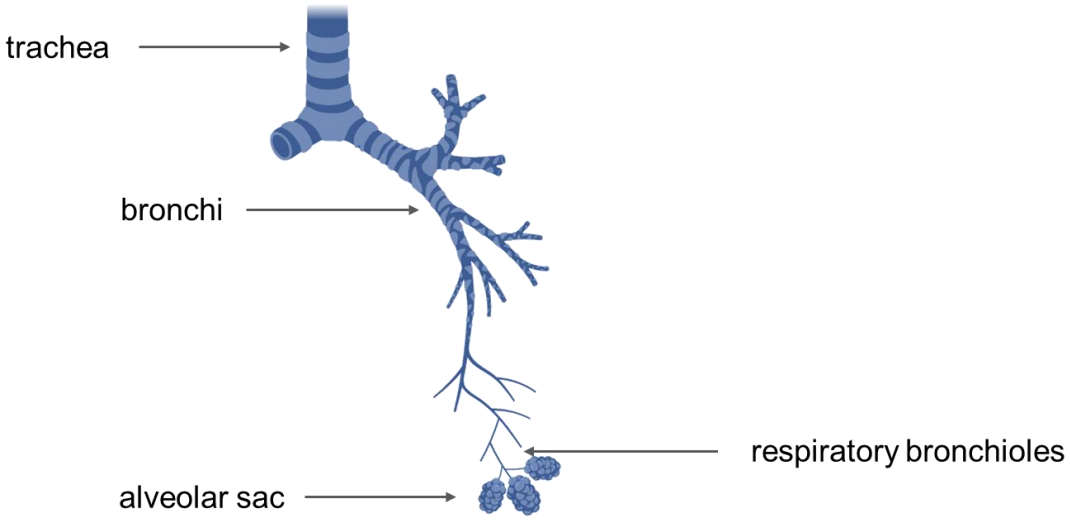

Figure 4: Bronchial tree; created with BioRender

In the pipe system, the surface area increases and the velocity of the air decreases with every bifurcation. In the alveoli, the actual gas exchange then takes place through diffusion. The oxygen from the air is absorbed and binds to the haemoglobin in the blood and the carbon dioxide from the blood is exhaled [32]. This gas exchange is driven by the differences in the partial pressure of the gases [32]. The alveolar region has a huge total surface area (100 m^2) and a highly permeable membrane [33].

Systemic and local treatment via pulmonary or nasal drug application is possible through the respiratory tract. Drug particles or droplets, therefore, have to be inhaled and separated from the airflow by contact and adsorption to the respiratory surfaces instead of being exhaled with the airflow [34]. This process is called deposition and is mainly caused by impaction, sedimentation and diffusion (Figure 5).

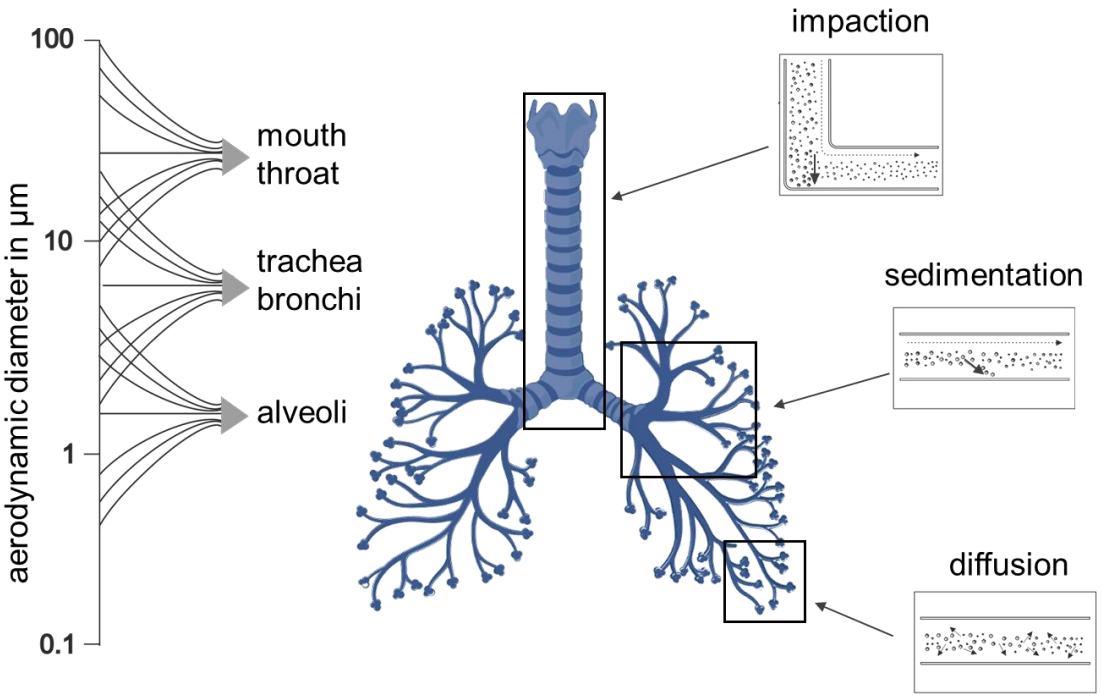

Figure 5: Mechanisms of airway deposition in the respiratory system; created with BioRender

Impaction happens when the air stream changes direction and particles cannot follow due to their moment of inertia [34]. It mainly happens in the upper airways and is more likely for larger particles with a particle size of around 10 µm and faster air streams [34]. Sedimentation velocity directly correlates with gravity, the aerodynamic diameter of the particle and the velocity of the air [35]. In the smaller airways and the alveolar region, the flow velocities are low and the dimensions are small. In these regions, sedimentation is dominant [34]. Increasing the residence time in the smaller airways by breath-holding increases sedimentation [34]. This leads to an adequate deposition for local treatment. In the smaller airways and alveoli diffusion occurs for particles < 1µm by random motion (Brownian motion). Although diffusion increases with decreasing particle size, particles < 0.5 µm are likely to be exhaled since the sedimentation for these particles is so low [34]. Furthermore, the mucociliary clearance pushes dust, microorganisms and insoluble particles out of the airways. It is a coordinated beating of the cilia in the nose and the airways towards the throat where they are swallowed [31].

The deposition of the drug particles or droplets highly depends on their mass median aerodynamic diameter (MMAD). The MMAD refers to the aerodynamic diameter at which half of the aerosol is above and half is below [35]. In general, particles with an MMAD of 5-10 µm mainly deposit in the large conducting airways and oropharyngeal region and particles with an MMAD of 1-5 µm in the small airways and the alveoli [33]. Drug particles should usually deposit in the smaller airways; therefore, they should have an MMAD < 5 µm. However, the

individual anatomy can influence the deposition profile [33]. In addition, lung diseases, like chronic obstructive pulmonary disease (COPD) or asthma, that cause airway narrowing, mucus hypersecretion and mucus plugging as well as incorrect inhalation techniques can affect particle deposition as well as the efficacy in the lungs [36].

3.4.2 Systemic and Local Targeting

Local targeting via pulmonary application is possible for all types of protein drugs, regardless of their molecular size. The local application seems to be especially interesting for the treatment of severe lung diseases. For these diseases, often no adequate treatment is available and the development of expensive drugs like biologics is reasonable. These diseases are mainly asthma and chronic obstructive pulmonary disease, all different kinds of lung cancer, infectious lung diseases like COVID-19 and rare lung diseases, like cystic fibrosis. Several antibodies against lung cancer and asthma are already on the market [37]. Against COVID-19 and COPD antibodies are in the later stages of clinical studies [38]. Those antibodies are all for parenteral application. Against cystic fibrosis, however, a protein drug is approved for pulmonary application for local treatment (see Section 3.4.3.2).

Systemic targeting via pulmonary application is an interesting non-invasive alternative for all parenteral systemic protein therapies. This alternative treatment is, however, only feasible for proteins that penetrate through the pulmonary epithelium well enough. The penetration properties of the proteins directly correlate to their molecular mass (Figure 6).

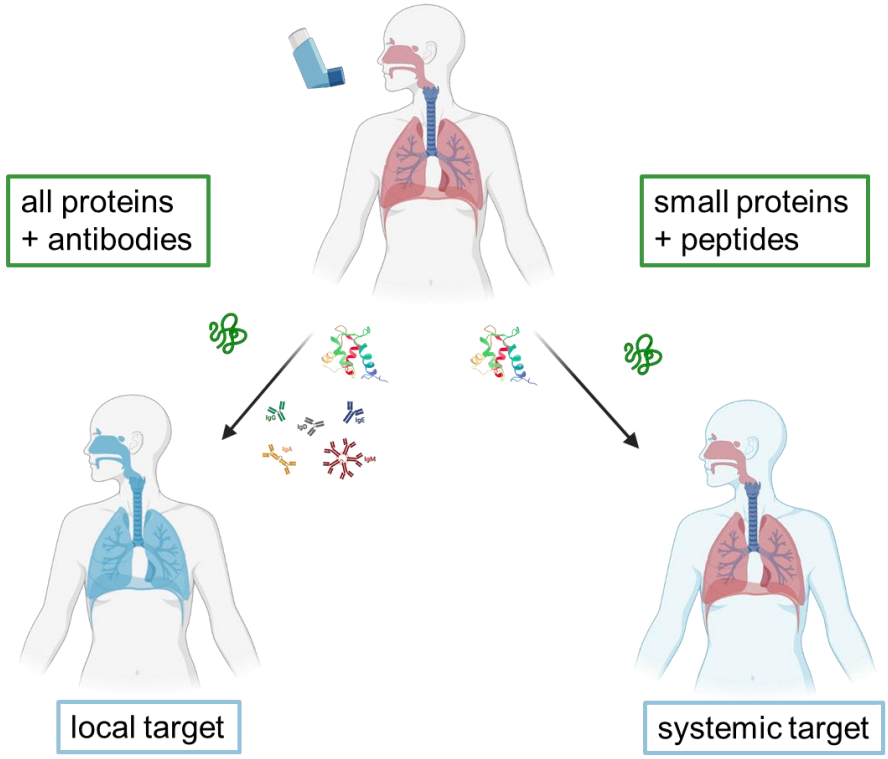

Figure 6: Pulmonary delivery of therapeutic proteins; created with BioRender

For low molecular weight proteins and peptides around 5 kDa or smaller, a systemic treatment is possible via the lung. The molecules can penetrate through the pulmonary epithelium in both ways. Therefore, also a parenteral administration for targeting the lungs is possible. Nevertheless, the inhaled proteins only have a bioavailability of 20-50% for systemic targeting after pulmonary application [31]. For inhaled insulin, for example, the bioavailability also highly depended on the formulation and was reported from 10% to 50% for different formulations [39].

Proteins with a higher molecular weight, like antibodies, cannot penetrate through the pulmonary epithelium easily [31]. A pulmonary delivery for systemic targeting is unlikely to work well due to poor bioavailability. A parenteral application for pulmonary targeting also comes with several challenges. The low penetration rate leads to an accumulation of the protein in the serum instead of at the pulmonary target. The concentration in the serum was found 500-10000 times higher than in the lung for examined antibodies [40]. This leads to an extreme increase in the needed dosages and increases the risk of systemic side effects. The other way around causes a pulmonary application of high concentrations of high-molecular-weight protein in the lung only a low concentration in the serum [31]. Therefore, the needed dosages for a therapeutic effect equivalent to the parenteral application could be lower and the risks for systemic side effects decreases [31]. Considering, especially, the comparably challenging and cost-intensive production method, lower needed doses are advantageous.

Reducing the overall cost of therapy could increase access to therapeutic proteins for patients who cannot afford the current costs.

The pulmonary application of protein drugs comes, however, with challenges due to the short residence time in the lung compared to the plasma [31]. The lungs have three clearance mechanisms that mainly cause this phenomenon. The first, mucociliary clearance is a coordinated beating of the cilia in the nose and the airways towards the throat. It pushes dust, microorganisms and insoluble particles out of the airways [31]. This would be especially problematic for aggregated proteins while monomers probably dissolve fast enough. To avoid mucociliary clearance the addition of low molecular PEGs is possible, which increases the mucus penetration of the drug, which can then avoid mucociliary clearance [31]. Second, alveolar macrophages can take up larger proteins > 40kDa. This may not influence smaller proteins and peptides < 25 kDa since they are absorbed rapidly [41]. Furthermore, the linkage of proteins with excipients (e. g. PEGylation) can reduce the uptake of larger proteins [31]. Third, small proteins and peptides around 5 kDa or smaller can be absorbed into the system circulation which is disadvantageous when local treatment is desired. For larger proteins, this mechanism is not as relevant since they are absorbed poorly [31]. Some particular proteins are cleared from the lung much faster than expected based on their size. This suggests the presence of special receptor-mediated-transport pathways [42].

3.4.3 Commercially Available Protein Drugs for Pulmonary Delivery

Of the 1836 FDA-licensed biological products (including different strength or application routes of the same biological active ingredient) only four are approved for pulmonary application: Afrezza® in 4 units, 8 units or 12 units dosage containing insulin as API and Pulmozyme® containing recombinant human DNAse 1 [26]. Nevertheless, several inhalable protein drugs against lung diseases (including COVID-19) were in clinical trials in 2020 [31]. Till today, the FDA approved none of these drugs.

3.4.3.1 Inhalable Insulin

A well-known example of systemic treatment with a small protein via the lung is the peptide hormone insulin. It is supposed to fully or partly replace parenteral insulin for diabetes mellitus patients. Both commercial products Exubera® and Afrezza® only contain short-acting insulin. They are therefore suitable for combination therapy for type 1 diabetes patients and a combination or monotherapy for type 2 diabetes patients [43].

Exubera® by Pfizer Inc. (New York, USA) was the first approved inhalable insulin in 2006. Exubera® came with a large inhalation device and was dosed in milligrams which was not

easy to adapt to the commonly used I.E. dosages for the patients. It was the first approved spray-dried protein drug and contained mannitol and glycine as stabilising excipients and a citrate buffer [44]. Due to poor sales, the company withdrew from the market in 2007.

In 2014 Afrezza® by MannKind Corporation (Danbury, Connecticut, USA) was approved. It comes with a small inhalation device and a more patient-friendly dosing system in units (commonly used for insulin dosages) [43]. Afrezza® is still available on the US market as 4 units, 8 units and 12 units dosed device. In Afrezza® the insulin is adsorbed onto carrier Technosphere® particles consisting of fumaryl diketopiperazine (FDKP) and polysorbate 80 [45]. The FDKP crystallises under acid conditions and forms the microparticles on which insulin can adsorb spontaneously during production [46].

Although the idea seemed promising, inhalable insulin has been discussed controversially. Contrary to the common subcutaneous application, no injection is necessary which can be beneficial for the patient. The need for several applications daily can be challenging for patients with needle phobia or with a semi-optimal injection technique which can lead to skin irritations or injuries. Nevertheless, the pulmonary application of insulin occasionally caused local side effects and irritations in the lungs as well as an increased risk for hyper- and hypoglycaemia in certain patients. This led to a contraindication for smoking patients or patients with specific lung diseases [43]. Furthermore, the pulmonary application was not suitable for long-acting insulin [43]. Last, the bioavailability of pulmonary insulin was reported to be significantly lower than for subcutaneous applications [39].

Exubera® and Afrezza® both contain fixed insulin doses. For Afrezza® three differently dosed products exist. Nevertheless, insulin therapy for diabetes mellitus is highly complex and the dosing scheme is extremely diverse. It varies with the individual patients and their concrete conditions or food consumption each day. Therefore, patients often have to inhale multiple times with differently-dosed inhalers. This makes the therapy potentially more complicated and less convenient than the commonly used dose-flexible insulin pens. To face this problem different companies tried to fabricate inhalable insulin with flexible doses, e.g. Dance Biopharm Holding Inc. (San Francisco, USA) with a vibrating mesh nebuliser [47]. However, no further inhaled insulin products were approved until today.

3.4.3.2 Dornase alfa

The other FDA-approved inhalable protein drug is Pulmozyme®. The API is Dornase alfa, a recombinant human DNAse 1. The enzyme is indicated for local pulmonary use in patients with cystic fibrosis. Before the approval of Dornase alfa around half of the patients with this

illness passed away before reaching adulthood [48]. Although the life expectancy increased for cystic fibrosis patients in the last years, it is still only 48.5 years even in high-income countries [49]. In patients with cystic fibrosis, the airways contain an abundance of neutrophils which release actin and DNA after necrosis. This increase in extracellular DNA increases the viscosity of the sputum resulting in mucus obstruction, impaired mucociliary clearance and an increase in infections and airway inflammation [50]. Dornase alfa, when inhaled, cleaves the extracellular DNA and reduces the sputum viscosity and the risk for resulting health issues [50]. Several studies found an improvement in lung function after treatment with Dornase alfa [49]. Furthermore, no serious side effects were reported [49].

In 1993 the FDA approved Pulmozyme® [50]. It was developed as the first inhalable protein formulation since achieving the required doses with the traditional parenteral delivery methods seemed unlikely [48]. It is an inhalable Dornase alfa formulation for nebulisation and formulated as an aqueous solution without stabilising excipients in single-dose ampules. Until today it is the only inhalable protein drug for local treatment available on the market.

For this reason, DNAse has been extensively studied as a model substance for protein inhalation formulations. Zijlstra et al. investigated dry powder formulations for inhalation of the protein and found stabilising influences the excipients of trehalose, sucrose and inulin [51].

3.5 Formulations for Pulmonary Delivery

Formulations for pulmonary drug delivery can be liquids or dry powders. Several inhalation devices with different techniques exist to deliver these formulations to the lungs. The devices can be grouped into metered dose inhalers, nebulisers or dry powder inhalers. These devices produce aerosols with reproducible drug doses in inhalable particle- or droplet sizes (MMAD < 5 µm). Ideally, the devices are easy to use, portable and inexpensive [34]. After adequate training, the patients themselves (or e. g. the family) can typically use them at home.

3.5.1 Dry Powders for Inhalation

Dry powder formulations for inhalation (DPI formulations) are a highly stable and easy-to-use option for inhalable drugs [44]. These formulations do not need to be sterile, isotonic or isohydric. This makes it possible to choose the excipients and their concentrations more freely to obtain the conditions needed for a stabilised protein.

The majority of dry powder protein formulations (for parenteral use) available on the market are freeze-dried [52]. This technique is well established and stress factors, process parameters and stabilising excipients are well investigated. The process produces powders with a water content of less than 1% which is significantly lower than the powders produced by other drying processes [17]. Lyophilisation is, however, not very suitable for DPI formulation production. To reach the lungs the dry particles, must have a mass median aerodynamic diameter smaller than 5 µm and a narrow size distribution. Both characteristics do usually not apply to freeze-dried powders since control of the particle properties is difficult [52]. Furthermore, freeze-drying is highly time and power-consuming [52]. However, rare examples of DPI formulations based on lyophilised materials exist in the literature. Hufnagel et al. investigated thin-film freeze-drying for the production of DPIs of monoclonal antibodies [53]. They formulated a stable thin-film lyophilisate of IGg2a which showed an aerosol performance suitable for inhalation. Furthermore, Pfeffer designed a lyophilised insulin DPI formulation with good aerodynamic performance [54].

Several devices for dry powder inhalation exist. Some devices are capable of dispensing multiple doses, either through a powder reservoir that dispenses the required amount of powder or by containing pre-loaded doses within a blister foil [55]. Other devices are loaded with a single dosage capsule immediately before use [55]. DPIs are environmentally friendly, easy to handle and stored as a dry powder which increases the physicochemical stability of the drug [34]. Although the exact handling of the devices differs, they usually rely on the same principle of passive inhalation-activated drug delivery. The drug, as a powder in an

inhalable particle size, is inhaled with the inspiratory air flow of the patient alone. This requires no coordination between actuation and inhalation [55] which makes the devices easier to use than devices that require this coordination like pressurised metered dose inhalers. To gain the required good powder flowability and dosage uniformity three techniques are established for the production of inhalable powders (Figure 7) [56].

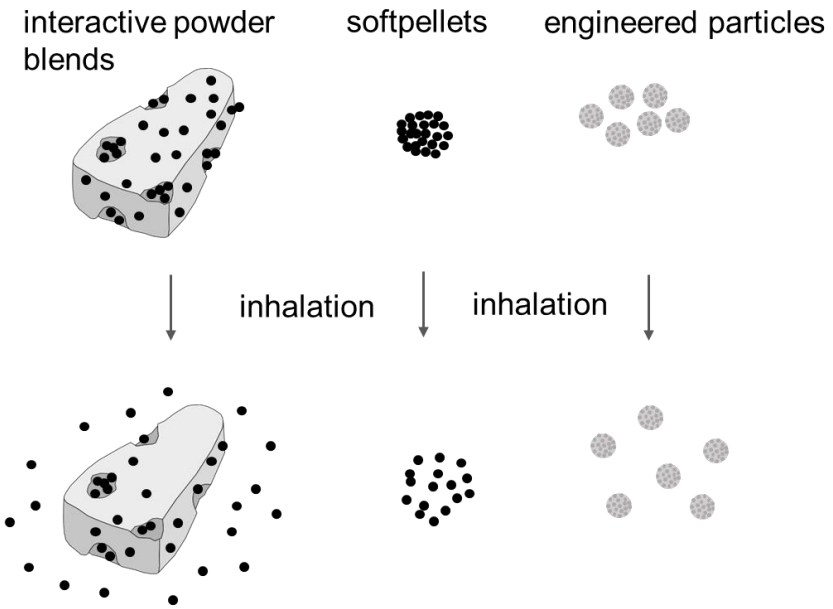

Figure 7: DPI formulation strategies (not to scale)

First, in interactive powder blends, the micronised API particles are attached to a larger carrier particle typically consisting of lactose or mannitol. The larger particles increase the flowability of the blends. They are, however, due to their size, not inhaled into the lungs, but separated from the active ingredient in the throat. The active ingredient is then inhaled into the lungs. Second, the micronised active ingredient particles loosely agglomerate as larger spherical soft pellets. During inhalation, these soft pellets disaggregate producing particles which can reach the lungs. Third, porous low-density drug particles are engineered, typically with spray drying. These particles are typically directly inhalable with an MMAD < 5 µm in a narrow size distribution and a round shape. Micronisation is usually not suitable for protein drugs due to the high energy input during the milling process which interferes with the protein's structural integrity [57]. Therefore, spray drying seems to be the most suitable production method for protein DPIs.

3.5.1.1 Spray Drying Process

Spray drying is a widely used drying technique in the pharmaceutical industry to produce dry powder formulations. It is a one-step technique and therefore suitable for continuous manufacturing. The powders can be produced in different particle sizes and the process is

relatively economical and fast [52]. Spray dryers atomise a liquid feed into small droplets which then rapidly dry in a heated airflow into particles. These particles are producible in a wide range of sizes [58]. The spray drying equipment comes in various sizes and technical configurations. They can be laboratory scale and produce a few milligrams of the product as well as industrial scale and produce several tons a day [58]. For atomisation, several nozzle types exist. The most common ones are rotary, pressure, two-fluid and ultrasonic nozzles [58]. The produced particles are usually round and can be produced in the range of a few micrometers allowing pulmonary administration. The particle shape and size depend on parameters like the inlet temperature, airflow velocity, concentration and composition of the liquid feed, the spray drying system and the nozzle. Those parameters can be controlled to engineer the particle fitting for pulmonary application [59].

The shape of the particles mainly depends on the temperature of the air stream during the drying process, the solvent and the solute. The temperature influences the evaporation rate of the specific solvent. Every solute has a specific diffusion coefficient in the solution. Typical examples of low diffusion coefficients are large molecules, like proteins or polymers. Smaller molecules, like small saccharides, usually have a high diffusion coefficient. Furthermore, surface-active molecules tend to accumulate at the droplet's surface, leading to an uneven distribution of the solutes within the droplet. The diffusion flux, caused by the increase in surface concentration during the drying process can, however, have a stronger effect and lead to a more even distribution. The ratio between the evaporation rate and the diffusion coefficient can be calculated as the Peclet number [59]. In systems with a Peclet number larger than 1 solutes enrich at the surface [59]. This leads to a shell formation and hollow particles. These can be hollow solid spheres if the shell becomes rigid quickly or collapsed, wrinkled particles if the shell is unstable (Figure 8b+c).

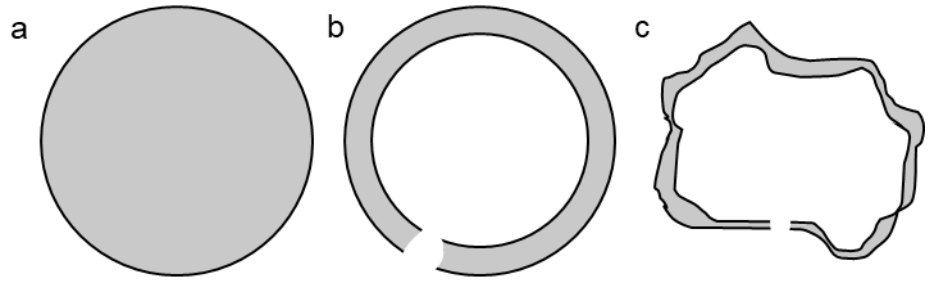

Figure 8: Schematic representation of particle morphologies: a) solid particle, b) hollow sphere with blow hole, c) collapsed hollow particle with blow hole

Often the shell contains blow holes caused by the solvent evaporating from the droplet core. In systems with a Peclet number lower than 1, the solutes remain evenly distributed in the droplet during evaporation and a solid particle forms (Figure 8a) [59].

The size of the particle highly depends on the choice of the nozzle. Furthermore, an increase in the concentration of the solids in the spray feed leads to larger particles and an increase in the spray gas flow to smaller droplets and therefore smaller particles.

3.5.1.2 Stress Factors During Spray Drying

Spray drying exposes proteins to different stress factors that can for example lead to denaturation or aggregation. The stress factors and their intensity depend on the phase of spray drying and the chosen parameters (Figure 9).

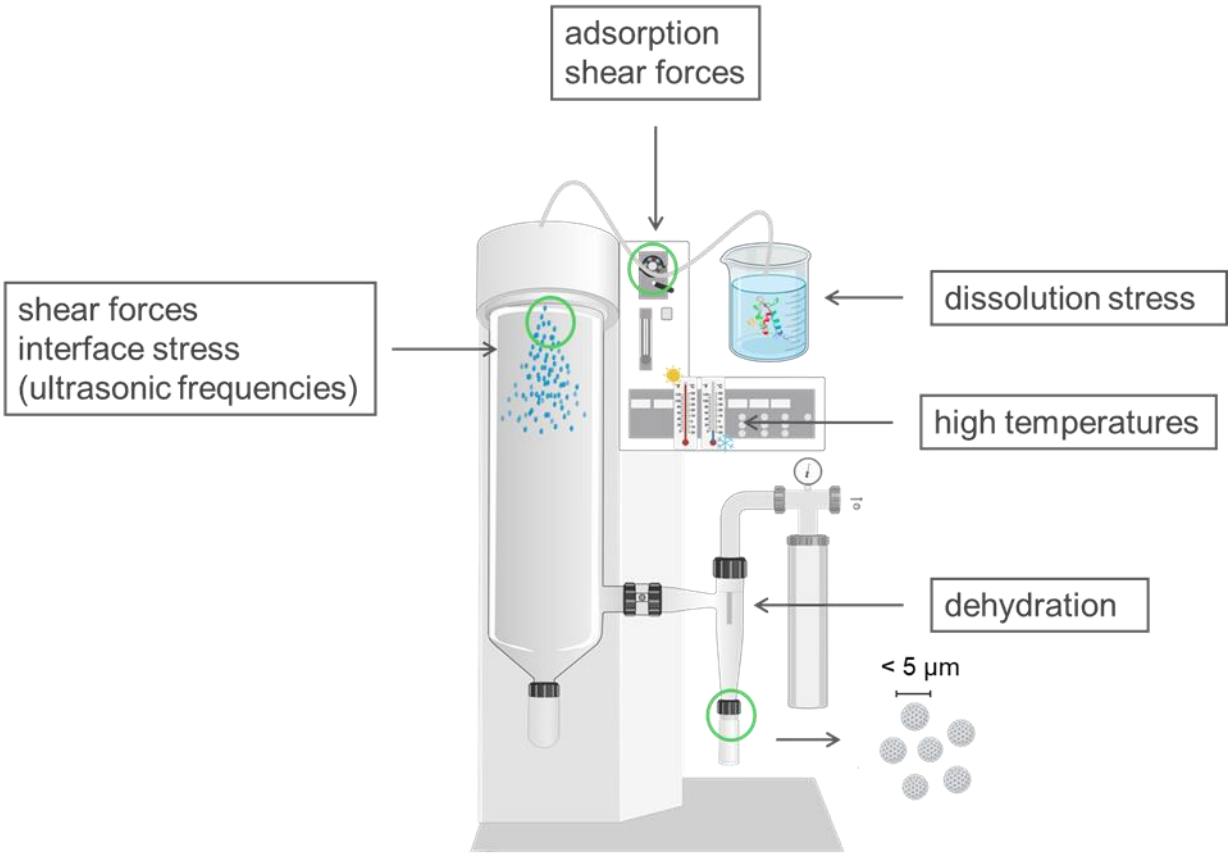

Figure 9: Stress factors for protein drugs during spray drying; created with BioRender

Before spray drying, the protein must be dissolved in the liquid feed media, usually water or an aqueous buffer. This can already lead to structural or physical changes. Especially, dissolving higher protein concentrations can lead to a challenging high viscosity or protein aggregation due to the increasing protein-protein interactions [60]. Consequently, choosing the right concentration and dissolution conditions like stirrer velocity and temperature is essential.

The first phase of the spray drying process is the pumping process. A peristaltic pump pumps the liquid feed through a tubing system towards the spray nozzle. Some spray dryers, such as the nano spray dryer (see Section 5.1.2), pump the solution through the spray head in a circle and expose it to additional thermal stress. The most common tubing material in all spray drying systems is silicone. The material exposes proteins to adsorption stress due to its high hydrophobicity [61]. As proteins in their native state tend to have the hydrophilic amino acid facing the outside and hydrophobic amnio acids the inside of the molecule, especially, partially unfolded protein molecules are prone to adsorption [11]. Adsorption of native proteins is, however, possible as well. The surface tension of the adsorption material can initiate structural changes in the adsorbed protein population, like aggregation, which might influence the structural integrity of the overall protein population [11]. The sensitivity to interfacial stress highly depends on the surface properties (e. g. hydrophobicity) and the overall structural stability of the specific protein [11].

In the following phase, the spray nozzle atomises the liquid feed into small droplets. This exposes the protein to high shear-related stress in combination with air-liquid interfaces [62]. As described above proteins tend to accumulate at interfaces which can cause aggregation. This also applies to the surfaces of the aqueous solution droplets, where the ambient air is more hydrophobic than the solution. The intensity of the interface stress, therefore, highly depends on the sizes of the produced droplets. Smaller droplets come with a larger overall surface, leading to an exposition of more protein molecules to the interface. Depending on the nozzle ultrasonic sound can occur as well. The intensity of the atomisation stress highly depends on the chosen nozzle and the process parameters, like spray rate, ultrasonic frequency or flow rate of the atomisation gas flow.

Last, in the actual drying phase the heated airflow dries the droplets into particles which are then separated from the airflow in a cyclone or an electric field. During this step, additional air-liquid-interface stress may destabilise the protein. The intensity of this stress highly depends on the chosen parameters. Furthermore, the protein is highly exposed to dehydration stress due to the loss of hydrogen bonds and different intensities of thermal stress depending on the chosen inlet- and outlet temperature [15]. In the drying droplet, also the protein concentration increases with increasing evaporation. The actual temperature the protein in the drying droplets is exposed to can, however, be lower than the outlet temperature due to evaporation cooling. Additionally, the temperature inside the droplet in a real spray dryer is also influenced by interactions with other droplets [59] or parts of the spray dryer and the temperature inside the spray dryer varies depending on the position.

The different phases and techniques of spray drying cause a variety of stress factors and their intensities (Figure 9). This may lead to a need for different protein-stabilising excipients. Therefore, investigating each of the different phases and systems comparably is necessary to optimise the production of protein drugs via spray drying.

3.5.1.3 Stabilising Excipients

Various stress factors can cause structural changes in proteins during spray drying. Stabilising the protein, therefore, requires considering all necessary parameters and finding the most suitable ones. Furthermore, several excipients are known to stabilise proteins during spray drying (Table 4).

Table 4: Excipient classes frequently used for stabilisation of proteins during spray drying

Excipient class	Addressed stress factor during spray-drying	Stabilising mechanism	Example suitable for inhalation
Sugars/ sugar alcohols	Thermal/ dehydration stress	Replacing molecular water/ steric hindrance?	Mannitol
Amino acids	Not defined	hydrogen bonds/ filling holes in the molecular structure?	Glycine
Surfactants	Interfacial stress, minimise aggregation in solution	Higher tendency of adsorption → replacing proteins from interfaces	Polysorbate 80
Salts and buffering agents	pH changes	Stabilisation of the pH optimal for the protein	Calcium carbonate
Excipient proteins	Interfacial stress	Higher tendency of adsorption → replacing proteins from interfaces	None approved
Others: further polymers, lipids, glycerol	Not defined	Diverse	None approved

Sugars and sugar alcohols are known to be good stabilisers against thermal or dehydration stress [15]. The phenomenon also occurs in nature, where some organisms utilise increased concentrations of sugars or sugar alcohols to safeguard proteins from freezing or desiccation [63]. The effect was then adapted for protein stabilisation during lyophilisation and is

now well-established in the biopharmaceutical industry [63]. This stabilising effect probably relies on the formation of hydrogen bonds between the sugar or sugar alcohol and the protein. The sugars or sugar alcohols are thereby replacing the molecular water which may be bound within the native protein by hydrogen bonds and causing a steric hindrance to the unfolding of the protein structure [15]. Reducing sugars can undergo a Maillard-reaction with the protein and thereby change the proteins´ structure [15]. These sugars should be used carefully. During the spray drying process sugars and sugar alcohol mainly stabilise the proteins against the stress factors during the drying stage.

The most used sugar alcohol in studies as well as in commercial products is mannitol [52]. Studies showed its stabilising effect on peptide hormones in spray drying formulations and it was also used in the formulation of Exubera® [52]. Nevertheless, some studies found a negative impact of mannitol crystallisation during spray drying when used alone or in higher concentrations. This led to a destabilising effect during the storage of certain antibodies [64]. To avoid a protein destabilising crystallisation a combination with other excipients seems to be possible. In Exubera®, for example, the formulation was specially designed to stabilise the protein in an amorphous glass [65].

Trehalose has the best stabilising effect of the disaccharides and is a widely used excipient during spray drying [15]. Studies suggest that this phenomenon relies on a superior ability to form hydrogen bonds with the proteins compared to other sugars. Furthermore, sucrose, lactose and melibiose have been frequently studied, although lactose and melibiose are reducing sugars and may undergo a Maillard-reaction with the protein [52]. Some oligosaccharides, like cyclodextrins or polysaccharides, like inulin, stabilised proteins during spray drying [52]. Polysaccharides might also cause additional effects like mucoadhesion or release modification, which must be kept in mind [52].

Optimal stabilising sugars or sugar alcohols should form as many interactions with the protein as possible, be miscible with the protein on a molecular level, have a low crystallisation tendency and be non-reducing [15]. Nevertheless, therapeutic proteins can vary enormously in their structure and stability and degradation pathways are highly complex. A general stabilisation recommendation or choice in excipient sugars is therefore not possible. Furthermore, other requirements for the excipients depending on the application route and the formulation have to be considered.

For inhalation, only mannitol and lactose are FDA approved as well as the polysaccharide carrageenan [66]. Lactose is a reducing sugar and might undergo a Maillard-reaction causing protein degradation. Carrageenan is not one of the well-investigated or preferred polysaccharides for protein stabilisation [52]. Mannitol, being well-investigated for inhalation as well as for protein stabilisation, seems to be the most suitable excipient of the group of sugars and sugar alcohols.

Amino acids have a good effect in protecting the protein structure. Although this effect has been widely studied, the mechanism behind it is not fully understood. The formation of hydrogen bonds or filling holes in the molecular structure and thereby being a steric hindrance to structural changes seems possible [52]. Studies investigated several amino acids as stabilising excipients during spray drying. Arginine, histidine and glycine showed protein stabilising effects, that often increased in combination with sugars [52]. This is probably due to a synergistic effect of the different stabilisation methods. Also, combinations of different amino acids increased the protein stabilising effect [67]. Some amino acids also have buffering effects during the spray drying process as well as technological advances, e.g. the improvement of the aerodynamic performance by L-leucine [68].

Glycine is the only amino acid FDA-approved for inhalation [66]. It was already used as a stabilising excipient in combination with mannitol in Exubera® [44].

Surfactants are effective stabilisers against air-liquid interface stress and can minimise the aggregation of the protein monomers in the solution. This is caused by the surfactant´s high tendency to adsorb at the interface, thereby expelling the protein from these interfaces and forming protein-surfactant complexes through hydrophobic interactions [52]. Surfactants are widely used in liquid as well as in solid protein formulations. In higher concentrations, ionic surfactants can denature proteins therefore non-ionic surfactants are preferred. Polysorbate 20, polysorbate 80 and poloxamer 188 are the most used ones for spray drying. These surfactants stabilise the protein while the spray-dried product is still an aqueous solution during the sample preparation, the pumping and the atomisation phase.

Polysorbate 80 is the only FDA-approved surfactant for inhalation [66] and is used in the formulation of Afrezza®.

A wide range of other excipients can stabilise proteins during production and storage. Several of them are investigated for spray drying.

Salts and buffering agents stabilise the pH of the feed solution before the drying phase and during concentration changes in the drying droplets. This can be useful, especially for pH-sensitive proteins. Several salts and buffering agents are FDA-approved for inhalation [66]. The increasing concentration of buffer salts during the drying process might, however, lead to a suboptimal environment for the protein´s stability.

Excipient proteins, like BSA, can stabilise against aggregation and denaturation of other proteins by protein-protein interactions [52]. Furthermore, those proteins can have a higher tendency to accumulate at surfaces and protect the drug-protein against interface stress [52]. Nevertheless, no excipient protein is FDA-approved for inhalation.

Also, several further polymers, glycerol and different lipids are frequently used for protein stabilisation during spray drying. Of the lipids, studies investigated dipalmitoyl phosphatidyl choline for inhalable protein formulations [52]. It is, however, not FDA-approved for inhalation.

3.5.2 Liquid Formulations for Nebulisation

Nebulisers nebulise liquids into inhalable droplets and can deliver high doses compared to other inhalation devices. Nebulisers can produce the aerosol by using gas flow, vibrating meshes, or ultrasonic frequencies.

Jet nebulisers are the most commonly used nebulisers [69]. They atomise the liquid drug with a gas (air) flow through a nozzle produced by a compressor or central air supply. The airflow thereby causes a vacuum in the nebuliser which leads to a flow of the drug solution towards the nozzle, following the Venturi principle. The primary spray particles are usually too large to be inhaled and therefore collide immediately with a baffle that causes further breakup by splashing [70]. This produces large and a few small droplets. The small droplets follow the gas flow while the large droplets are recycled in the drug reservoir [40]. The aerosol produced is usually cooler than the ambient temperature [70].

Ultrasonic nebulisers contain piezoelectric crystals that vibrate in ultrasonic frequencies producing large and small droplets. Here as well, the small droplets are inhaled and the large droplets are recycled in the drug reservoir [40]. These nebulisers especially show heating of the formulation during the nebulisation process which is especially challenging for heat-sensitive drugs [70].

Mesh nebulisers have several advantages compared to the other types of nebulisers. They are comparable quiet, portable, and have a high throughput rate and a low residual volume

[69]. Furthermore, they are supposed to expose the drug to low shear-related stress and have been employed for administering extremely costly and delicate medicinal compounds, such as biologics [69]. The devices use either a static or vibrating mesh for nebulisation of the liquid. In static mesh nebulisers, an ultrasonic transducer vibrates the liquid. The produced droplets are pushed through a mesh with small holes (3-6 µm). In vibrating mesh nebulisers the mesh vibrates and pumps the liquid through its small holes [40]. The droplets produced in mesh nebulisers are all within the inhalable size range [69] since the process allows close control of the droplet size [71].

Patients can inhale the droplets with a mask or mouthpiece as a fine mist with normal breathing. Traditional devices are large, need to be plugged in and can be used only at home or in hospitals [70]. Nowadays, many devices are small and portable, with batteries as their energy source [70]. With the nebuliser, the drug can be inhaled with the tidal breath and does not require the patients to actively participate, inhale in a certain way at a certain time point or have any specific lung volume [55]. Therefore, nebulisers can also be used for babies and small children, patients whose health conditions do not allow them to take action or patients with poor lung function. In addition, nebulisation is frequently required for inhaled therapies of mechanically ventilated patients in critical care [72]. Compared to other inhalation devices, the dose delivery is time-consuming, however, newer devices are becoming faster and they can easily deliver high doses to the lungs [70].

Nebuliser formulations are liquids. A protein formulation as an aqueous solution is comparably easy and cheap in production [31]. This, however, can decrease storage stability. Furthermore, the nebulisation exposes the protein to stress factors during application. During droplet production, the protein is exposed to shear-related stress factors. Numerous air-liquid interfaces occur at the large surfaces of the small droplets. The concentration in the solution remaining in the reservoir can change due to evaporation, which might interfere with the stability depending on the protein's properties. Additionally, ultrasonic frequencies occur in some devices and especially in these devices the temperature can increase during the process leading to additional thermal stress.

To increase the storage stability, storing the formulation as a dry powder and then redispersing it directly before the application might be possible and will be explored in this thesis.

3.5.3 Pressurised Metered Dose Inhaler Formulations

Pressurised metered dose inhalers are the most widely used inhalation devices for treating lung diseases, like asthma and COPD, and have been on the market since the 1950s [73].

They consist of a metal container containing the drug as liquid formulation e. g. ethanolic solutions or as suspension together with a liquefied gas propellant under pressure [55]. The metering valve inside of the devices enables the dispensation of a precise drug dose, the actuator contains the nozzle which forms the spray and defines the droplet size and the plastic mouthpiece permits the patient the oral inhalation of the drug [55]. The device uses the propellants for aerosol production. The droplets usually have a velocity higher than the velocity of the inspiratory flow. Therefore, numerous droplets impinge the throat and only a part of the drug reaches the deeper lung [34]. This might be problematic since protein drugs tend to be complicated and expensive in production. The storage as the solution comes with a decrease in protein stability. Also, a negative influence of the propellant on protein stability seems possible [31]. Currently, there is no protein-metered-dose inhaler formulation available on the market. Furthermore, the commonly used propellants have a high global warming potential [74]. Pressurised metered dose inhalers were, therefore, found to have a larger carbon footprint than DPIs and have a significant impact on the carbon footprint of the health sector [75]. Due to these well-documented environmental benefits, the equal effectiveness, the lower costs and the patient-friendly device a switch from metered dose inhalers to DPIs is recommended by some experts [75]. This, however, requires the patient to have a certain lung volume and might not be suitable for emergency therapy e. g. during an asthmatic attack.

4 Materials

4.1 Proteins

In the experiments, different model proteins were used. All proteins were commercially available as lyophilised powders. The proteins were used in the experiments depending on their analytical properties and their sensitivity to the different stress factors.

4.1.1 Bovine Serum Albumin

Bovine Serum Albumin (BSA) is the blood serum albumin of cattle with a molecular weight of 66.4 kDa and consisting of 583 amino acids. The isoelectric point is 5.1 to 5.5 [76, 77]. Due to its low cost and broad availability, it is a well-established model protein. Furthermore, it can be used as an excipient protein to stabilise the actual protein drug (see Section 3.5.1.3).

BSA was used as an off-white to yellow lyophilised powder with a purity of ≥96% (agarose gel electrophoresis) (Merck KGaA, Darmstadt, Germany).

4.1.2 Chymotrypsin

Chymotrypsin is a serine peptidase consisting of three polypeptide chains with a molecular weight of 25 kDa, consists of 241 amino acids and has an isoelectric point of 8.75 [78]. The protein is most stable at pH 9 and most active at pH 7.5-8 [79]. At a pH lower than 5.5 it forms an inactive dimer and denatures under a pH of 3.

Chymotrypsin was used as a lyophilised off-white powder with a minimum purity of 85% with an enzyme activity of >40 u/mg (Merck KGaA, Darmstadt, Germany). One unit hydrolyses 1.0 µmole of benzoyl-L-tyrosine ethyl ester (BTEE) per minute at pH 7.8 and 25 °C.

4.1.3 Insulin

Insulin is a peptide hormone with 51 amino acids in two side chains linked with disulphide bridges, a molecular weight of 5.8 kDa and an IEP of 5.3 [80]. It is soluble in a pH from 2.0 to 2.5 [81]. Insulin is well-established in the treatment of all types of diabetes mellitus. The common application is the subcutaneous injection with an insulin pen or injection with an insulin pump. It was, however, the first protein drug approved for inhalation (see Section 3.4.3.1).

Insulin was used as a lyophilised off-white powder (Merck KGaA, Darmstadt, Germany).

4.1.4 Lysozyme

Lysozyme is an antibacterial enzyme and part of the innate immune system of mammals. It hydrolyses glycosidic bonds in peptidoglycans. Therefore, it is especially effective against gram-positive bacteria due to the high amount of peptidoglycan in the cell walls [82].

Lysozyme has a molecular weight of 14.3 kDa, consists of 129 amino acids and has an isoelectric point of 11.35 [82]. The enzyme is active over a pH range of 6.0 to 9.0 [82].

This thesis used lysozyme from chicken egg white as a lyophilised powder with a purity of ≥90 % and enzyme activity of ≥40,000 units/mg. One unit is defined as a ΔA_{450} of 0.001 per min at pH 6.24 at 25 °C in a suspension of *Micrococcus lysodeikticus* as substrate in a 2.6 mL reaction mixture (1 cm light path). Lysozyme was procured from Merck KGaA, Darmstadt, Germany for the stability experiments. Due to supply difficulties, the lysozyme for the other experiments was procured from Carl Roth GmbH + Co. KG, Karlsruhe, Germany.

4.1.5 Ovalbumin

Ovalbumin is the chicken egg´s albumin and the major protein component of the egg white. It is a phosphorylated glycoprotein with an amino acid sequence consisting of 385 residues and a molecular weight of 42.7 kDa [83]. The isoelectric point is 4.54 [83].

It was used as an off-white lyophilised powder with a purity of ≥98% (agarose gel electrophoresis) (Merck KGaA, Darmstadt, Germany).

4.2 Excipients

4.2.1 Mannitol

Mannitol ($C_6H_{14}O_6$) is a hydrophilic and non-reducing sugar alcohol. It is highly water-soluble and can be obtained from plants, algae or fungi as well as industrial synthesis. It is a well-known and widely used excipient. Being approved for inhalation [66] and suitable for spray drying it is also a suitable excipient for DPIs. As a non-reducing sugar alcohol, it will not undergo a Maillard-reaction with the protein [15]. It can, therefore, easily be used for protein stabilisation. This stabilising effect was already used in spray-dried peptide-hormone-formulations like Exubera®, Trelstar® LA, and Somatuline®. Nevertheless, some studies with monoclonal antibodies found a negative influence on the stability caused by the crystallinity of the co-spray-dried mannitol (see Section 3.5.1.3).

Furthermore, mannitol is used as an active ingredient to liquidise the sputum in the lungs of cystic fibrosis patients [84].

It was used as the spray-agglomerated mannitol Parteck® M DPI (Merck KGaA, Darmstadt, Germany) which is an inhalation-grade excipient.

4.2.2 Glycine

Glycine is the smallest and simplest proteinogenic amino acid with only a hydrogen atom as a side chain and a molecular weight of 75.07 g/mol. It has no asymmetric centre and is therefore not chiral. The carboxyl group has a pKa of 2.34 and the protonated amino group has a pKa of 9.60.

Glycine is a well-established protein-stabilising excipient that has also been studied in several spray drying experiments. It is the only amino acid FDA-approved for inhalation [66].

It was used as a white powder (Merck KGaA, Darmstadt, Germany).

4.2.3 Polysorbate 20 and Polysorbate 80

Polysorbate 20 and polysorbate 80 are non-ionic hydrophilic surfactants with an HLB value of 16.7 and 15.0. Both are well-investigated excipients in spray drying studies for protein stabilisation against interface stress (see Section 3.5.1.3). Both excipients are FDA-approved for several application routes, including nasal and parenteral applications [66]. Nevertheless, only polysorbate 80 is FDA-approved for inhalation [66].

Due to their amphiphilic character polysorbate 20 and polysorbate 80 can directly interact with the proteins and most fluorescence dyes. This interferes with most of the used analytics and therefore minimises the use of polysorbate 20 and polysorbate 80 in this work.

Polysorbate 20 and polysorbate 80 were used as yellowish-brownish liquids with the brand names Tween® 20 and Tween® 80 (Croda International, Snaith, United Kingdom).

4.2.4 Lactose

Lactose is a widely used excipient in the pharmaceutical industry. In inhalable formulations, it is one of the most established excipients for dry powder formulations. Several DPI products use it as a carrier material [56].

As a reducing sugar, however, it can undergo Maillard-reactions with proteins and is, therefore, not suitable as a stabilising excipient. It was used as a comparison to mannitol as excipient sugar/ sugar alcohol.

This study used lactose in the form of InhaLac® 230 a white powder carrier material and an inhalation-grade excipient (Meggle Group, Wasserburg am Inn, Germany).

All further materials with their quality and source are summarised in Table 30 (Annex).

4.3 Inhalation Devices

4.3.1 Nebulisers

Nebulisation can be used to administer APIs to patients who are, e.g., due to their health conditions, unable to use DPI devices. Jet-, ultrasonic- and mesh nebulisers exist (see Section 3.5.2).

Each type exposes the protein to different stress factors and it is likely, that they influence the protein structure differently. Therefore, the nebulisation experiments investigated one nebuliser of each type. The eFlow® *rapid* (Pari GmbH, Starnberg, Germany) is a membrane nebuliser with a short nebulisation time. The inhalation device can be used plugged in as well as battery-powered and is comparably quiet. The device is small, lightweight and therefore portable. The TurboBoy® (Pari GmbH, Starnberg, Germany) is a jet nebuliser that nebulises with compressed air. The Multisonic® (Otto Schill GmbH & Co KG, Probstzella, Germany) uses ultrasonic frequencies to nebulise liquid formulations. Both the TurboBoy® and the Multisonic® are comparably large plugged-in devices and are not portable.

4.3.2 Dry Powder Inhaler

The RS01® (Plastiape, Onsago, Italy part of the Berry Global Group Evansville, Indiana USA) is a dry powder inhaler based on single-dosed capsules in capsule size 3. This thesis used the device with an airflow resistance of 40 mL/min. To deliver the powder, the patient has to place the capsule inside the device, close it and press the two buttons at the device´s side containing the needles puncturing the capsule. Patients can then inhale the powder with their breath.

5 Preparative Methods

5.1 Spray Drying

Different experiments were performed to characterise the spray drying process's influence on the proteins. The proteins were therefore dissolved in a 4.0 mg/mL concentration in water and then processed by spray drying under different parameters and with and without the addition of excipients.

5.1.1 Mini Spray Dryer

The Mini Spray Dryer B-290 (Büchi Labortechnik AG, Essen, Germany) (mini spray dryer) is a well-established laboratory-scale spray dryer (Figure 10).

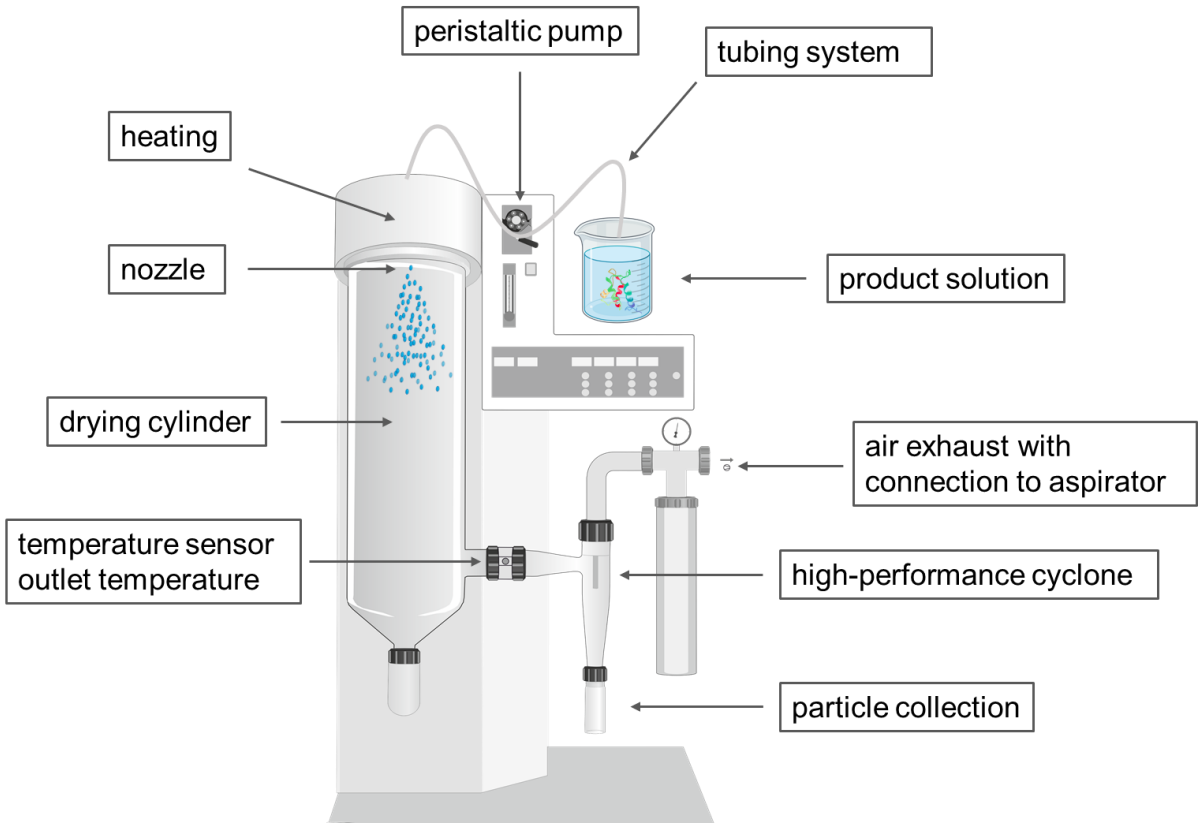

Figure 10: Schematic overview Mini Spray Dryer B-290

It can be operated with different nozzles. This work used the two-fluid nozzle with a diameter of 0.7 mm and the ultrasonic nozzle (Figure 11).

Figure 11: Spray drying nozzles used for the mini spray dryer in this work; two-fluid nozzle (left), ultrasonic nozzle (right)

The two-fluid nozzle in the mini spray dryer atomises the solution via airflow and leads typically to a particle size of 2-25 µm [85]. The ultrasonic sound nozzle uses an ultrasonic frequency for atomisation. It produces larger droplets leading typically to a particle size of 10-60 µm [86]. Those larger droplets need a longer drying period, so the airflow velocity needs to be lower. This exposes the protein longer to thermal and dehydration stress. It also results in lower outlet temperatures than spray drying with the same inlet temperature and the same spray rate with the two-fluid nozzle and, therefore, lower thermal stress. The spray-dried particles are then separated from the airflow using a high-performance cyclone and collected.

5.1.2 Nano Spray Dryer

The Nano Spray Dryer B-90 HP (Büchi Labortechnik AG, Essen, Germany) (nano spray dryer) is a laboratory-scale spray dryer (Figure 12).

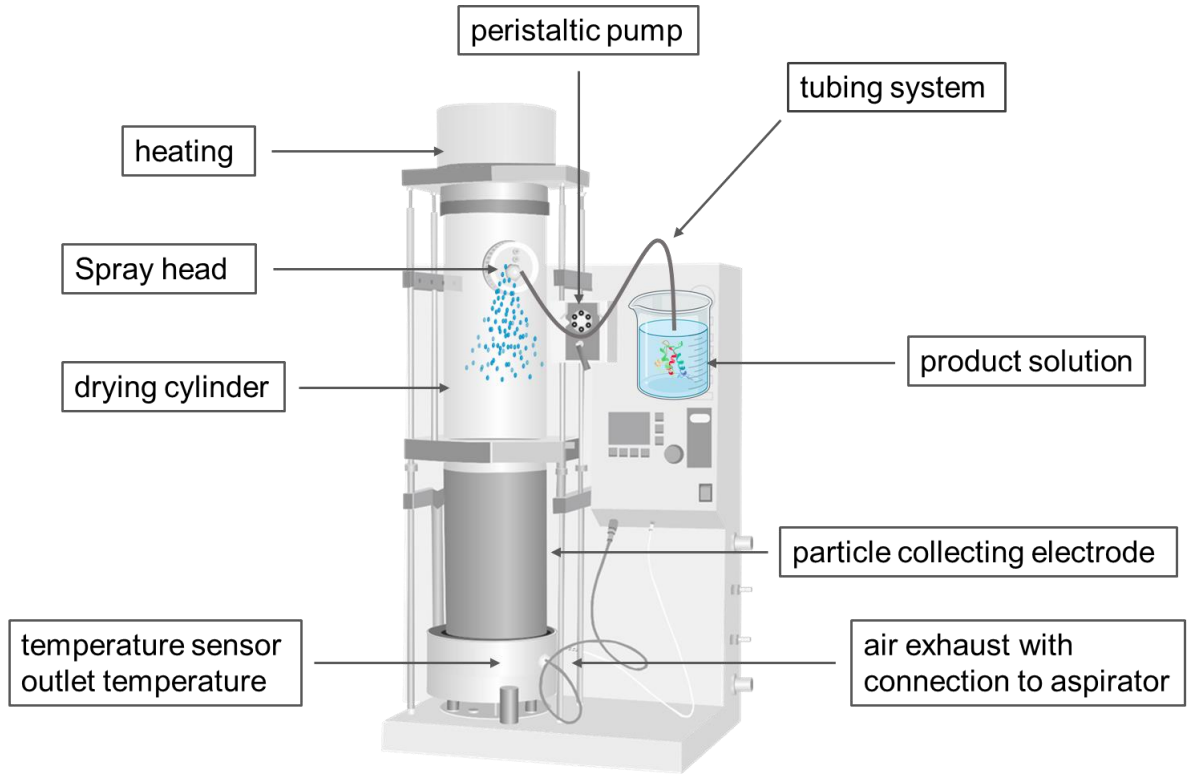

Figure 12: Schematic overview of the Nano Spray Dryer B-90 HP

It is specially designed for the production of very small particles and very low amounts of product. The system pumps the solution in a circle through the tubing material and the spray head (Figure 13), which shortly exposes the protein to thermal stress.

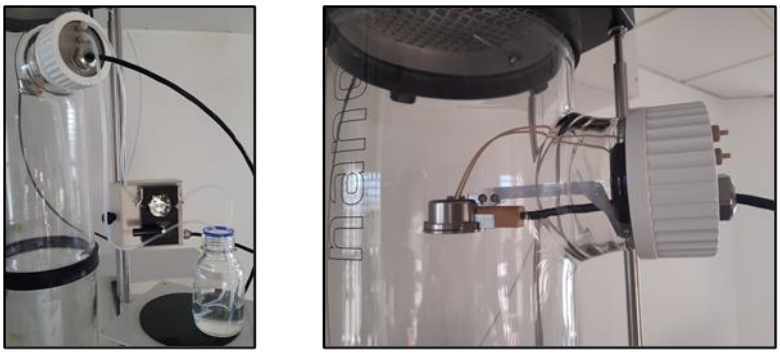

Figure 13: Spray head inside the nano spray dryer; including pumping system (left), only spray head (right)

This can be an additional stress factor to the interfacial stress during the pumping process. The nano spray dryer uses a piezoelectric spray head (Figure 13), which nebulises via a membrane with an ultrasonic frequency for atomisation. Last, the collecting electrode collects the spray-dried particles. The typically produced particles have a size of 0.2-5 µm [87].

Both systems have been used for the production of various samples of different compositions. A comprehensive summary of all spray-dried batches is given in Table 31, Table 32, and Table 33 (Annex).

6 Analytical Methods

6.1 Dissolution Behaviour

6.1.1 Dissolution Velocity

The Franz cell dissolution method is a method to detect changes in the dissolution velocity through a moistened membrane (Figure 14).

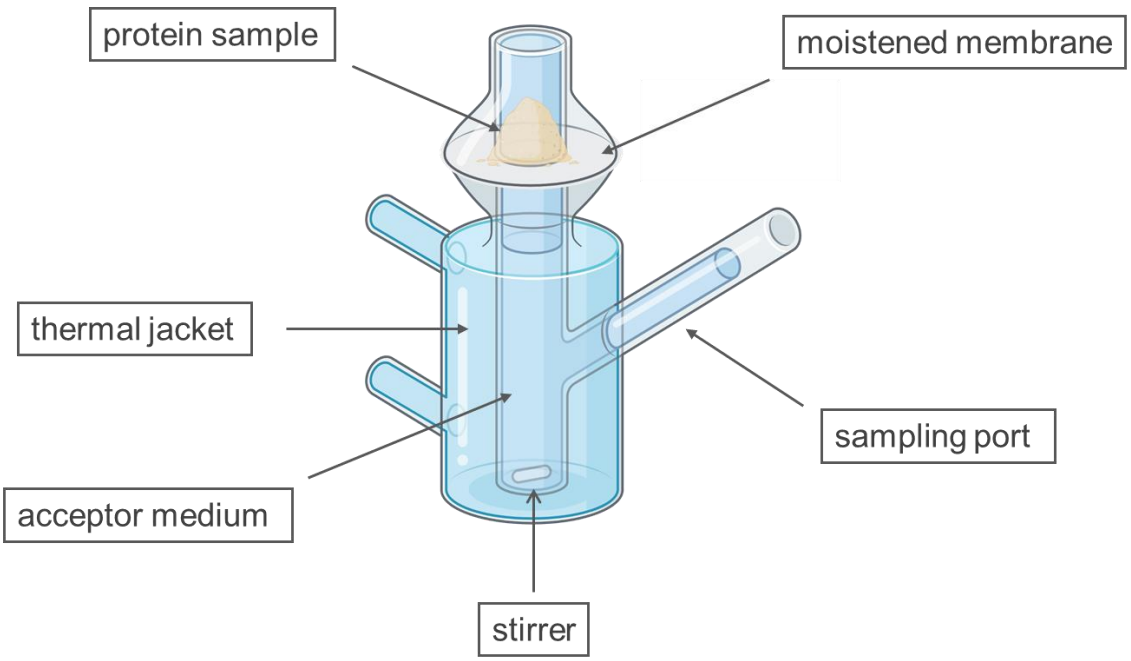

Figure 14: Franz diffusion cell; created with BioRender

The Franz cell consists of a donor compartment and an acceptor compartment. The last contains an acceptor medium in a thermal jacket and a moistened membrane as a barrier between the compartments. The sample is placed on top of the moistened membrane in the donor compartment and therefore, does not have direct contact with the medium. It allows a slow dissolution since the sample is only moistened by the medium that penetrates through the membrane and is, therefore, only exposed to a small amount of dissolution medium. This makes the detection of dissolution behaviour changes in protein dry powders simpler since they tend to dissolve too fast to measure when exposed to larger amounts of dissolution medium.

This set-up was used to compare the dissolution velocity of ovalbumin dry powder samples before and after spray drying in this work. The measurements were performed at a temperature of 37 °C ± 0.5°C, 7.9 mL mQ water as acceptor medium, a cellulose acetate membrane

(0.45 µm) and a sample mass of 50 mg ± 5 mg. 0.2 mL samples were taken every 6 min from the acceptor compartment for 90 minutes and immediately replaced with 0.2 mL of mQ water. Triplicates of each sample were measured three times and the concentration in the acceptor medium was measured via UV/Vis using the NanoDrop (see Section 6.5.3).

The dissolution profiles were compared using the similarity factor f_2. In the following equation (Equation 1), R_t is the dissolution value of the reference batch at time t and T_t is the dissolution value of the test batch at time t.

$$f = 50 * log(1 + \frac{1}{n} * \sum_{t=1} n * (R_t - T_t)^2) - 0.5 * 100$$

Equation 1: Similarity factor f_2

Values over 50 show equivalence of the dissolution profiles whereas values under 50 show differences.

6.1.2 Solubility

Due to the very high solubility of used model proteins and the increasing viscosity in the aqueous solution, the solubility could not be measured directly in water. Low salt concentrations increase the solubility. High concentrations of certain salts, like ammonium sulphate, however, decrease the solubility of proteins in water by precipitation [88]. This effect is generally referred to as salting-out. This work used this effect to measure the solubility of the dry powder ovalbumin samples. It used a 33.3% ammonium sulphate aqueous solution where the salt precipitated this protein. The concentration of the salt was chosen due to preliminary experiments (not shown) where other salt concentrations tested were not able to precipitate ovalbumin. Therefore, 1 mL of the salt solution was added to 50 mg of each sample and shaken for 72 h. The samples were prepared in triplicates. The concentration of each solution was determined via UV/Vis using the NanoDrop (Section 6.5.3).

6.2 Physicochemical Characterisation

6.2.1 Contact Angle

To detect changes in the hydrophilicity, the wettability of the protein powder was determined via contact angle measurement of water drops. Angles < 90° thereby show a favourable wettability. Increasing contact angles indicate an increase in hydrophobicity. With the hydraulic press, PW10 (Paul-Otto Weber GmbH, Remshalden, Germany) at a force of 20 kN for 1 min comprimates of the protein powders were produced. The contact angle of the water drops at one comprimates of each sample type was then measured seven times with the Goniometer type G1 (Krüss GmbH, Hamburg, Germany) (Figure 15).

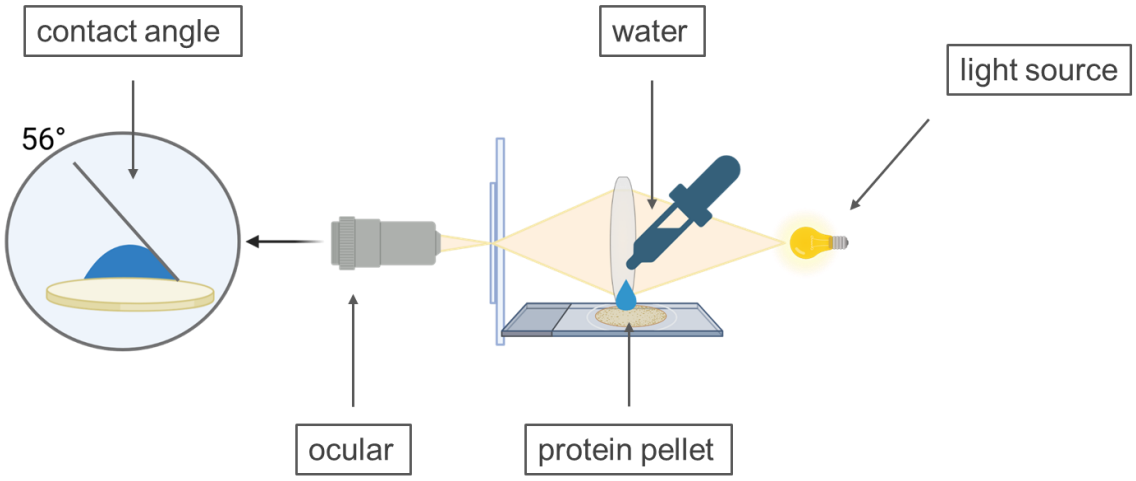

Figure 15: Schematic illustration of the contact angle measurement at the goniometer; created with BioRender

6.2.2 Surface Tension

The interface activity of the proteins was compared by measuring the surface tension of aqueous protein solutions with a concentration of 4 mg/mL. Furthermore, the influence of the excipient addition was investigated. The surface tension was measured with the Tensiometer K12 (Krüss GmbH, Hamburg Germany) using the standard measuring probe for the Du-Noüy-ring method. Measurements were performed three times for each sample at room temperature.

6.2.3 Particle Morphology

The particle morphology of the spray-dried particles was visualised with scanning electron microscopy (SEM). This microscope guides finely bundled electron beams over the sample in a specific pattern. The interaction of these electrons with the material causes a specific pattern of the backscattered electrons which is detected and creates the image of the sample. The measurement is performed under a vacuum to avoid interactions with air molecules.

The samples were first attached to an aluminium stub with double-sided carbon tapes and then coated with a thin gold layer (BAL-Tec SCP 050 Sputter Coater, BAL-Tec, Pfäffikon, Switzerland). The Phenom World XL (Phenom-World BV, Eindhoven, The Netherlands) scanning electron microscope uses an acceleration voltage of 10 kV, a vacuum of 10 Pa and a spot size of 3.3 nm for particle visualisation.

6.2.4 Crystallinity

Possible changes in the crystallinity of ovalbumin were detected in diffractograms of one sample of the lyophilisate and one sample of the protein spray-dried with the two-fluid nozzle. X-ray powder diffraction was performed using a Mythen 1K-detector Stadi-Ps (STOE & CIE GmbH, Darmstadt, Germany) with the following parameters: Mo-Ka1-radiance, 2-35° 2 Theta, 60 sec/step. It detects the differences between crystalline and amorphous powders. While the diffractograms of crystalline substances show clear peaks amorphous substances scatter diffuse and gives no clear signal.

6.2.5 Particle Size

The HELOS system with the RODOS module (Sympatec GmbH, Clausthal-Zellerfeld, Germany) determines the geometric diameter of particles using laser diffraction. The particles of the powders dispersed with pressured air at 3 bars thereby scattering the monochromatic laser light leading to an interference pattern. Based on this the Paqxos 5.2.1 software (Sympatec GmbH, Clausthal-Zellerfeld, Germany) calculates the particle size based on the Fraunhofer theory. It shows the particle size distribution as a cumulative curve and the x_{10}, x_{50} and x_{90} values. X_{10}, x_{50} and x_{90} mean that 10%, 50% or 90% of the particles have a smaller diameter than the value x. The mean of the x_{10}, x_{50} and x_{90} values of three particle size measurements was used to compare spray-dried powders. Measurements were taken three times with lens 3 for the smaller particles and lens 5 for the larger particles. The span value was used to evaluate the width of the particle size distribution: the larger the span, the broader the distribution and vice versa. It can be calculated using Equation 2.

$$Span = \frac{x_{90} - x_{10}}{x_{50}}$$

Equation 2: Calculation of the span value

6.3 Aerodynamic Behaviour

Cascade Impactors are generally used as an in-vitro method to characterise the aerodynamic performance of inhalable drugs. Impactors can be used to analyse dry powder as well as liquid formulations. The principle relies on an air stream's particle or droplet size classification. Each stage of the cascade impactor comprises nozzles through which a fixed volume of air flows [89]. As the size of the nozzles decreases, the flow rate increases, which allows the separation of particles or droplets based on their aerodynamic size [89]. While larger particles are more prone to being impacted smaller particles can travel a greater distance and are only separated at higher air velocities. Due to the lack of physiological conditions as in the human lung, they cannot be considered lung models.

6.3.1 Fast Screening Impactor

The Fast Screening Impactor (FSI) (Copley Scientific Limited, Nottingham, UK) separates the dose of DPI formulations by the aerodynamic particle size. For a correct separation, the airflow must be set to the flow rate defined for each insert plate and inhalation device. In this work, the flow rate was set to 40 L/min, leading to a collection time of 6 sec for a total air volume of 4 L. The measurement was performed three times with five capsules each containing 10 mg of the spray-dried product. Particles with a not inhalable aerodynamic particle size impact in the corpus and the throat. Particles with an aerodynamic diameter smaller than 5 µm can pass the holes of the insert plate and are collected in the glass fibre filter. For sample collection, water was added to the different stages (5 mL each to the throat with the mouthpiece, the capsules and the inhaler, 10 mL to the filter and 15 mL to the corpus). The protein concentration was then determined using size exclusion chromatography (SEC) (see Section 6.5.3).

6.4 Collection of the Nebulised Samples

Nebulisation is a widely used inhalation method (see Section 3.5.2). It is also suitable for protein drugs. Nevertheless, it exposes the protein to different stress factors. First, the drug must be either a liquid formulation or redispersed before nebulisation. Usually, those formulations are aqueous liquids in which proteins in general tend to be less stable. The nebulisation process exposes the protein to enormous air-liquid-interface stress and shear-related stress while producing very small droplets in the inhalable size range. Also, thermal stress can occur as some nebulisers produce heat during the processing time [90]. In ultrasonic nebulisers, the ultrasonic frequencies can be another stress factor.

In the nebulisation experiments, the chymotrypsin lyophilisate or the chymotrypsin DPI formulation was redispersed to a 4.0 mg/mL protein concentration in water. Each nebuliser was filled with 5 mL of the protein solution for nebulisation and turned on for 4 min (eFlow® rapid) or 6 min (TurboBoy® and Multisonic®). The nebulised samples were then collected for analysis (see Section 6.4.1).

6.4.1 Next Generation Impactor

The Next Generation Impactor (NGI) (Copley Scientific Limited, Nottingham, UK) is a cascade impactor consisting of seven stages with decreasing nozzle size and a terminal micro-orifice collector (MOC). In this work, it was mainly used for the collection of aerosols produced by nebulisers to investigate the protein structure changes (Figure 16).

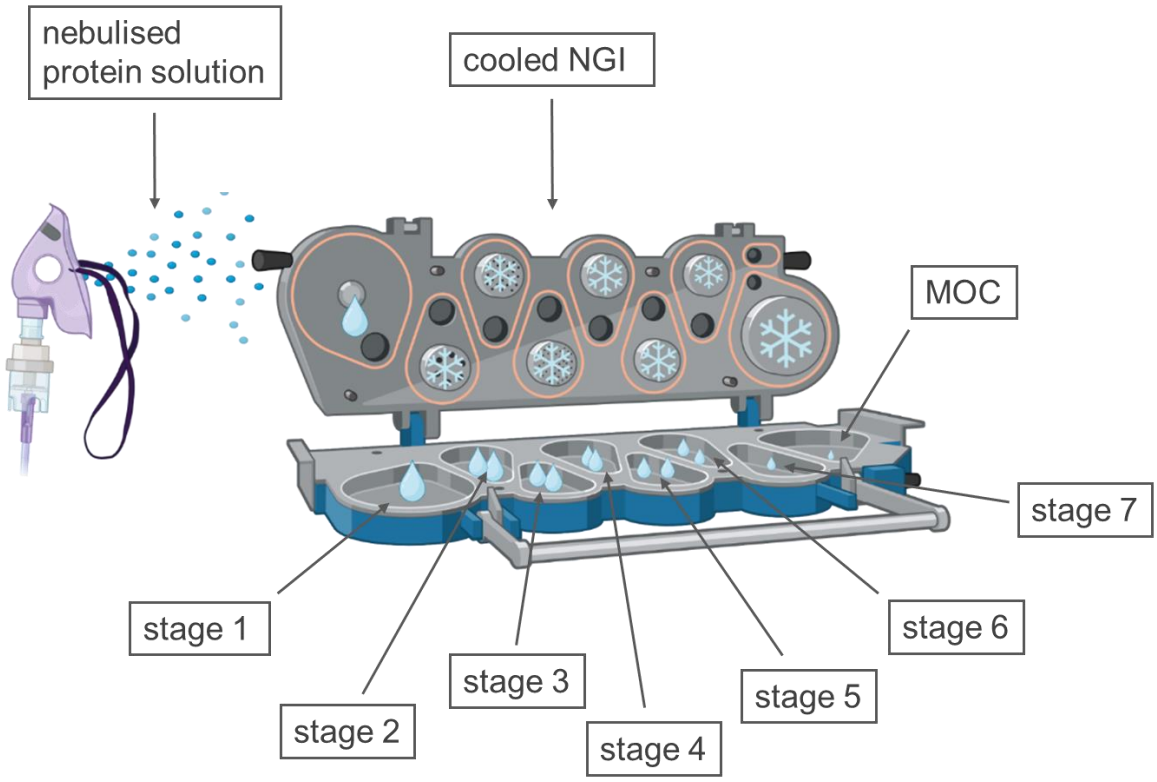

Figure 16: NGI as a collection device for nebulised protein solutions; created with BioRender

A method, close to the one described in the Ph. Eur 2.9.44 [91], was performed for the collection step. Therefore, the NGI was cooled at 2-8°C for 90 min, the airflow was set to 15 L/min and the collection was started immediately. The nebuliser was connected to the NGI using the standard introduction port and individualised mouthpiece. The collection time was set experimentally to receive enough material for the analysis depending on the nebuliser's performance. Furthermore, only stages containing enough solution (>0.1 mL) for protein stability analysis were considered for analysis. Depending on the nebuliser, some stages did not contain (enough) solution and were, therefore, not included in the measurement.

6.4.1.1 Determination of the Concentration of Brilliant Blue and Mannitol

The brilliant blue concentration was determined via UV/Vis using the NanoDrop (see Section 6.5.3) at a wavelength of 540 nm. The concentrations of the samples were calculated using a calibration curve with seven data points between 0.0144 mg/mL and 0.048 mg/mL and an R^2 of 0.9997. The concentration of the mannitol solution was determined using the freezing point osmometer (Osmomat® 3000 Basic, Gonotec GmbH, Berlin Germany). This measures the freezing point depression as a colligative property and calculates the osmolarity. Using the calibration curve with eight data points between 0.0026 mg/mL and 0.078 mg/mL and an R^2 of 0.997 the mannitol concentration of a solution can be calculated.

6.5 Protein Analytics

6.5.1 Native and Denatured Protein Samples

First, the starting material was dissolved in mQ water at a concentration of 4.0 mg/mL. In the following, these samples were referred to as “native samples”. Nevertheless, no further purification steps were performed. Therefore, there might be a minor part of degradation contamination in these samples.

Second, the material was dissolved in mQ water to a 4.0 mg/mL concentration and then heated at 100°C for 3 h in a closed centrifuge tube. These samples were referred to as “denatured samples”. Although theoretically further degradation might be possible the samples showed the highest degraded protein structure measured with the following methods and were, therefore, defined as fully denatured.

In several experiments, the produced samples were compared to the native and the denatured protein reference samples.

6.5.2 Size Exclusion Chromatography for the Detection of Structural Changes

Size exclusion chromatography (SEC) is a common analytical technique to measure the size distribution of protein samples [92]. The separation mechanism relies on the different hydrodynamic radii. The SEC column´s material consists of cross-linked porous polymer microspheres with pores of defined sizes. Proteins with a smaller hydrodynamic size penetrate more of the pores while proteins with a larger hydrodynamic radius penetrate fewer pores. Larger species, therefore, eluate faster and are detected earlier in the SEC. The hydrodynamic size is highly influenced by the molecular weight but also by the protein’s shape and dissolution properties [92]. Elongated proteins eluate before globular proteins with the same molecular weight. Furthermore, the method can detect differences in the integrity of the protein structure (Figure 17).

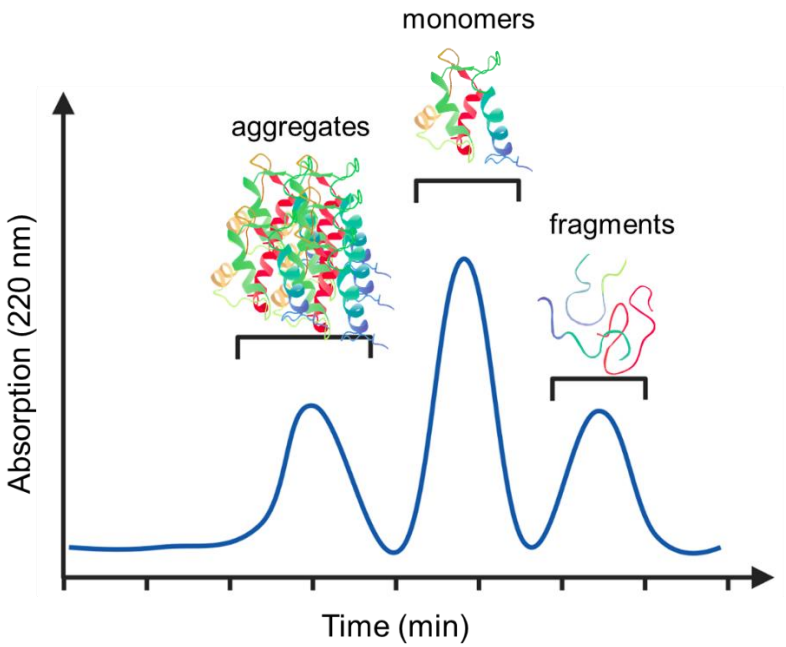

Figure 17: Schematic SEC chromatogram of protein species; created with BioRender

Larger aggregates can elute before the smaller monomer. However, the changing molecular weight of protein aggregation can be masked by the changes in the shape of the proteins. Fragments of degraded proteins elute after the monomer. Those fragments can be of various sizes and therefore might not occur in one clear peak. Peak detection is possible at a wavelength of 190-230 nm (peptide bond) and 280 nm (aromatic amino acids). While the peak at 280 nm is more specific to proteins, absorption at the lower wavelength can lead to higher signals and therefore be suitable for the detection of lower concentrations.

For the detection of protein degradation, this work used a TSKgel G2000 SWxl 300mm SEC column (Tosoh Bioscience GmbH, Griesheim, Germany) at 30 °C column temperature with a 25 mM potassium phosphate and 150 mM sodium chloride buffer at pH 5.5 on a Waters HPLC. The peaks were detected at a wavelength of 280 nm and the flow rate was 1.0 mL/min for chymotrypsin. Before analysis, samples were diluted to a concentration of 0.5 mg/mL in mQ water and filtered through a 0.2 µm membrane since larger particles tend to interfere with the integrity of the sensitive column.

6.5.3 Protein Concentration

The protein concentration of samples that were not contaminated with UV-absorbing material, in this work this applied for all samples besides the FSI samples, was determined using the NanoDrop One (Thermo Fisher Scientific, Waltham, Massachusetts). The used protein-analysis profile measures concentration at 280 nm and takes a baseline at 340 nm. This

excludes interference caused by possible protein aggregation or other light-scattering contaminations. The concentrations were calculated using a calibration curve. Therefore, the absorption of seven samples in the range of 0.05 mg/mL and 10.00 mg/mL was measured and the calibration curve with an R^2 of 0.9999 was calculated.

Furthermore, the SEC was used to determine ovalbumin concentrations for samples contaminated with UV-absorbing materials. In this work, this was only the case for the FSI samples. With the separation of materials by their size, those UV-absorbing substances were separated from the protein which could then be detected. Before the measurements the samples were filtrated through a 0.2 µm filter. The protein concentration could then be calculated using a calibration curve. Therefore, the absorption of seven samples in the range of 0.05 mg/mL and 10.00 mg/mL was measured and the calculation curve with an R^2 of 1.00 was calculated. For the concentration determination, the peaks were detected at a wavelength of 220 nm. A Yarra SEC 2000 3 µm column with a 25 mM potassium phosphate and 150 mM sodium chloride buffer at pH 5.5 on a Waters HPLC and a flow rate of 0.35 mL/min at 30°C column temperature was used.

6.5.4 Tertiary Structure and Aggregation

The fluorescence emission spectra assay detects changes in the tertiary structure as well as the formation of aggregates [92]. The plate reader Tecan Spark (Tecan Trading AG, Männedorf, Switzerland) recorded two types of emission spectra in a black 96 well plate suitable for fluorescence measurements. Two different assays based on these emission spectra could be run.

The first assay used the extrinsic fluorescence emission of the fluorescent dye 8-Anilino-1-Naphthalene Sulfonic acid (ANS). This assay is theoretically suitable for all kinds of proteins. ANS, being almost non-fluorescent in water, shows an increasing fluorescence and a blueshift of the maximum with increasing hydrophobicity of the solvent [92, 93]. It also interacts with the hydrophobic parts of a protein, which increases the fluorescence emission of ANS. The quantity of these binding sites usually increases with increasing protein degradation since most proteins have a hydrophobic core and a hydrophilic outside in the native state. Increasing the protein´s hydrophobicity by changes in the tertiary structure or protein aggregation, therefore, changes the fluorescence emission of ANS. It usually leads to a blueshift and an increase in the emission intensity (Figure 18).

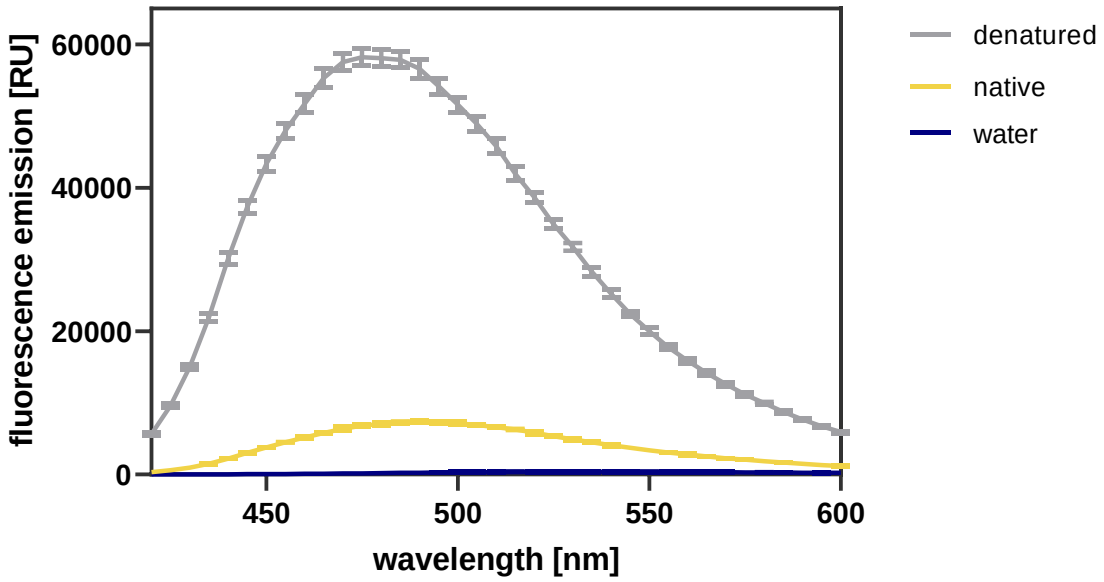

Figure 18: Spectra of chymotrypsin native and denatured as an example for the extrinsic fluorescence assay; the mean curve of n=3, error bars=CI

The extrinsic fluorescence emission (Figure 19) was recorded every 5 nm from 400 to 600 nm using an excitation wavelength of 375 nm.

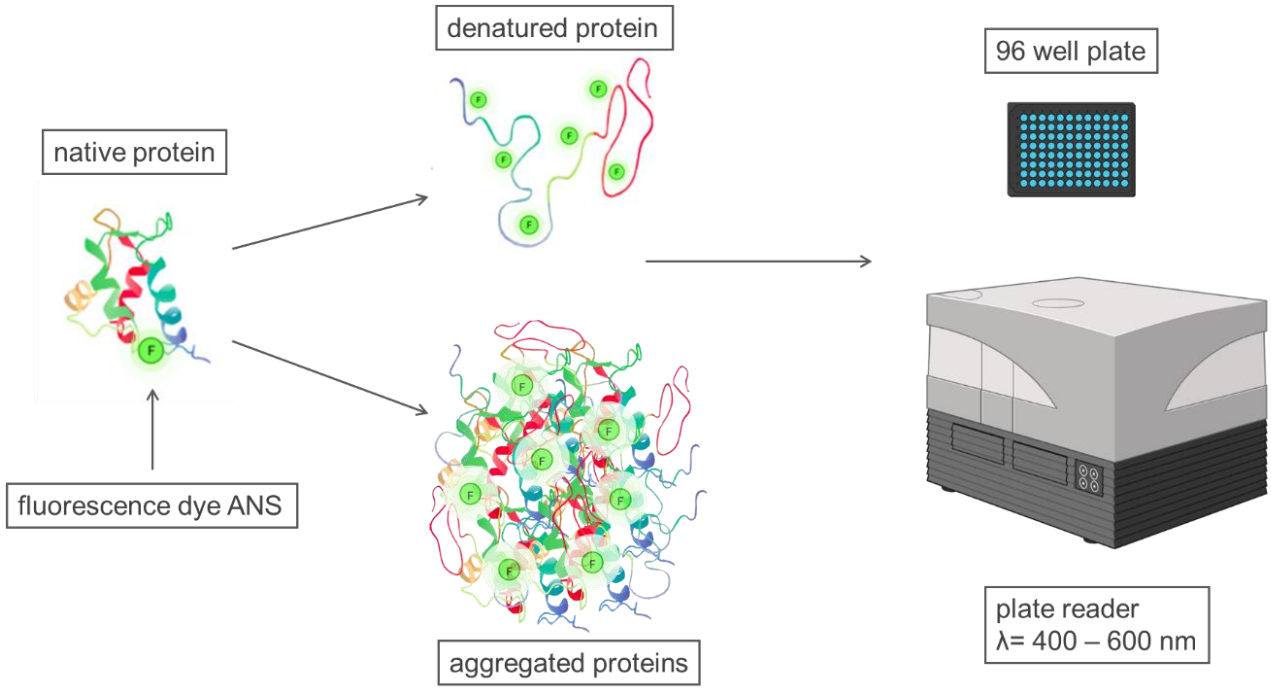

Figure 19: Measurement of extrinsic fluorescence; created with BioRender

Protein characterisation with an intrinsic fluorescence assay is theoretically possible for proteins containing at least one of the naturally fluorescent amino acid residues [92]. These are phenylalanine, tyrosine and tryptophan with an absorption maximum of 258 nm, 275 nm and 280 nm and an emission maximum of 282 nm, 304 nm and 350 nm. Tryptophan shows the

highest fluorescence intensity and the fluorescence spectrum highly depends on its local environment [92]. It can change if the protein structure changes depending on the position(s) of the tryptophan(s) in the native structure and thereby detect protein degradation (Figure 20).

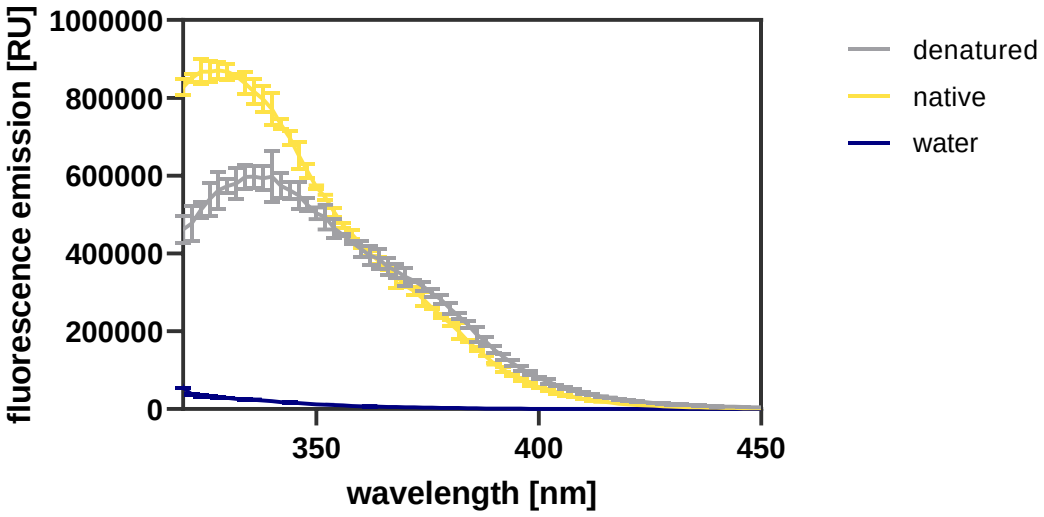

Figure 20: Spectra of chymotrypsin native and denatured as an example for the intrinsic fluorescence assay; the mean curve of n=3, error bars=CI

Nevertheless, the fluorescence intensity is interfered by intrinsic quenching and is usually lower than that of extrinsic fluorescence [92].

This intrinsic fluorescence assay (Figure 21) used the intrinsic fluorescence of tryptophan.

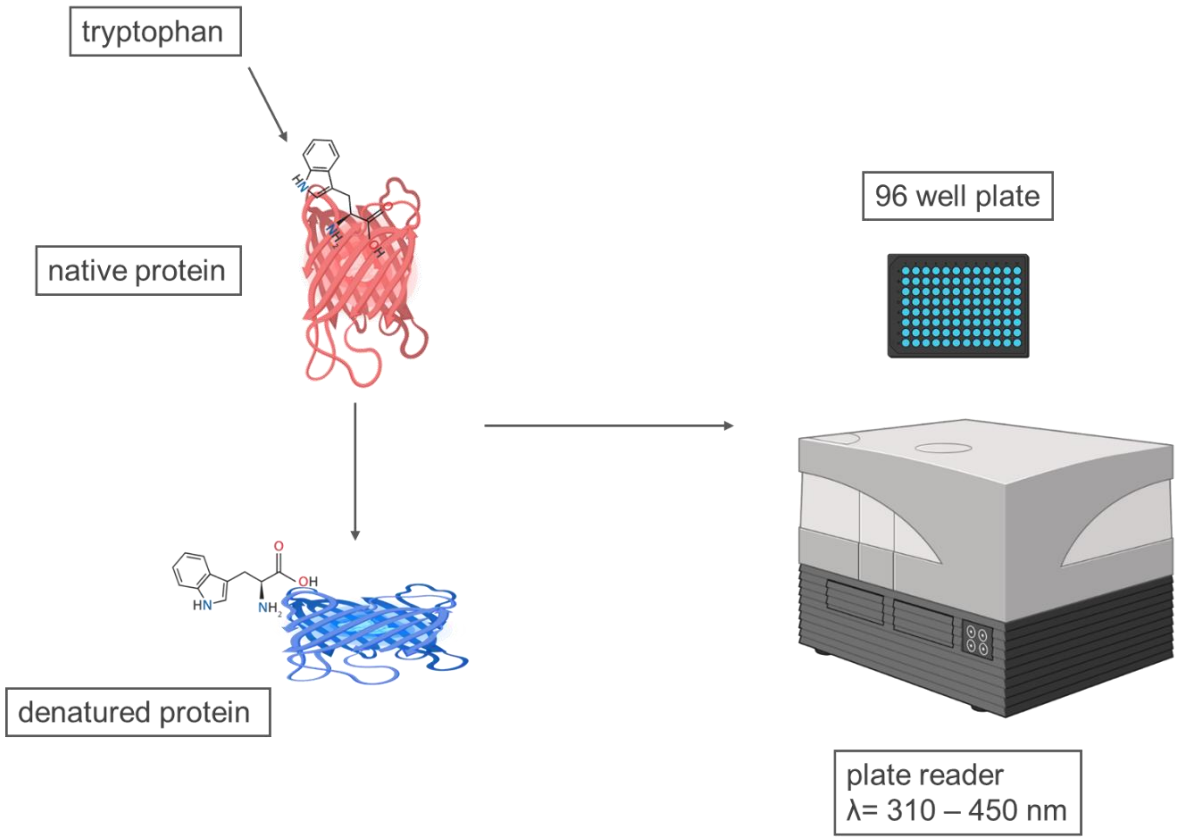

Figure 21: Measurement of the intrinsic fluorescence of tryptophan; created with BioRender

Tryptophan is known to be the amino acid that gives the most accurate results in the intrinsic fluorescence assay [92]. Furthermore, it was not possible to measure the fluorescence absorption and emission maxima of phenylalanine and tyrosine since the excitation wavelength needed would be too low for the used plate reader. The spectra of the intrinsic fluorescence of tryptophan were recorded at wavelengths from 310 nm to 450 nm in 2 nm steps with an excitation wavelength of 280 nm.

Before analysis, the protein samples were dissolved to a 4.0 mg/mL concentration. In addition, a sample of the native protein functioned as a positive control and a sample of the denatured protein as a negative control at a 4.0 mg/mL concentration. For the intrinsic fluorescence measurements, 200 µL of each sample was pipetted into three wells. For the measurement of the extrinsic fluorescence, 100 µL of each sample was pipetted into three wells. 100 µL of saturated ANS solution (about 0.015 mg/mL) was added to each plate and the plate was carefully shaken to ensure mixing of the solutions.

6.5.4.1 Maxima Ratio and Blueshift

The emission intensity maxima ratio and the blueshift in nanometers were calculated to better compare the different spectra. The blueshift is the difference in the wavelength of the

spectra maximum between the sample and the native protein. The maxima ratio was calculated with Equation 3.

$$Maxima\ ratio = \frac{maximum\ sample - maximum\ native}{maximum\ denatured - maximum\ native}$$

Equation 3: Maxima ratio for the intensity of the fluorescence emission spectra

A maxima ratio close to 0 indicates a protein structure close to the native structure. Increasing changes in the protein structure led to an increase in the maxima ratio. A value around 1 indicates a fully denatured protein.

6.5.5 Enzyme Activity of Chymotrypsin

The chymotrypsin enzyme activity assay detects the enzymatic functionality of chymotrypsin. Therefore, a change in enzyme activity indicates changes in the parts of the protein structure important for the enzyme activity. In case of minor changes in other parts of the tertiary structure that are not directly associated with enzyme activity, the assay may not detect these changes.

The assay was performed close to the one described by Worthington Biochemical Corporation, Lakewood, USA [94]. Before the measurement, the sample was diluted to an estimated concentration of 2.75 u/mg with 0.001 N HCl. Then the following was mixed in a quartz cuvette: 50 µL of the prepared sample, 50 µL of 0.001 N HCl and 2.9 mL of a freshly prepared mixture of 135 mL 0.08 M Tris-HCl buffer pH 7.8 containing 0.1 M calcium chloride (Merck KGaA, Darmstadt, Germany) and 126 mL 0.00107 M Benzoyl-L-tyrosine ethyl ester (BTEE) (Merck KGaA, Darmstadt, Germany) in 63% V/V methanol/ mQ water. The UV-Vis-spectrometer (UV-1280 by Shimadzu, Kyoto, Japan) directly calculated the ΔA 256/min by measuring the increasing absorption at 256 nm for 5 minutes. With this, Equation 4 (964 being the extinction coefficient of BTEE and concentration being the chymotrypsin concentration in the reaction medium in mg/mL) can calculate the enzymatic activity of chymotrypsin in u/mg.

$$enzyme\ activity\ u/mg = \frac{1000 * \Delta A256}{964 * concentration}$$

Equation 4: Calculation of the chymotrypsin activity

Each sample was produced in triplicate (n=3) and measured three times. Results are given as the mean enzyme activity of these measurements calculated using Equation 4.

For an easier comparison of different chymotrypsin batches, the enzyme activity of the commercial product was set to 100%. The enzyme activity of the samples was then calculated as remaining activity in % using Equation 5.

$$remaining\ activity = \frac{enzyme\ activity\ (sample)\ *\ 100\%}{enzyme\ activity\ (commercial\ product)}$$

Equation 5: Calculation of the remaining activity

6.5.6 Nano Differential Scanning Fluorimetry, Turbidity and Dynamic Light Scattering

The model proteins were analysed with the Prometheus Panta® during a device demonstration by the company NanoTemper Technologie GmbH (München, Germany). The Prometheus Panta® measures the unfolding of the proteins as well as the formation of small and large aggregates. Furthermore, it can heat the samples in a temperature ramp from 15°C to 95°C, thereby analysing the protein's thermal stability.

The measurement relies on a simultaneous measuring of dynamic light scattering (DLS), static light scattering (SLS), nano differential scanning fluorimetry (nano DSF) and back reflection. The size of the molecules directly correlates with the intensity of the backscattered light. Therefore, proteins that form larger aggregates give multiple peaks with different hydrodynamic radii compared to the monomer after degradation. Due to the minimal size differences between the monomer and the dimer, however, discrimination between these two species is not possible. The nanoDSF measures the intrinsic fluorescence of tryptophan detecting the temperature-dependent unfolding of the protein (equally to the method described in Section 6.5.4). The turbidity, measured by the intensity of the reflected light, increases with increasing size and number of the aggregates.

The samples were measured in a concentration of 1 mg/mL in three buffers: pH 3.5 (68 g/L KH_2PO_4), pH 5.5 (13.12 g/L KH_2PO_4, 1.29 g/L Na_2HPO_4) and pH 7.4 (NaCl 8 g/L, KCl 0.2 g/L, 0.2 g/L KH_2PO_4, 1.42 g/L Na2HPO4) one time. The results were used to get an idea about the thermal and pH-dependent stability of the model proteins.

6.6 Statistics

To evaluate the data different statistical methods have been used. Data was presented as mean value ± the 95% confidence interval of each sample type.

The David test (Equation 6) was used to test for normal distribution. The value was then compared to table values depending on the number of samples.

$$T_{David} = \frac{range}{standard\ deviation}$$

Equation 6: David test on normal distribution

The statistical tests were performed with Excel. The t-test shows the statistical significance of a difference between the two sample groups. The test requires a normal distribution. Furthermore, an f-test on equality or inequality of the variances is necessary. The data analysis tool in Excel then calculates the p-value for the t-test depending on the considered equality of the variances. A p-value < 0.05 hereby indicates a significant difference, a p-value < 0.01 indicates a very significant difference and a p-value < 0.001 indicates a highly significant difference. In this work, most experiments were performed in triplicates, allowing statistical tests.

7 Results and Discussion

The experiments performed in this work aim to identify factors that stress proteins during the production and application of inhalable dry powder (DPI) formulations and suggest suitable stabilising measures. The first experiments investigated the suitability of different well-established model proteins as model proteins for spray drying stability studies. Furthermore, they studied the overall stability of the chosen model protein chymotrypsin. The next part aimed to discriminate the influence of stress factors and the effects of stabilising excipients during the protein DPI production by spray drying. It investigated structural as well as physical changes in the proteins. The last part studied the possibility of redissolving the DPI formulation to nebulise it. This idea aimed at broadening the usability of protein DPIs that target severe lung diseases since some of the patients might not be able to use a DPI device.

7.1 Comparison of the Model Proteins

This section compared the different model proteins regarding their properties and usability for further studies. The present work required proteins that are economically and commercially available in amounts required for the spray drying experiments (multiple grams). Therefore BSA, chymotrypsin, insulin, lysozyme and ovalbumin were chosen as potential model substances. The proteins need to be unstable enough to present structural changes after processing, but stable enough to denature only partly. This would make it possible to investigate differences between destabilising factors and process parameters as well as stabilising influences of excipients. Furthermore, analytical methods to identify these changes need to be available. Last, the proteins must be technically suitable for spray drying and nebulisation since proteins that tend to form very large particles may plug sensitive spray drying nozzles or nebuliser parts.

This work, therefore, compared the model proteins regarding their dissolution characteristics after spray drying as a physical change in protein dry powder formulations. Furthermore, it investigated the tendency of proteins to accumulate at interfaces. This might correlate with their sensitivity to interfacial stress during pumping and atomisation. Next, it compared the melting point of the model proteins as an indicator of their liability to the thermal stress during spray drying. Last, the investigation of tertiary structure changes after spray drying showed the sensitivity to the specific experimental set-up. The proteins being most liable to the stress factors investigated were selected as model proteins for the later performed stability studies.

7.1.1 Dissolution Characteristics of the Model Proteins

During the different studies, some proteins displayed specialities in the conditions under which they dissolved or exhibited changes in their solubility after processing. While BSA and lysozyme exhibited no observable dissolution specialities, the other model proteins did. The used insulin was only soluble in dissolution media with a pH between 2.0. and 2.5. Chymotrypsin tended to form insoluble gel-like structures when dissolved in too high (> ca. 10 mg/mL) concentrations which could not be dissolved by further dilution afterwards. This limited the usability of chymotrypsin for dissolution studies. Chymotrypsin as well as ovalbumin displayed an observable change in dissolution behaviour and seemed to dissolve worse and slower after spray drying which was later analysed for ovalbumin (Section 7.5).

7.1.2 Interface Activity

The surface tensions of the protein solutions were different depending on the model protein. The concentrations of the samples were chosen equally to the spray feed during the spray drying experiments. The solution, therefore, showed the interface activity of the proteins equally to these experiments. In a concentration of 4 mg/mL, the surface tension decreased in the following sequence: lysozyme, BSA, ovalbumin and chymotrypsin (Table 5).

Table 5: Surface tension of protein aqueous solutions with and without 0.1% polysorbate 20 and polysorbate 80, n=3, +/- CI

	Surface tension [mN/m]
Ovalbumin	42.82 (± 0.58)
BSA	51.89 (± 0.17)
Chymotrypsin	35.93 (± 0.38)
Lysozyme	57.54 (± 1.23)

A lower surface tension in this experiment indicated a higher interface activity of the protein. Proteins that tend to accumulate more on surfaces might be more influenced by interfacial stress. The differences in the surface tension most likely rely on the differences in protein structure. For the spray drying experiments, this kind of protein might, therefore, be a more promising model protein.

7.1.2.1 Influence of Denaturation on Surface Tension

Denaturation might influence the surface activity of proteins. This was, therefore, tested for the protein with the highest surface activity, chymotrypsin. Denaturation of chymotrypsin, however, affected the surface tension of the 4 mg/mL protein solution only slightly. The mean

value changed from 35.93 N/m ± 0.43 N/m to 37.88 N/m ± 0.26 N/m. Although the difference is measurable, it is too small for further interpretation and could be due to chance. It does, therefore, not seem likely that the difference is relevant.

7.1.3 Structural Stability in Different Dissolution Media

7.1.3.1 Size Homogeneity

The measurement of the hydrodynamic radius by means of three measurements differed for the examined proteins (Table 6).

Table 6: Hydrodynamic radius of the model proteins in different buffers, n=1

Sample	pH	Cumulant Radius (nm)	Cumulant PDI	Peak 1 (nm)	Peak 2 (nm)
BSA	3.5	3.41 ± 0.06	0.07 ± 0.04	3.41 ± 0.06	
	5.5	3.86 ± 0.02	0.02 ± 0.02	3.86 ± 0.02	
	7.4	3.87 ± 0.07	0.07 ± 0.03	3.87 ± 0.07	
Chymotryp-sin	3.5	2.79 ± 0.02	0.03 ± 0.02	2.79 ± 0.02	
	5.5	2.99 ± 0.09	0.48 ± 0.04	2.51 ± 0.07	19.31 ± 4.35
	7.4	2.75 ± 0.03	0.38 ± 0.03	2.50 ± 0,08	19.30 ± 5.16
Lysozyme	3.5	1.88 ± 0.15	0.44 ± 0.07	2.10 ± 0.05	47.56 ± 17.62
	5.5	2.83 ± 0.35	0.53 ± 0.16	1.87 ± 0.04	40.43 ± 8.30
	7.4	2.10 ± 0.16	0.35 ± 0.15	1.80 ± 0.03	60.57 ± 8.47
Ovalbumin	3.5	36.57 ± 3.34	0.95 ± 0.04	4.64 ± 1.71	76.16 ± 20,75
	5.5	15.07 ± 0.49	0.91 ± 0.01	3.93 ± 0.50	37.80 ± 3.96
	7.4	9.17 ± 0.42	0.93 ± 0.03	3.67 ± 0.30	31.90 ± 9.53

BSA exhibited only one peak equalling the monomer/dimer. The protein was highly mono-dispersed in all buffers. Chymotrypsin was monodispersed at a pH of 3.5. At pH 5.5 and 7.4 aggregates with a size of 19.3 nm occurred. They did not highly influence the cumulant radius. It is, therefore, likely that only small amounts of these aggregates form at higher pHs. Lysozyme formed large aggregates at all examined pHs. Here as well, no higher influence on the cumulant radius occurs. For ovalbumin large aggregates occurred at all examined pHs. These aggregates decreased with increasing pH. They highly influenced the cumulant

radius. It is, therefore, likely that a high number of aggregates occurs after thermal degradation.

7.1.3.2 Colloidal and Thermal Stability

The thermal unfolding experiments showed varying thermal stability of the proteins in three buffers (Table 7).

Table 7: Melting temperature of the model proteins in different buffers, n=1

	Melting Temperature (C°)			
pH	**BSA**	**Chymotrypsin**	**Lysozyme**	**Ovalbumin**
3.5	Not found	54.23	75.35	70.44
5.5	56.52	57.96	76.43	74.82
7.4	60.62	54.15	71.90	77.59

Lysozyme and ovalbumin had a comparable high melting temperature above 70 °C while chymotrypsin and BSA had a much lower one (between 50°C and 60 °C). The exact melting temperature, however, varied with the buffer for all four proteins.

Moreover, the proteins exhibited colloidal stability that depended on the buffer (Table 7). BSA did not unfold at pH 3.5. At pH 5.5 and 7.4 it did unfold. While it formed aggregates during unfolding at pH 5.5, at pH 7.4 it unfolded without forming aggregates. Chymotrypsin unfolded and formed aggregates at all three pHs. However, only at pH 3.5, their amount was high enough to increase the turbidity. Lysozyme unfolded in all three buffers but had high colloidal stability and did not form any aggregates. Ovalbumin unfolded and formed aggregates in all three used buffers. Its colloidal and thermal stability, however, increased with increasing pH.

The melting temperature is an indicator of the sensitivity of proteins against thermal stress. Since the experiments require proteins that are influenced by the spray drying process, proteins with lower melting temperatures seem to be more promising model proteins. Of the proteins tested in this section chymotrypsin and BSA have the lower melting temperature and may, therefore, be more sensitive to the spray drying process.

7.1.4 Stability of the Tertiary Structure

This section investigated the different model proteins' structural stability and the suitability of the fluorescence assays to detect. Therefore, it compared the fluorescence emission spectra of the native and the denatured proteins. It analysed the intrinsic fluorescence of the

proteins´ tryptophan residues and the extrinsic fluorescence of the fluorescence dye ANS. Furthermore, it aimed to identify the protein with the optimal usability for spray drying experiments. To effectively show the influence of different spray drying conditions and stabilising excipients, the proteins needed to exhibit larger structural changes after spray drying. The section, therefore, compared the changes of the model proteins after spray drying at the mini spray dryer with the two-fluid (150°C inlet and 80-90°C outlet temperature) and the ultrasonic nozzle (150°C inlet and 50-60°C outlet temperature). For the proteins that changed structurally during spray drying with the mini spray dryer, the section additionally analysed the sensitivity for spray drying with the nano spray dryer. Depending on their structural or physical changes, the proteins were chosen for spray drying experiments concerning protein stability or dissolution behaviour changes.

7.1.4.1 BSA

Figure 22 illustrates the intrinsic fluorescence emission spectra of BSA's tryptophan residues.

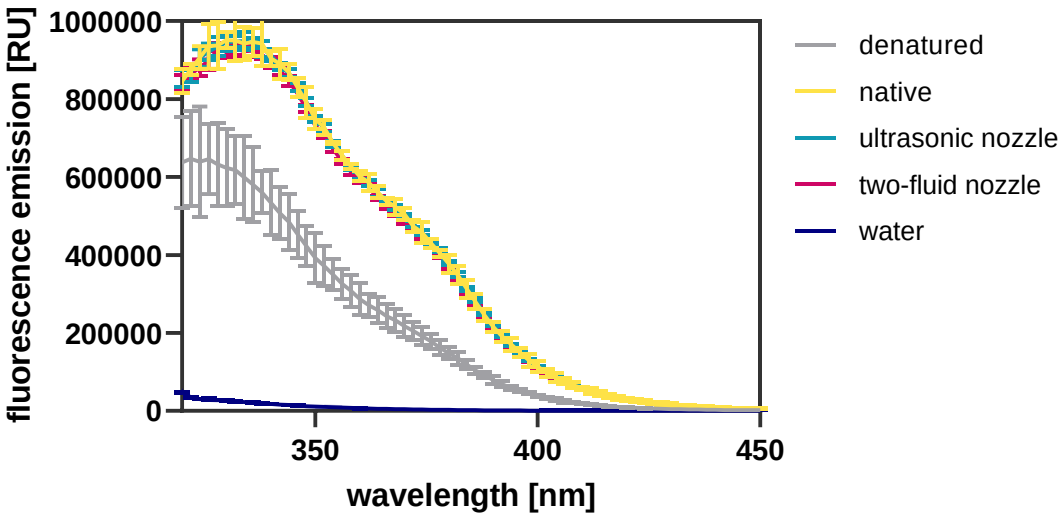

Figure 22: Fluorescence spectra of the intrinsic fluorescence of tryptophan of BSA native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The native protein had an emission intensity of 951716 at the maximum wavelength of 330 nm. With denaturation, emission intensity decreased to 645657 and a blueshift to a maximum wavelength of 326 nm occurred. The detection of denaturation of BSA is generally possible using intrinsic fluorescence. The spectra of the ultrasonic nozzle and the two-fluid nozzle both overlaid with that of the native protein. Therefore, the spectra did not indicate denaturation after spray drying.

Figure 23 shows the fluorescence emission spectra of BSA in the presence of the fluorescence dye ANS.

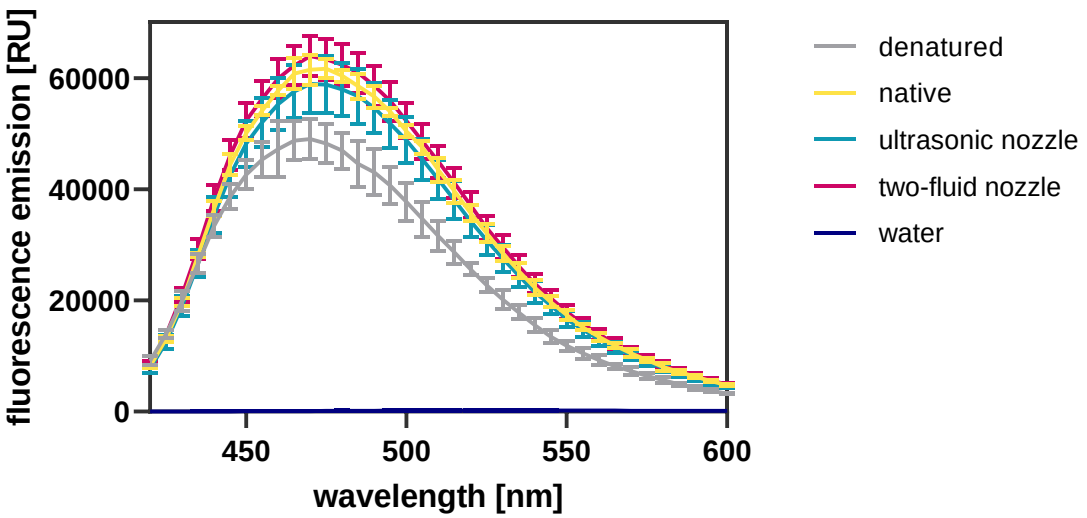

Figure 23: Fluorescence spectra with the fluorescence dye ANS of BSA native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The native protein had an emission intensity of 61701 and a wavelength of 475 nm of the maximum. Contrary to all other examined proteins, emission intensity decreased with denaturation to 49075 and a blueshift to a wavelength of 470 nm of the maximum occurred. These results were unexpected since BSA as a serum albumin should be more hydrophilic in the native stage. Furthermore, this difference was rather small making further investigation very difficult. After spray drying with the ultrasonic nozzle and the two-fluid nozzle the spectra of both samples overlaid with the spectrum of the native protein. This indicated no structural change.

The intrinsic fluorescence of tryptophan of BSA changed highly after denaturation. The extrinsic fluorescence, however, showed only minor differences between the native and the denatured protein. Spray drying did not cause detectable differences for both the intrinsic as well as extrinsic fluorescence.

7.1.4.2 Chymotrypsin

Figure 24 displays the fluorescence emission spectra of chymotrypsin's intrinsic fluorescence of the tryptophan residues.

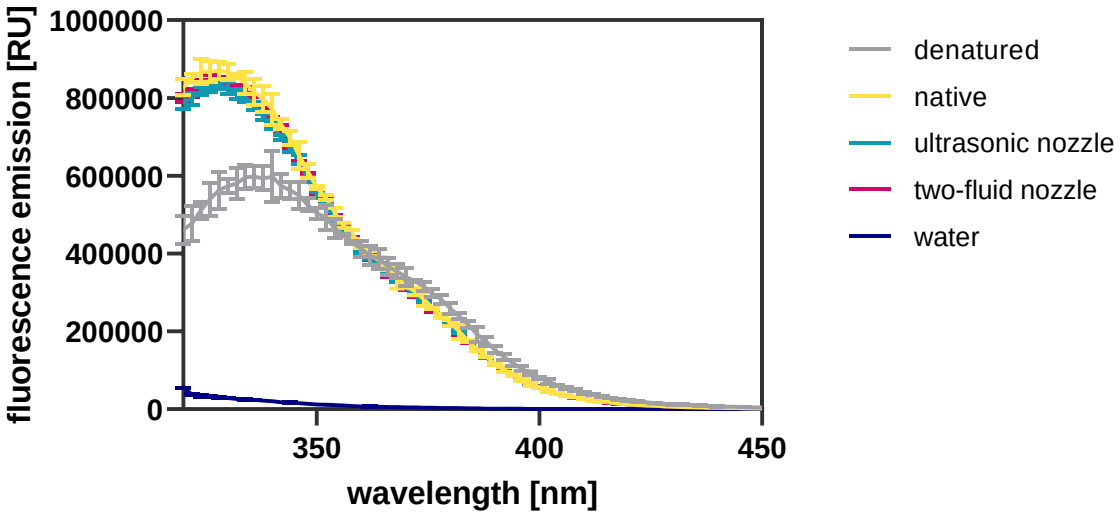

Figure 24: Fluorescence spectra of the intrinsic fluorescence of tryptophan of chymotrypsin native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The native protein exhibited a maximum wavelength of 326 nm and an emission intensity of 857736. In contrast, the denatured protein displayed a lower emission intensity maximum of 646568 and a redshift to a wavelength of 336 nm. The intrinsic fluorescence assay, therefore, detected the difference between the native and the denatured chymotrypsin. The spectra of the spray-dried samples overlaid with that of the native. The assay did not detect structural after spray drying.

Figure 25 displays chymotrypsin's fluorescence emission spectra of the fluorescence dye ANS.

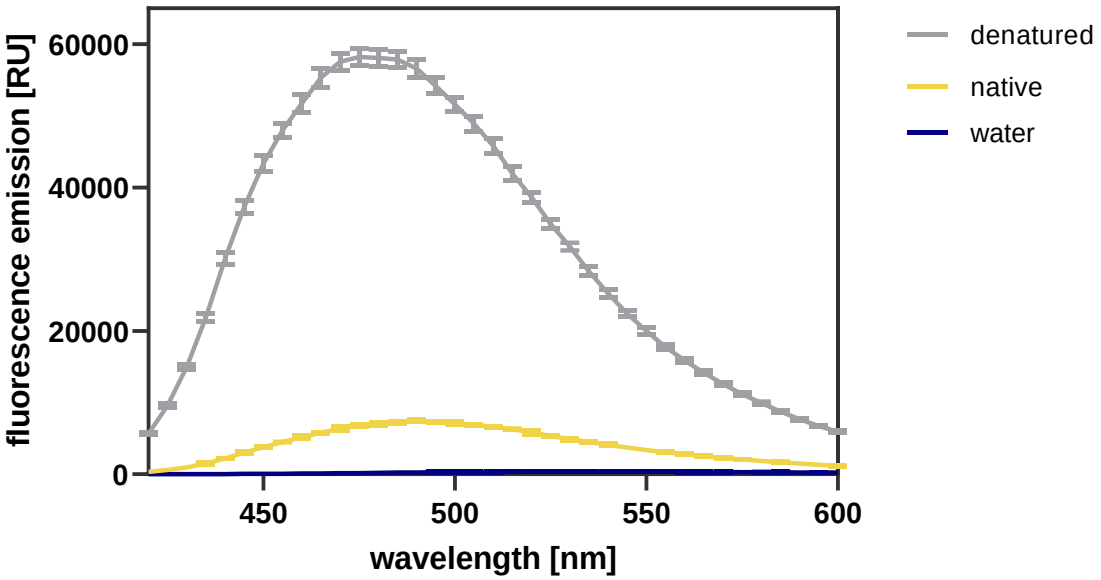

Figure 25: Fluorescence spectra of the fluorescence dye of chymotrypsin native and denatured; the mean curve of n=3, error bars=CI

The native protein presented an emission intensity of 7435 and a wavelength of 490 nm of the maximum. After denaturation, a decrease in emission intensity of the maximum to 58223 and a blueshift to a wavelength of 475 nm occurred. The large difference between the native and the denatured samples indicated the assay's usability for the detection of smaller structural changes.

For chymotrypsin spray drying with both spray dryers was feasible and caused a change in protein structure (see Sections 7.3.1, 7.3.2). The changes were, however, only detectable with the more sensitive extrinsic fluorescence assay. The intrinsic fluorescence assay was not able to detect smaller structural changes and was, therefore, not used for further studies.

7.1.4.3 Insulin

The used insulin only dissolved in acidic solutions (pH <2.5). While all other used model proteins did not aggregate to visible aggregates, insulin formed very large, visible aggregates (Figure 26).

Figure 26: Aggregated insulin after cooking for 3 hours

Due to the high sensitivity of the instrument, spray drying at a nano spray dryer at low pHs was not recommended. The large aggregates can additionally clog the delicate tubing system in the spray head. Therefore, insulin was not spray-dried at the nano spray dryer. To increase the technical feasibility of the spray drying at the mini spray dryer, glycine and mannitol were added in equal concentrations as stabilising excipients. To protect the sensitive spray drying nozzles against plugging with large aggregates insulin was not spray-dried without the excipients.

For insulin, a measurement of the intrinsic fluorescence was not possible due to the lack of tryptophan. Only the fluorescence emission in the presence of the fluorescence dye ANS was measured (Figure 27).

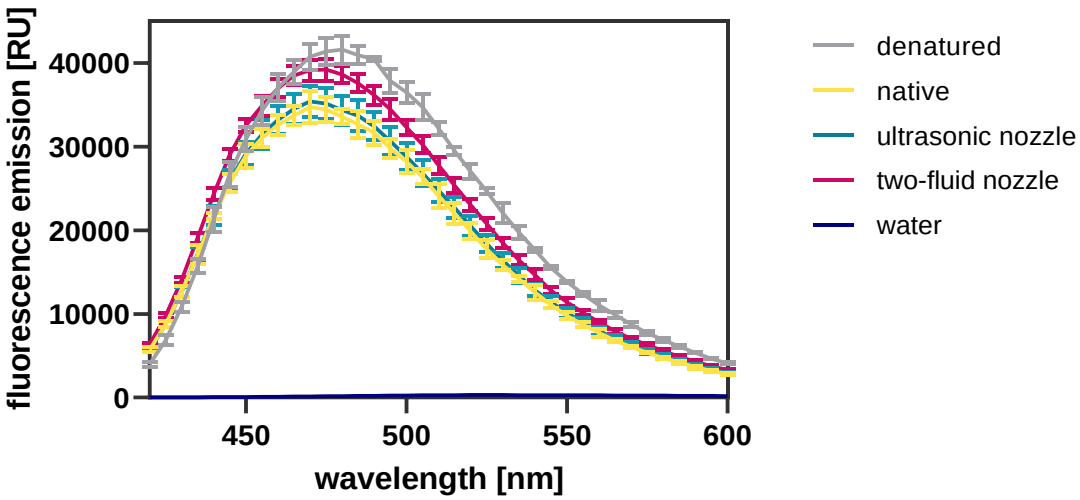

Figure 27: Fluorescence spectra of fluorescence dye ANS of Insulin native, denatured and spray-dried with glycine and mannitol; the mean curve of n=3, error bars=CI

A minor increase in the fluorescence intensity from 34744 to 41629 and a redshift of 10 nm occurred after denaturation. The spectrum of the sample spray-dried with the two-fluid nozzle overlaid with that of the native protein. The spectrum of the samples spray-dried with the

ultrasonic nozzle displayed a small emission increase compared to the native protein. Both spectra indicated no or only minor structural changes after spray drying.

The small differences in the spectra of the native and the denatured make a structural interpretation more difficult. Small changes in the tertiary structure are not likely to be detected. Furthermore, the tendency to form large aggregates and the need for a low pH dissolution medium limits the usability of insulin for several experiments. Due to the absence of detectable changes after spray drying, no further stability studies for spray drying were performed.

7.1.4.4 Lysozyme

For all examined lysozyme samples including the denatured protein, the fluorescence emission spectra of the intrinsic fluorescence of tryptophan overlaid (Figure 28).

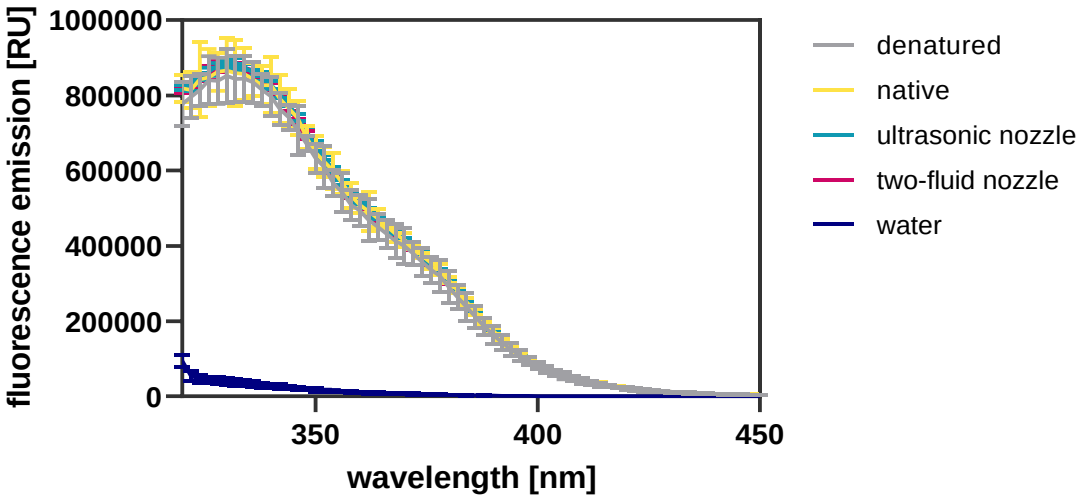

Figure 28: Fluorescence spectra of the intrinsic fluorescence of tryptophan of lysozyme native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The intrinsic fluorescence spectra assay is therefore not suitable to detect structural changes in lysozyme.

Figure 29 displays the fluorescence emission spectra of lysozyme in the presence of the fluorescence dye ANS.

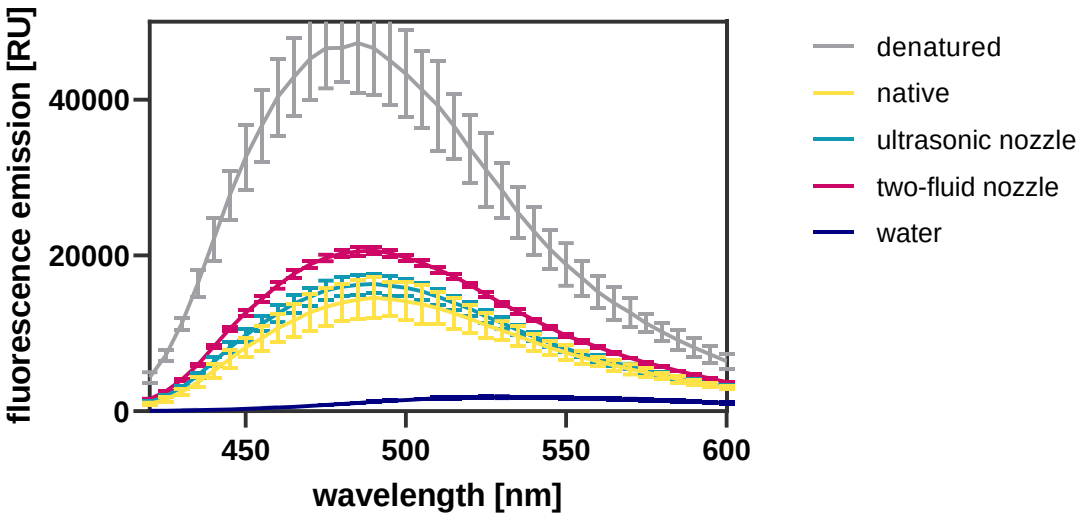

Figure 29: Fluorescence spectra of fluorescence dye ANS of lysozyme native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The native protein had an emission intensity of 14560. The fluorescence emission intensity highly increased after denaturation to 47278. The maximum of the denatured protein shifted 5 nm to blue. Due to the large difference between the native and the denatured sample, the assay seems to be suitable for detecting structural changes in lysozyme. After spray drying with the ultrasonic nozzle and the two-fluid nozzle, the emission increased a little to 17926 and 20624. None of the spray-dried samples showed a blueshift compared to the native protein and a wavelength of the emission maxima of 490 nm. The assay, therefore, detected very minor structural changes in lysozyme after spray drying.

Since the mini spray dryer caused minor changes in lysozymes' tertiary structure, also the influence of spray drying with the nano-spray-dryer was tested. Figure 30 illustrates the fluorescence emission spectra of these lysozyme samples.

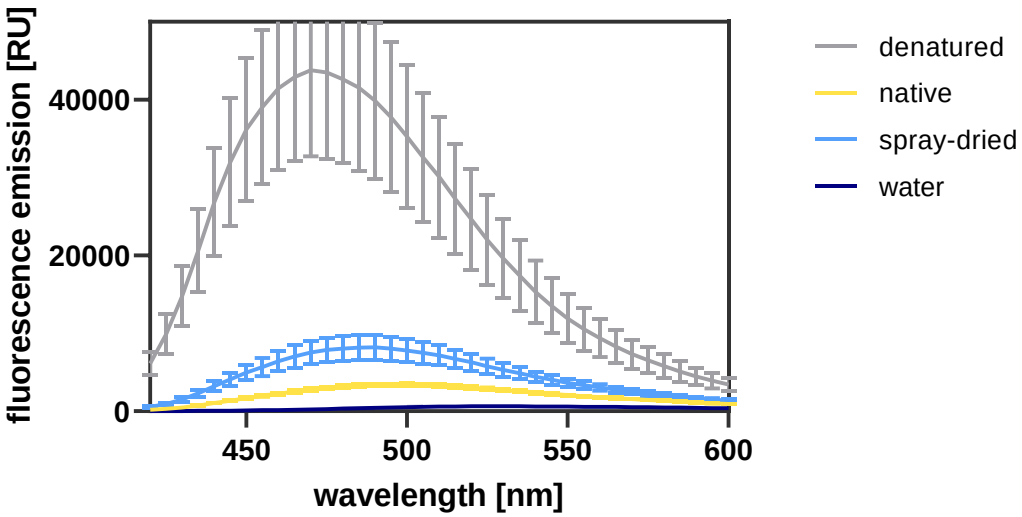

Figure 30: Fluorescence spectra of fluorescence dye ANS of lysozyme spray-dried with the nano spray dryer; the mean curve of n=3, error bars=CI

A very minor increase in emission intensity and no blueshift after spray drying with the nano spray dryer, indicating a minor change in protein structure, occurred.

While the intrinsic fluorescence of lysozyme did not change after denaturation, the extrinsic fluorescence did change. Due to only minor changes after spray drying with both the nano- and the mini-spray-dryer, no further stability studies were performed.

7.1.4.5 Ovalbumin

Figure 31 shows the fluorescence emission spectra of ovalbumin´s intrinsic fluorescence of tryptophan.

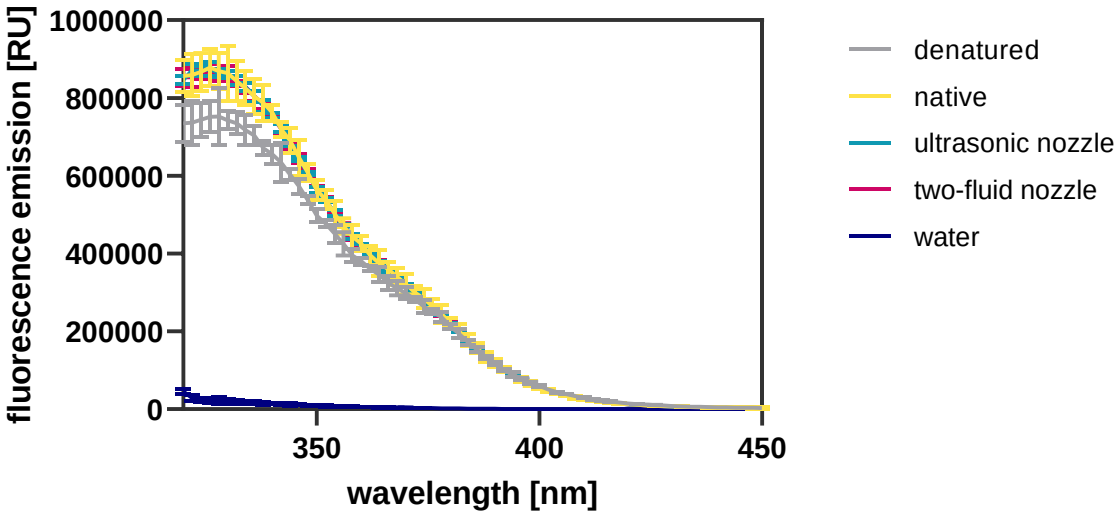

Figure 31: Fluorescence spectra of the intrinsic fluorescence of tryptophan of ovalbumin native, denatured and spray-dried; the mean curve of n=3, error bars=CI

The native protein exhibited an emission intensity of 877687 and a maximum wavelength of 326 nm. Denaturation caused a minor change in the fluorescence spectra to a lower emission intensity of 752332 and a maximum wavelength of 328 nm. The intrinsic fluorescence assay might, therefore, not be the most suitable assay for the detection of structural changes in ovalbumin. The spectra of the protein after spray drying with the ultrasonic nozzle and the two-fluid nozzle were overlaying with the native protein indicating no changes after spray drying.

The fluorescence emission spectra in the presence of the fluorescence dye ANS of the ovalbumin samples did not change after spray drying (Figure 32).

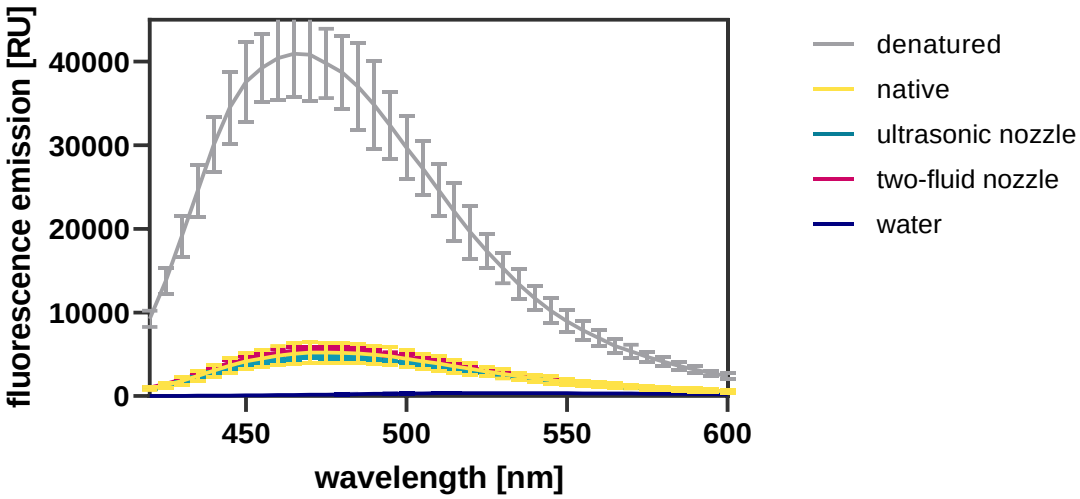

Figure 32: Fluorescence spectra of the fluorescence dye ANS of ovalbumin native, denatured and spray-dried; the mean curve of n=3, error bars=CI

Nevertheless, the extrinsic fluorescence seems to be suitable for the detection of structural changes in ovalbumin since it found a large difference between the native and the denatured sample. The native protein had a maximum emission intensity of 5227 and a wavelength of 475 nm (Figure 32). After denaturation, the emission intensity increased to 40932 and shifted 10 nm to blue (Figure 32).

For ovalbumin spray drying with the nano-spray-dryer was not feasible since the spray head started dropping under all examined conditions. The most likely explanation would be the high foaming tendency of the ovalbumin [95] in the spray head of the nano spray dryer. It could, however, not be further investigated.

While the intrinsic fluorescence in ovalbumin only showed a minor change after denaturation, the extrinsic fluorescence changed enormously. For the spray-dried samples, however, no change occurred in the assay. Ovalbumin was, therefore not used for further studies of tertiary structure changes.

7.1.5 Protein Aggregation

For the protein investigated in the further stability studies, chymotrypsin, the occurrence of protein aggregates after denaturation was detected with the SEC. The native chymotrypsin had only one peak at 8.87 min (Figure 33).

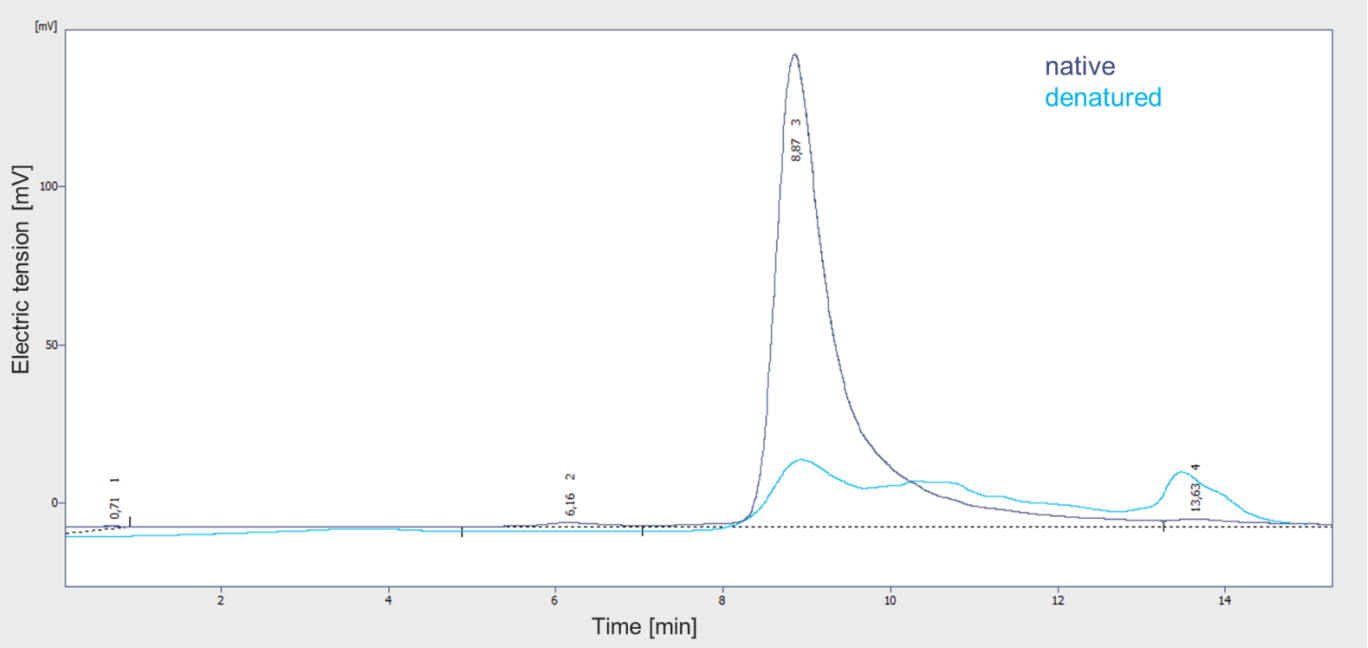

Figure 33: SEC Chromatogram of chymotrypsin native and denatured; n=1

Minor peaks eluted before and after the main peak (Figure 33). The commercial product is not 100% pure chymotrypsin; therefore, it is possible that either impurities or minor parts of already degraded protein cause these peaks. After denaturation, the protein showed no real peak but a broad area starting at the elution time of the native peak and ending at the final elution time of the run (Figure 33). The peaks of the native and the denatured protein were overlapping. This complicates the analysis of protein stability. No aggregation peaks occurred in the chromatogram. This indicates the fragmentation of chymotrypsin into pieces with a broad range of hydrodynamic radii and no aggregation into soluble aggregates. This, further, highly complicates interpreting the analysis. Last, the filtration step before the analysis and the measurement of only the soluble sample parts exclude insoluble aggregates from the analysis. Although these aggregates were not visible for the denatured sample, they may occur in a sub-visible concentration. Furthermore, degradation caused by spray drying might lead to different degradation products. Therefore, the SEC was not used for the stability studies with chymotrypsin.

7.1.6 Selection of Model Proteins based on their Characteristics

The comparison of the model proteins revealed different properties of the proteins and different sensitivities for the examined stress factors. Furthermore, the available analytical methods were of different suitability for the protein samples.

BSA had the lowest interface activity. Although BSA did not have a high melting temperature, neither the tertiary structure nor the physicochemical properties seemed to have changed

after spray drying with the mini spray dryer. In this work, BSA was, therefore, not used for further stability experiments. Nevertheless, BSA is a frequently used model protein for spray drying experiments and other studies found changes caused by the process. Three studies found a monomer loss of BSA indicating a multimer formation after spray drying with the mini spray dryer [96–98]. The effect found in the studies was, however, rather small.

Chymotrypsin had the highest interface activity and a low melting temperature indicating a low thermal stability. It could be spray-dried without any technical problems with both spray drying systems and was the most process-sensitive protein. The protein showed large differences between extrinsic fluorescence emission spectra of the native and the degraded protein. In the SEC and the intrinsic fluorescence emission spectra assay, the changes were not as well detectable or analysable. Chymotrypsin was chosen for the stability studies due to its high sensitivity to stress factors during spray drying. It was then analysed with the extrinsic fluorescence emission spectra assay which detected the differences in the protein structure the best.

Insulin formed large visible aggregates after denaturation that could easily block the tubing inside the nozzle of the spray dryer. This can cause an interruption in production or even damage sensitive and expensive parts of the spray dryer. The fluorescence emission assay could not detect these aggregates very well and no changes were detected after spray drying with the mini spray dryer. Insulin was not used for further experiments for these reasons.

Lysozyme showed a low interface activity. The protein had a high melting temperature indicating a high thermal stability and high colloidal stability. It could also be spray-dried with both spray dryers but exhibited only very minor structural changes. In this work, it was, therefore, not investigated in further experiments. Nevertheless, also lysozyme is a frequently used model protein for spray drying and other studies found influences of spray drying under different conditions. Two studies found a loss in enzyme activity after spray drying which increased with increasing inlet and outlet temperature [99, 100]. Furthermore, they found an increase in activity loss with an increase in fine particle collection [99, 100]. They explained this phenomenon with the increased stress the smaller droplets were exposed to during the drying stage. Ji et al. identified the negative effects of spray drying in ethanol-water mixtures on the secondary structure of lysozyme [101].

Ovalbumin had a medium interface activity. It showed high thermal stability, but lower colloidal stability. The DLS measurements detected aggregates even in the native sample, the number of those increased with degradation. Although the fluorescence emission spectra

assay detected no changes after spray drying with the mini spray dryer, the dissolution behaviour visibly changed. The protein was, therefore, chosen for dissolution behaviour investigations.

The section showed that finding the most suitable model protein for more complex experiments requires considering various factors. Spray drying exposes the protein amongst other stress factors to interfacial and thermal stress. Nevertheless, a direct correlation between structural degradation, interface activity and melting temperature was not found. Only chymotrypsin, which had a low melting temperature and a high interface activity, was sensitive to the spray drying process. For BSA with an equally low melting temperature but a low interface activity, no structural change occurred. Ovalbumin with a high melting temperature and a high interface activity showed no structural but a physical change. Lysozyme with a high melting temperature and a low interface activity surprisingly changed structurally during the spray drying process. The change was, however, rather small. Furthermore, different analytical methods can show different sensitivities of proteins against certain stress factors. In the present work, the intrinsic fluorescence assay detected no structural changes after spray drying in any of the model proteins. The extrinsic fluorescence assay, however, exhibited structural changes for chymotrypsin and lysozyme. For some of the proteins examined in this section, spray drying studies of others found slightly different effects on the protein structure. This again demonstrates that for the evaluation of a stability study the used analytical methods are highly important.

7.2 Influence of the Excipients on the Model Proteins

Various excipients exist to stabilise proteins during spray drying (see Section 3.5.1.3). This section investigated the influence of selected excipients on the model proteins. It investigated the thermal stability of chymotrypsin in different concentrations with and without stabilising excipients. As a stabiliser against thermal stress, it compared the impact of mannitol and lactose on the thermal stability of chymotrypsin. Furthermore, the stability of the protein in different dissolution media was detected. Last, the section evaluated the surfactants polysorbate 20 and 80 as stabilisers against interfacial stress and their influence on the interface activity of BSA, chymotrypsin, lysozyme and ovalbumin. Additionally, it examined whether the addition of glycine and mannitol has an impact on the surface tension of the protein solutions.

7.2.1 Thermal Stability Study with Mannitol and Lactose

An initial thermal stability study investigated the influence of storing the model protein chymotrypsin in aqueous solutions at 50°C to get an idea of the general stability of the protein. It analysed the remaining enzyme activity of solutions containing a chymotrypsin concentration of 1.5 mg/mL, 3.0 mg/mL and 7.5 mg/mL after 1, 4, 7 and 14 days. Furthermore, it investigated the stabilising influence of storage in a saturated mannitol solution and a saturated lactose solution (Table 8).

Table 8: Thermal stability study of chymotrypsin at 50°C with and without mannitol or lactose for 14 days; n=3, +/- CI

	1 day	4 days	7 days	14 days
Protein concentration	**activity [%]**	**activity [%]**	**activity [%]**	**activity [%]**
1.5 mg/mL	53.2 (± 2.12)	37.9 (± 1.86)	22.8 (± 4.59)	14.4 (± 1.34)
3.0 mg/mL	43.4 (± 1.09)	32.3 (± 1.58)	23.1 (± 2.05)	6.88 (± 1.08)
7.5 mg/mL	23.8 (± 1.49)	17.5 (± 1.60)	9.20 (± 2.26)	1.80 (± 1.97)
1.5 mg/mL + lactose	62.0 (± 3.20)	36.7 (± 3.40)	20.5 (± 1.27)	10.2 (± 0.11)
3.0 mg/mL + lactose	55.1 (± 0.86)	35.6 (± 0.87)	23.6 (± 0.89)	13.0 (± 0.67)
7.5 mg/mL + lactose	28.6 (± 0.88)	21.1 (± 0.87)	17.2 (± 1.24)	7.21 (± 0.47)
1.5 mg/mL + mannitol	75.8 (± 2.12)	61.4 (± 1.86)	54.6 (± 4.59)	28.1 (± 1.34)
3.0 mg/mL + mannitol	71.3 (± 1.09)	59.2 (± 1.58)	41.0 (± 2.05)	28.3 (± 1.08)
7.5 mg/mL + mannitol	44.2 (± 1.49)	35.5 (± 1.60)	27.7 (± 2.26)	19.3 (± 1.97)

The enzyme activity at day 0 was 46.51 u/mg ± 2.66 u/mg. This value was set to 100% and the remaining activity was calculated for the later time points. The pure chymotrypsin samples showed a prominent decrease in enzyme activity with increasing storage time. Furthermore, a concentration dependency occurred: the higher the concentration, the lower the remaining activity (Table 8). This effect probably relied on the self-degrading effect of the peptidase. The sample with 7.5 mg/mL showed values that were around half of the activity of the 1.5 mg/mL samples for almost all measurement points. After 14 days almost no activity remained for the 7.5 mg/mL samples. The values of the 3.0 mg/mL samples were in between the values of the other two concentrations.

The addition of mannitol led to a higher remaining activity for all chymotrypsin concentrations and all time points which indicated a stabilising effect. The effect occurred in a comparable amount for all examined protein concentrations (Figure 34, Table 8).

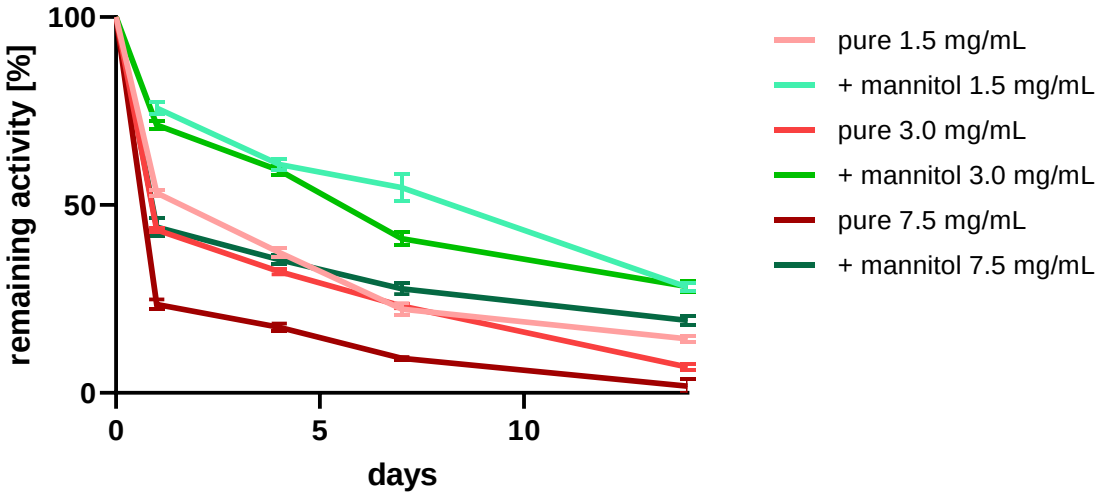

Figure 34: Thermal stability study of chymotrypsin at 50°C with and without mannitol for 14 days; the mean curve of n=3, error bars=CI

The addition of lactose only led to a remaining activity similar to or only very slightly higher than the pure chymotrypsin samples indicating no stabilising effect. The effect occurred in a similar amount for all examined protein concentrations (Figure 35, Table 8).

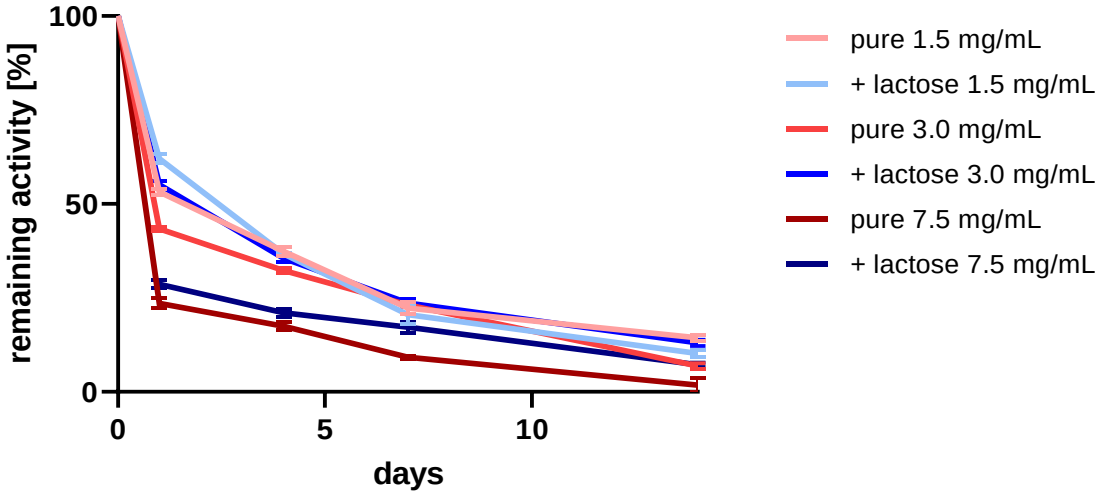

Figure 35: Thermal stability study of chymotrypsin at 50°C with and without lactose for 14 days; the mean curve of n=3, error bars=CI

In a second thermal stability study the influence of different temperatures was investigated. It compared the remaining activity of chymotrypsin samples with a concentration of 3.0

mg/mL dissolved in water to samples dissolved in a saturated mannitol solution. The solutions were stored at 40°C, 45°C and 50°C for 7, 14, 21 and 28 days. Furthermore, samples were stored at 60°C for 1, 4 and 7 days. The remaining activity decreased with increasing temperature and time, thereby demonstrating a negative influence of these parameters on protein stability. A stabilising effect of storage in saturated mannitol solution was found for all examined samples.

For the samples stored at 40°C, the activity decreased the least (Figure 36).

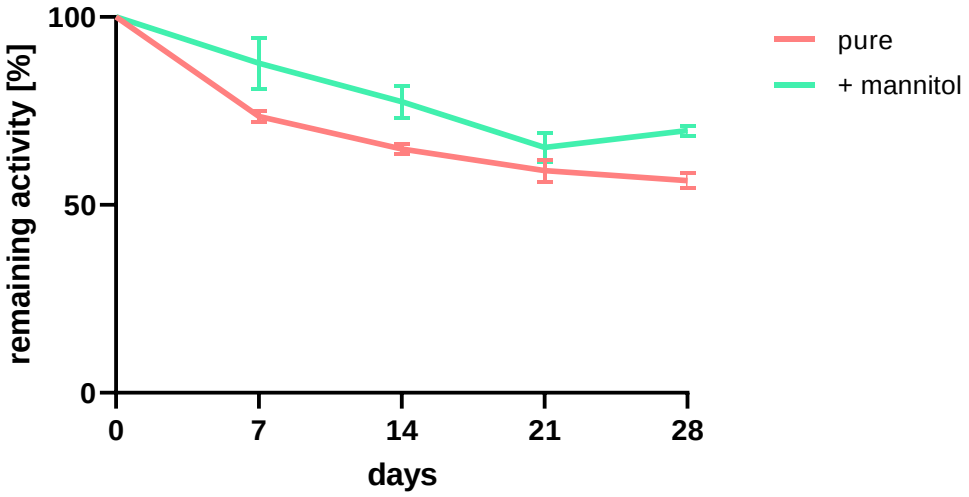

Figure 36: Thermal stability study of chymotrypsin at 40°C for 28 days; mean curve of n=3, error bars=CI

Only a small difference between the samples stored with and without mannitol with a remaining activity after 28 days of 69.8% and 56.5% occurred (Table 9).

Table 9: Thermal stability study of chymotrypsin at 40°C, 45°C and 50°C with and without mannitol as stabilising excipient for 28 days; n=3, +/- CI

	7 days	14 days	21 days	28 days
Sample conditions	**activity [%]**	**activity [%]**	**activity [%]**	**activity [%]**
40°C	73.5 (± 1.75)	64.9 (± 1.13)	59.1 (± 2.51)	56.5 (± 1.78)
40°C + mannitol	86.1 (± 5.80)	77.4 (± 3.60)	65.3 (± 3.21)	69.8 (± 1.15)
45°C	54.4 (± 0.97)	38.3 (± 6.32)	33.9 (± 1.12)	16.8 (± 5.38)
45°C + mannitol	73.1 (± 1.17)	66.9 (± 1.58)	60.2 (± 2.02)	55.4 (± 2.99)
50°C	20.7 (± 6.94)	11.7 (± 3.92)	0.12 (± 0)	0 (± 0)
50°C + mannitol	50.2 (± 3.14)	41.5 (± 1.88)	30.9 (± 0.28)	11.4 (± 1.01)

Storage at 45°C led to a medium decrease in activity. At this temperature, the storage in saturated mannitol solution highly stabilised the protein (Figure 37).

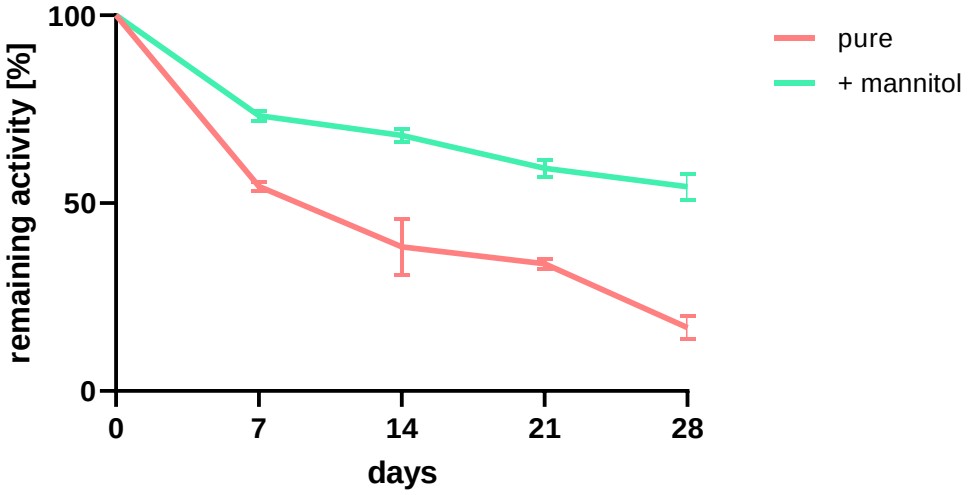

Figure 37: Thermal stability study of chymotrypsin at 45°C for 28 days; mean curve of n=3, error bars=CI

After 28 days 16.8% of the activity remained for the samples without mannitol and 55.4% for the samples with mannitol (Table 9). After storage at 50°C for 28 days, no activity remained for the samples without mannitol (Figure 38).

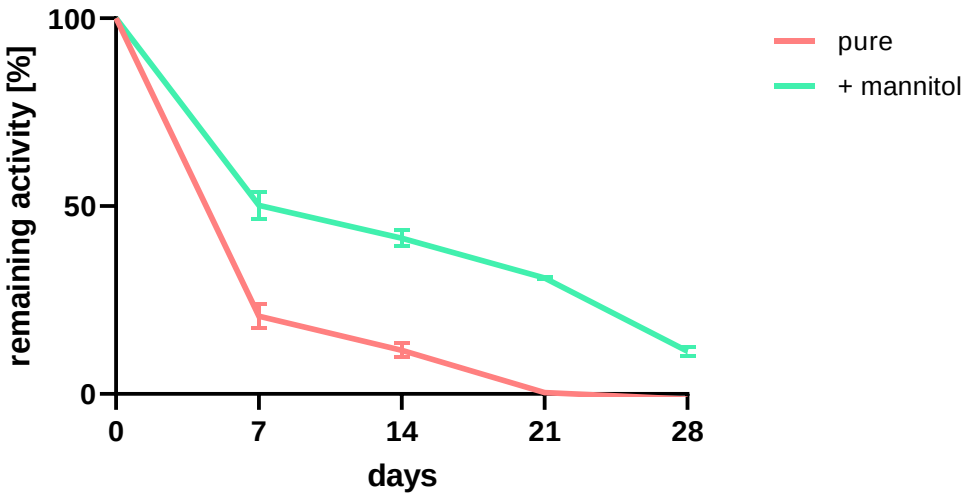

Figure 38: Thermal stability study of chymotrypsin at 50°C for 28 days; mean curve of n=3, error bars=CI

The samples with mannitol, however, still showed an activity of 11.4% (Table 9).

Furthermore, the samples containing additional mannitol exhibited a prolonged half-life of 37.4 days compared to 35.7 days at 40°C, 37.0 days to 12.1 days at 45°C and 10.0 days to 2.3 days at 50°C.

For the samples stored at 60°C without mannitol, after 1 day, no activity remained (Figure 39). For the samples stored at 60°C with mannitol, the activity decreased to 59.49% after 1 day, 16.77% after 4 days and to almost no activity (4.44%) after 7 days (Figure 39).

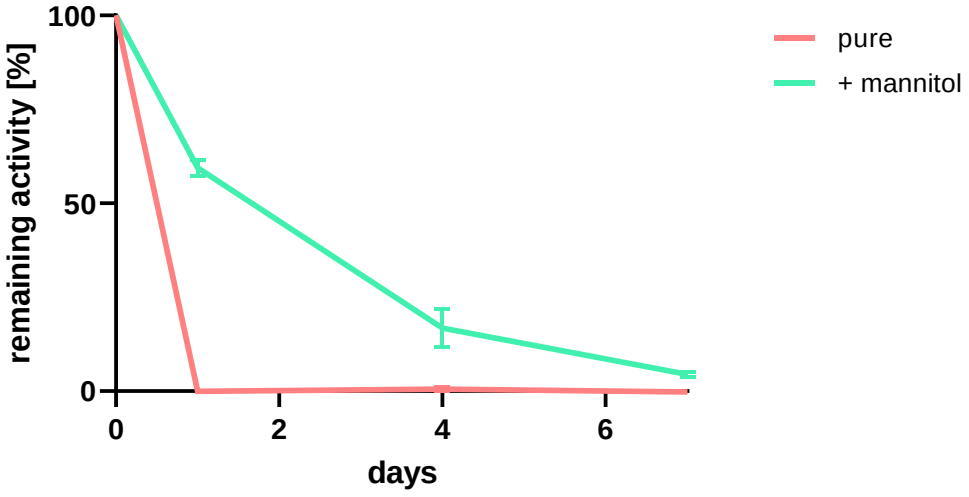

Figure 39: Thermal stability study of chymotrypsin at 60°C for 7 days; mean curve of n=3, error bars=CI

Therefore, no longer-lasting studies were performed at this temperature.

The thermal stability study demonstrated a negative influence of the stress factors heat and time. Increasing this stress increased the destabilisation resulting in a lower remaining enzyme activity. Furthermore, the data demonstrated a stabilising effect of mannitol on the model protein chymotrypsin. This can be explained by the well-investigated protein-stabilising effect of sugars and sugar alcohols against thermal stress [15]. Lactose did not show this stabilising effect. This probably relies on the Maillard-reaction which reducing sugars, like lactose, can undergo with proteins and which can have a destabilising effect [15]. Nevertheless, none of the present samples turned brown, which would have been a strong indicator of the Maillard-reaction. Furthermore, the study found a decreasing stability of chymotrypsin with increasing concentration. Protein stability in general can be influenced by the protein concentration of the solution since proteins tend to aggregate more easily in higher concentrations. Chymotrypsin in particular is a peptidase and can catalyse the hydrolysis of peptide bonds [102]. This effect is very likely not specific to certain kinds of proteins and therefore also happening between chymotrypsin molecules. The enzymatic destruction of chymotrypsin is likely concentration-dependent.

Mannitol was found to be an effective stabiliser for chymotrypsin against thermal stress and might be a suitable stabiliser during the drying phase of the spray drying process (see Section 7.3.2.2).

7.2.2 Influence of Dissolution Media on Chymotrypsin

This study investigated the effect of dissolving chymotrypsin in potentially degrading dissolution media in order to get an idea of how sensitive chymotrypsin is to these stress factors. Furthermore, the stabilising influence of mannitol and lactose against this stress was examined. It compared the remaining enzyme activity of protein samples dissolved in the pure dissolution media and samples dissolved in dissolution media that were saturated with mannitol or lactose. The measurements were taken immediately after dissolution under room conditions. The ethanol-water mixtures were examples of degrading solvents. With HCl 3.6% the influence of acidic conditions on the protein was analysed.

In ethanol-water-mixtures, the protein stability decreased with increasing ethanol concentration. This led to a remaining activity of 73.2% in 10% EtOH and 48.1% in 20% EtOH (Table 10).

Table 10: Remaining activity of chymotrypsin in different dissolution media with and without mannitol or lactose, n=3, +/- CI

	Pure chymotrypsin	Chymotrypsin + mannitol	Chymotrypsin + lactose
Solvent	**activity [%]**	**activity [%]**	**activity [%]**
HCl 3.6%	47.2 (± 2.26)	56.7 (± 1.50)	49.4 (± 1.45)
EtOH 10%	73.2 (± 2.17)	78.0 (± 1.47)	82.7 (± 1.88)
EtOH 20%	48.1 (± 1.22)	56.0 (± 1.09)	57.4 (± 1.25)

Mannitol and lactose both showed a small stabilising effect with a remaining activity of 78.0% (10% EtOH), 56.0% (20% EtOH), 82.7% (10% EtOH) and 57.4% (20% EtOH) (Table 10).

The highest impact on the protein stability had the dissolution in 3.6% HCl (remaining activity 47.2% (Table 10)). In HCl, only mannitol slightly stabilised the protein with a remaining activity of 56.7% while lactose showed results similar to the pure protein with 49.4% (Table 10).

Although some examples of spray drying proteins in ethanol-water mixtures [101] exist, in the present work those mixtures highly degraded chymotrypsin. Therefore, water was chosen as dissolution media as storage in aqueous solution only decreased chymotrypsin´s enzyme activity after several days in heightened temperature (see above). Since it was only slightly influenced by storage at 40°C for one week, it is very likely that the protein is not significantly degraded in the spray drying feed solution at room temperature during the process time.

7.2.3 Influence of the Excipients on the Surface Tension

This section compared the influence of the surfactants on the surface tension of the protein solutions. All protein solutions containing additional polysorbate 20 had surface tensions comparable to that of the pure 0.1% polysorbate 20 solution (Table 11).

Table 11: Surface tension of protein aqueous solutions with and without 0.1% polysorbate 20 and polysorbate 80, n=3, +/- CI

	Polysorbate 20 0.1%	**Polysorbate 80 0.1%**
	Surface tension [mN/m]	**Surface tension [mN/m]**
Ovalbumin	35.23 (± 0.10)	39.01 (± 0.32)
BSA	34.94 (± 0.27)	42.32 (± 1.08)
Chymotrypsin	31.92 (± 0.19)	36.98 (± 0.19)
Lysozyme	34.15 (± 0.47)	37.88 (± 0.31)
Surfactant solution	35.04 (± 0.31)	42.14 (± 0.55)

The solutions containing ovalbumin, BSA or lysozyme and polysorbate 20 showed very similar values (Table 11). For the solution containing chymotrypsin and polysorbate 20, the surface tension was slightly lower (Table 11).

The solutions containing additional polysorbate 80 influenced the protein differently (Table 11). For the solution containing BSA and polysorbate 80 the surface tension was very similar to that of the pure polysorbate solution. The solutions containing ovalbumin or lysozyme and polysorbate 80 showed values slightly lower. The solution containing chymotrypsin and polysorbate 80 had a surface tension very similar to the surface tension of the pure chymotrypsin solution.

The addition of polysorbate 20 to the protein solution led to an alignment of the surface tension close to the pure polysorbate 20 solutions. This may be caused by the surfactant´s higher tendency to adsorb at the surface and expel the proteins from it. Although polysorbate 80 should show a comparable behaviour it did not seem to influence the surface tension of the chymotrypsin solution.

Begum et al. found a similar effect [103]. They also compared the influence of polysorbate 20, polysorbate 80 and additional poloxamer P188 on the surface activity of different proteins. Their results demonstrated that the type of protein has an impact on the competitive surface adsorption of different surfactants [103]. The surfactants were not able to expel the more surface-active proteins from the surface as they were able to do with less surface-active proteins. The intensity of this phenomenon was also different for the different surfactants.

It is, therefore, possible that polysorbate 80, despite being generally known to do so with other proteins, did not expel the more surface-active chymotrypsin from the surface. Polysorbate 80 might not stabilise this protein against air-liquid interface stress during spray drying. Polysorbate 20, which also influenced the surface activity of the most surface-active protein, chymotrypsin, might expel the protein from the surface and protect it against air-liquid interface stress during spray drying. Polysorbate 20 was, therefore, chosen as an excipient during the spray drying process of the proteins.

The previous section identified mannitol as a suitable stabiliser for chymotrypsin against thermal stress. Glycine is generally known to be an unspecific but very suitable stabiliser for the spray drying of proteins. Both excipients were also tested as stabilisers in this work (see Section 7.3.2.2). Also, both excipients are not known to be interface active their impact on the solution surface was tested. The addition of 4 mg/mL glycine and 4 mg/mL mannitol sightly influenced the interface activity of the proteins (Table 12).

Table 12: Surface tension of protein aqueous solutions with the addition of glycine and mannitol, n=3, +/- CI

	mQ with the addition of glycine and mannitol	**Polysorbate 20 0.1% with the addition of glycine and mannitol**
	Surface tension [mN/m]	**Surface tension [mN/m]**
Ovalbumin	48.56 (± 0.40)	38.73 (± 0.14)
BSA	56.18 (± 0.21)	39.90 (± 0.51)
Chymotrypsin	40.15 (± 0.17)	35.53 (± 0.08)
Lysozyme	65.83 (± 1.04)	35.48 (± 0.07)
Excipient solution	71.68 (± 0.11)	36.47 (± 0.23)

The surface tension of glycine and mannitol in water (Table 12) was very close to that of pure water which is generally known to be 72.75 mN/m at 20°C. Also, the value of glycine and mannitol in 0.1% polysorbate 20 was very close to that of the 0.1% polysorbate 20 solution (Table 12). Nevertheless, the surface tension of all protein solutions with and without polysorbate increased a few mN/m with the addition of glycine and mannitol (Table 5, Table 12).

Glycine and mannitol are not surface active on their own. They do, however, interact with the proteins, which seemed to slightly influence the interface activity of the proteins. Furthermore, dissolving the excipients in water makes the solution slightly less hydrophilic, this might lower the tendency of the proteins to accumulate at the surface.

7.2.4 Selection of Excipients based on their Stabilising Potential

The section identified different stress factors that negatively influence the enzymatic activity of chymotrypsin. Furthermore, it found that stabilising excipients possessed the potential to preserve the protein's activity after exposition to these stress factors. It also exhibited that the tested excipients have a different influence on the interface activity of the proteins.

Thermal stress decreased enzyme activity with increasing time and intensity. Furthermore, organic solvents and acids highly degraded chymotrypsin. Mannitol had a good stabilising effect against thermal stress and a small effect against acidic conditions. In both cases, it increased the remaining activity of the stressed protein. Lactose, being a reducing sugar, did not stabilise the protein against these stress factors. Both excipients, however, showed a similar stabilising effect against stress caused by ethanol-water mixtures. Last, a decreasing remaining activity and therefore an increasing degradation occurred for all samples with increasing protein concentration. This indicates a self-degrading effect of chymotrypsin. The section found mannitol to be capable of stabilising the model protein chymotrypsin against thermal stress. It was, therefore, chosen as a stabilising excipient against the thermal stress during the spray drying process.

Surfactants are generally known to expel proteins from the surface and thereby protect them from interfacial stress but their ability to do so highly depends on the certain protein. This was also found in the present work. Polysorbate 20 seemed to influence the interface activity of all model proteins and was chosen for the spray drying experiments. It does, however, interfere with the extrinsic fluorescence assay and could not be investigated in the stability studies.

7.3 Discrimination of Influences during the Spray Drying Process

This section investigated the influence of different stress factors during the spray drying process. It compared the different phases of the spray drying process, different spray drying systems, various parameters and the influence of stabilising excipients. It used the model protein chymotrypsin in a concentration of 4.0 mg/mL in an aqueous solution and compared the native and denatured protein with the produced samples.

7.3.1 Nano Spray Dryer

The study compared three different phases of spray drying with the nano spray dryer (Figure 40).

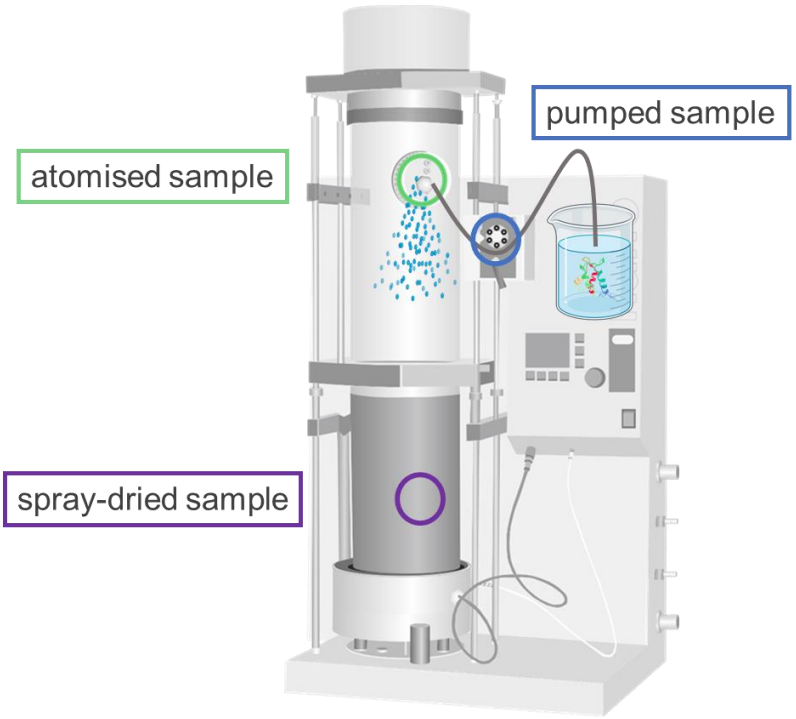

Figure 40: Samples were taken at the phases of spray drying at the nano spray dryer; created with BioRender

The first phase was the pumping process. The nano spray dryer pumped the samples through the tubing system and the spray head which atomised a part of the sample solution while the rest was pumped in a circle. During the pumping process, the protein may have been exposed to adsorption to the silicone tubing material, shortly to thermal stress and shear-related stress. The second phase was atomisation. A special construction allowed the collection of the solution right after atomisation (Figure 41).

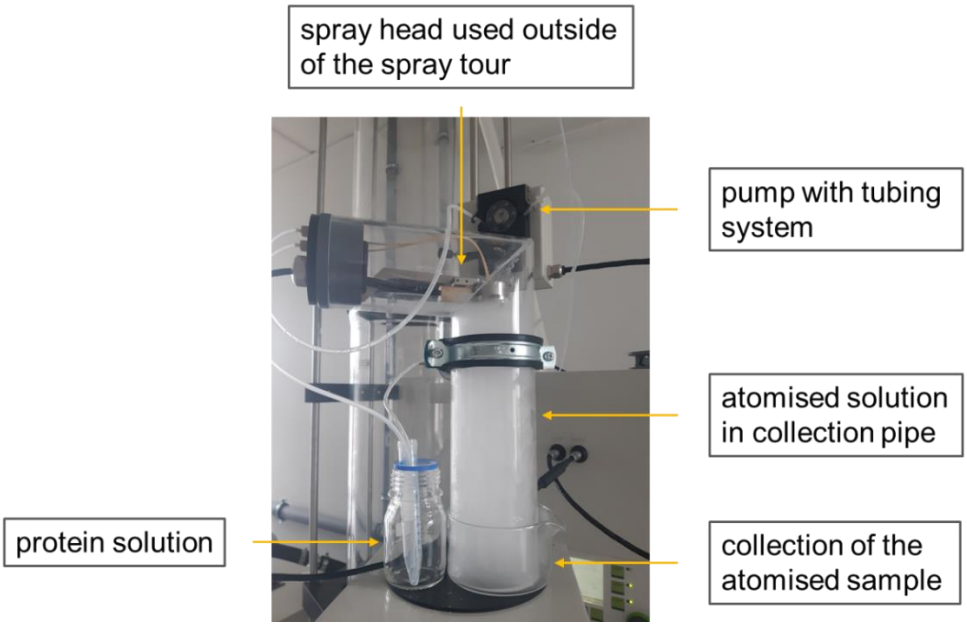

Figure 41: Construction for sample collection after atomisation before spray drying for the nano spray dryer

The atomisation in the spray head exposed the protein to shear-related stress, air-liquid interface stress and ultrasonic frequencies. The last phase was the actual drying phase. This additionally exposed the protein to thermal and dehydration stress.

This study used the piezoelectric spray head with the medium-sized membrane, a pump rate of 100%, a spray rate of 80% and an ultrasonic frequency of 125 Hz. Spray drying was performed with an inlet temperature of 120°C and an outlet temperature of 45°C.

The enzyme activity was determined for the chymotrypsin samples after pumping, atomisation and drying. Compared to the original activity the pumped and atomised samples did not change significantly in activity. A small activity decrease was found in the spray-dried samples with a remaining activity of 86.7% ± 1.97%.

The ANS fluorescence emission spectra showed distinct differences for the investigated samples (Figure 42).

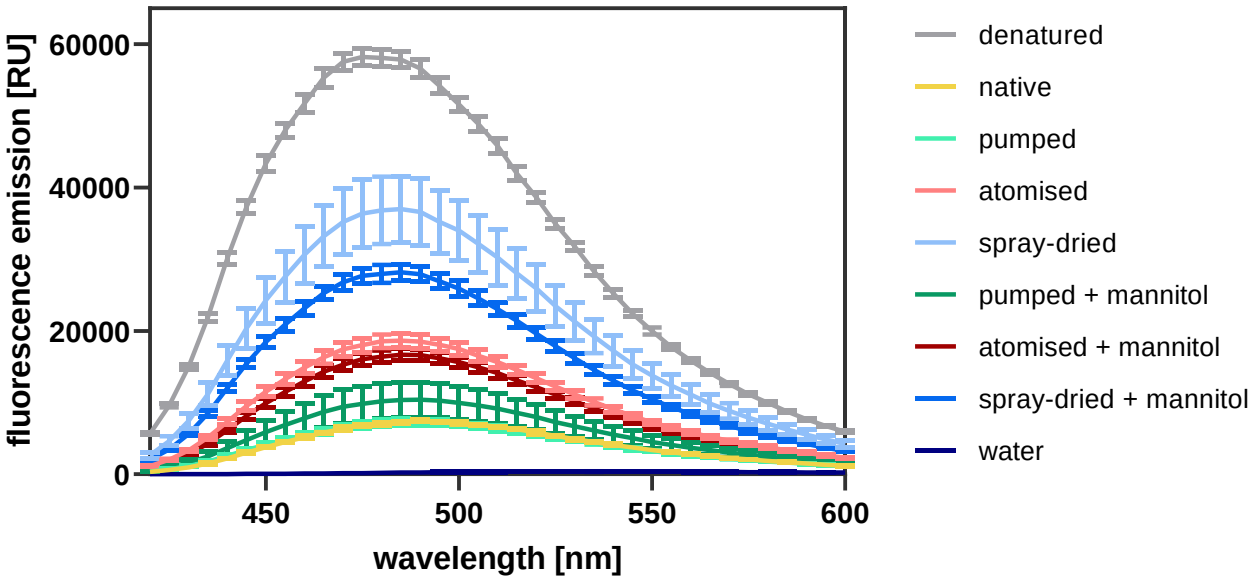

Figure 42: Fluorescence emission spectra of chymotrypsin in the different spray drying phases of the nano spray dryer with and without mannitol as stabilising excipients; the mean curve of n=3, error bars=CI

The atomised and the spray-dried samples displayed different emission spectra compared to the native protein. For these samples, the emission intensity was between the denatured and the native protein (Figure 42). However, for the spray-dried samples, it increased even higher to a maxima ratio of 0.58 compared to the atomised samples with a maxima ratio of 0.22 (Table 13).

Table 13: Influence of the different phases of spray drying of the nano spray dryer on the tertiary structure of chymotrypsin with and without mannitol as stabilising excipient; maxima ratio and blueshift of each mean curve

Sample	Maxima ratio	Blueshift
Chymotrypsin spray-dried	0.58	5 nm
Chymotrypsin atomised	0.22	5 nm
Chymotrypsin pumped	0.03	0 nm
Chymotrypsin + mannitol spray-dried	0.41	5 nm
Chymotrypsin + mannitol atomised	0.18	5 nm
Chymotrypsin + mannitol pumped	0.06	5 nm

Almost no changes in the emission spectra occurred for the pumped samples with a maxima ratio of 0.03 (Figure 42, Table 13). For the native protein and the pumped samples, the

emission maximum was at a wavelength of 490 nm, for the atomised and spray-dried samples 485 nm and for the denatured protein 475 nm (Figure 42). The data, therefore, indicated no influence of the pumping phase, a small influence of the atomisation phase and a high influence of the drying phase on the protein structure of chymotrypsin.

The second part of the experiment investigated the influence of the excipient mannitol on protein stability. 4.0 mg/mL mannitol was added to the chymotrypsin solution and samples were taken after the three phases of spray drying.

The changes in the enzyme activity were similar to the changes in the samples without mannitol. While no changes occurred for the pumped and atomised samples, a small decrease occurred after spray drying with a remaining activity of 81.8% ± 5.67%. Although this remaining activity was slightly lower than that of the samples spray-dried without mannitol, the difference is not significant (p-value 0.128). The measurement of the remaining activity did, therefore, not indicate differences between the two samples.

In the ANS fluorescence emission spectra, the emission intensity increase of the spray-dried sample was slightly lower than that after spray drying without mannitol with a maxima ratio of 0.41 (Figure 42, Table 13). This indicates a small stabilising effect. For the pumped and the atomised samples, similar emission intensities with a maxima ratio of 0.06 and 0.18 occurred (Figure 42, Table 13), indicating no effect of mannitol during these spray drying phases. For the spray-dried and atomised samples, a blueshift of 5 nm occurred (Figure 42, Table 13). No blueshift occurred for the pumped samples (Figure 42, Table 13).

The different phases of spray drying influenced the protein in different ways. The spray drying phase caused the major changes, the atomisation phases caused a lower change and the pumping phase had no or only minor effects. This demonstrates that the drying phase with the combination of all stress factors had the highest impact on the protein structure. The difference in the emission maxima between the spray-dried and the atomised samples is highly significant with a p-value of $1.01*10^{-5}$. The changes cannot only be explained by thermal and dehydration stress but also seem to be caused by the combination of ultrasonic frequencies, shear-related and high interfacial stress as some changes were detected directly after atomisation.

The samples with additional mannitol showed a similar trend. For the spray-dried samples, however, a small stabilising effect, compared to the samples without mannitol, occurred. The change from a maxima ratio of 0.58 to 0.41 also was very significant with a p-value of 0.0023.

This seems reasonable since sugars and sugar alcohols are known to be good protein stabilisers against heat and dehydration stress [15]. For the atomised and the pumped samples, only minor effects occurred with a change from 0.22 to 0.18. The change was very significant with a p-value of 0.0034 but due to the very small value possibly not relevant.

7.3.2 Mini Spray Dryer

This part of the work compared the different spray drying phases using the mini spray dryer (Figure 43).

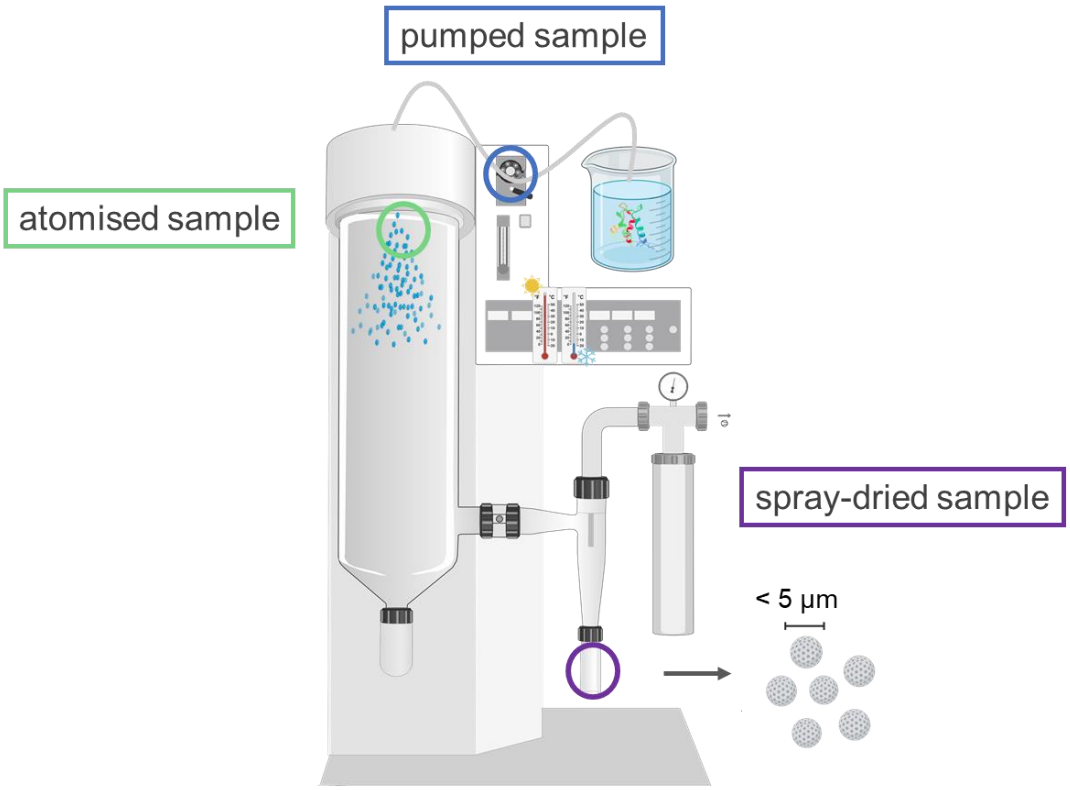

Figure 43: Samples were taken at the phases of spray drying using the mini spray dryer; created with BioRender

The mini spray dryer uses other spray drying nozzles than the nano spray dryer and collects the dried particles using a cyclone instead of a collection electrode.

Samples were taken after the pumping, the atomisation and the drying phase. Protein structure changes were detected using the extrinsic fluorescence assay. Two common nozzles were used: a two-fluid and an ultrasonic nozzle. For the atomised samples, a construction that allowed collecting directly after atomisation was used (Figure 44).

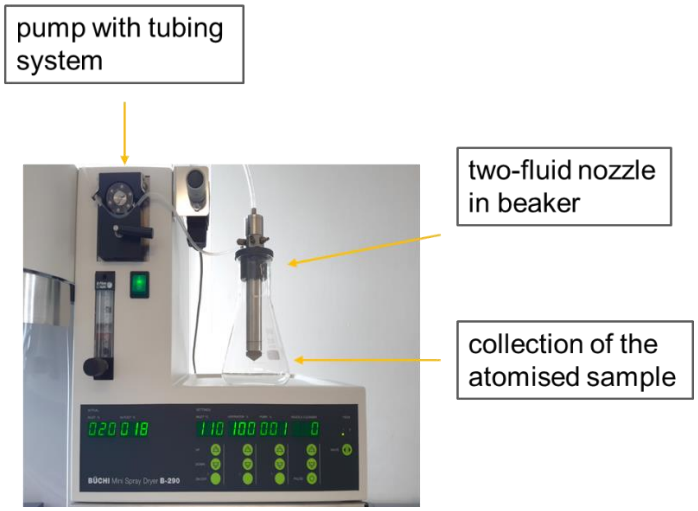

Figure 44: Construction for sample collection after atomisation before spray drying for the mini spray dryer

The two-fluid nozzle exposed the protein to shear-related stress and air-liquid interface stress. The ultrasonic nozzle additionally exposed it to ultrasonic frequencies. Due to technical variations different spray drying conditions had to be chosen (Table 14).

Table 14: Spray drying parameters of the mini spray dryer

Parameter	Two-fluid nozzle	Ultrasonic nozzle
Inlet temperature	150°C	150°C
Outlet temperature	80-90°C	55-65°C
Airflow	100%	50%

The larger droplets produced with the ultrasonic nozzle in this work required a longer drying period than the smaller droplets of the two-fluid nozzle. This led to the need for a slower airflow and a lower outlet temperature. For the pumped samples the fluorescence spectra overlaid with the spectra of the native proteins (Figure 45).

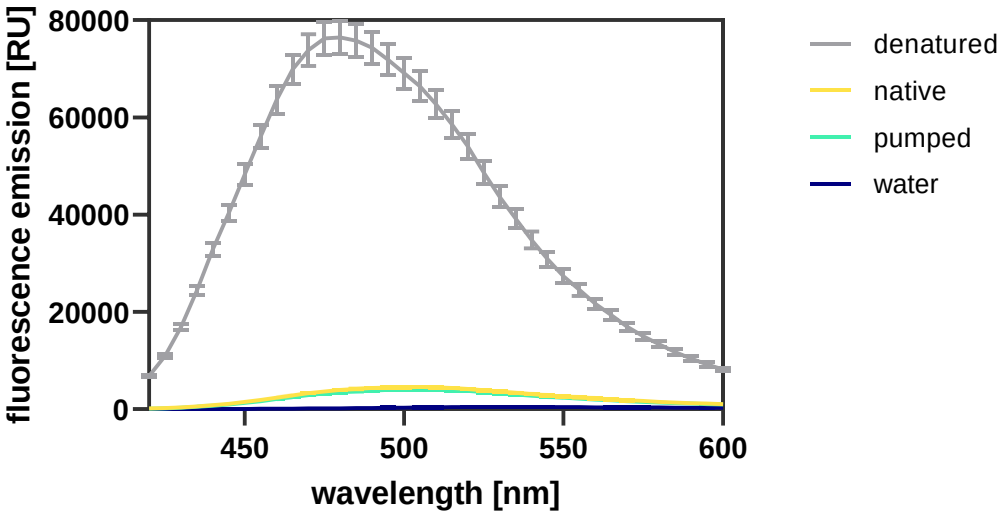

Figure 45: Fluorescence emission spectra of the pumping phase with the mini spray dryer; the mean curve of n=3, error bars=CI

The pumping phase does not seem to change the protein structure of chymotrypsin.

Almost no change occurred in the fluorescence emission spectra of the atomised samples. They had a maxima ratio of 0.08 (two-fluid nozzle) and a maxima ratio of 0.04 (ultrasonic nozzle) (Figure 46, Table 15).

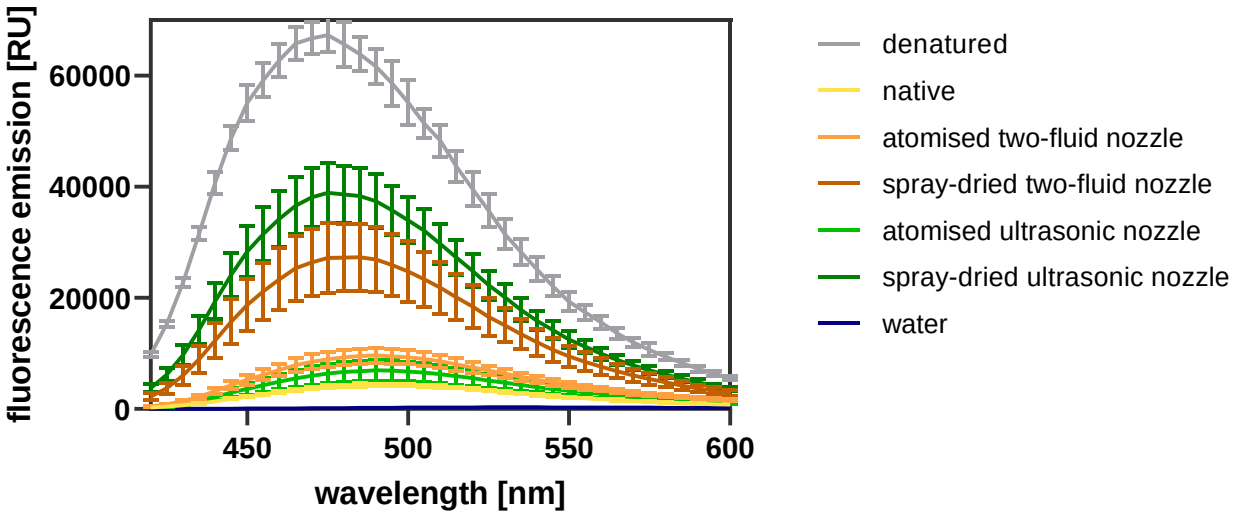

Figure 46: Fluorescence emission spectra of the atomisation and drying phase with the mini spray dryer; the mean curve of n=3, error bars=CI

Table 15: Influence of the different spray drying phases of the two-fluid and the ultrasonic nozzle on the tertiary structure of chymotrypsin; maxima ratio and blueshift of each mean curve

Sample	Maxima ratio	Blueshift
Chymotrypsin spray-dried two-fluid nozzle	0.36	5 nm
Chymotrypsin atomised two-fluid nozzle	0.08	0 nm
Chymotrypsin spray-dried ultrasonic nozzle	0.55	5 nm
Chymotrypsin atomised ultrasonic nozzle	0.04	0 nm
Chymotrypsin pumped	Overlay with the curve of the native protein	

Both spectra showed no blueshift (Figure 46). This indicates that atomisation with these nozzles causes no or only minor changes in the structure of chymotrypsin. For both spray-dried samples, the emission spectra changed. The emission intensity and the emission maxima lay between the native and the denatured protein (Figure 46). After spray drying with the two-fluid nozzle, the maxima ratio was 0.36 and after spray drying with the ultrasonic nozzle 0.55 (Table 15). Both maxima shifted 5 nm to blue (Table 15). For both samples, a small decrease in enzyme activity occurred with a remaining activity of 85.0% ± 1.50% for the two-fluid nozzle and 83.8% ± 0.95% for the ultrasonic nozzle. The data demonstrates that the full spray drying process interferes with the protein structure of chymotrypsin. Although the decrease in enzyme activity was similar after spray drying with both nozzles, the fluorescence assay indicates a larger influence of the ultrasonic nozzle.

7.3.2.1 Spray Drying Temperatures

This part of the study investigated the influence of the spray drying temperature on the model protein chymotrypsin. Therefore, spray drying was performed at the lowest temperature possible with the two-fluid nozzle at the Mini-Büchi. 50°C inlet and 35°C outlet temperature was the lowest temperature profile at which the spray drying process was still working without technical difficulties for the protein solution. At an outlet temperature of 30°C and inlet temperature of 22°C, the process did not fully work. The major part of the product was not fully dried and adhered to the walls of the spray dryer. Despite the semi-functional process, enough spray-dried product was produced for analysis.

Figure 47 illustrates the spectrum of chymotrypsin spray-dried at 50°C inlet and 35°C outlet temperature compared to the native and denatured protein.

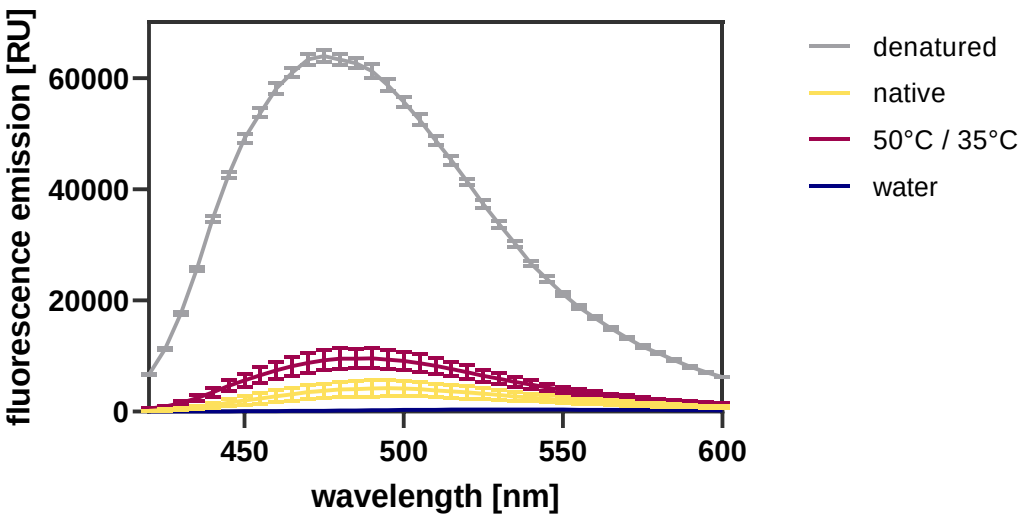

Figure 47: Influence of spray drying with 50°C inlet and 35°C outlet temperature on the tertiary structure of chymotrypsin; mean curve of n=3, error bars=CI

Although the maxima ratio was very small (0.09) a blueshift of 5 nm occurred (Table 16) which still indicates a small change in the protein structure.

Table 16: Influence of the spray drying temperature on the tertiary structure of chymotrypsin; maxima ratio and blueshift of each mean curve

Sample	Maxima ratio	Blueshift
Chymotrypsin spray-dried inlet 150°C/ outlet 90°C	0.36	5 nm
Chymotrypsin spray-dried inlet 50°C/ outlet 35°C	0.09	5 nm
Chymotrypsin spray-dried inlet 30°C/ outlet 22°C	0.21	10 nm

The second spectra show the fluorescence emission of chymotrypsin after spray drying at 30°C inlet and 22°C outlet temperature in comparison to the native and denatured protein (Figure 48).

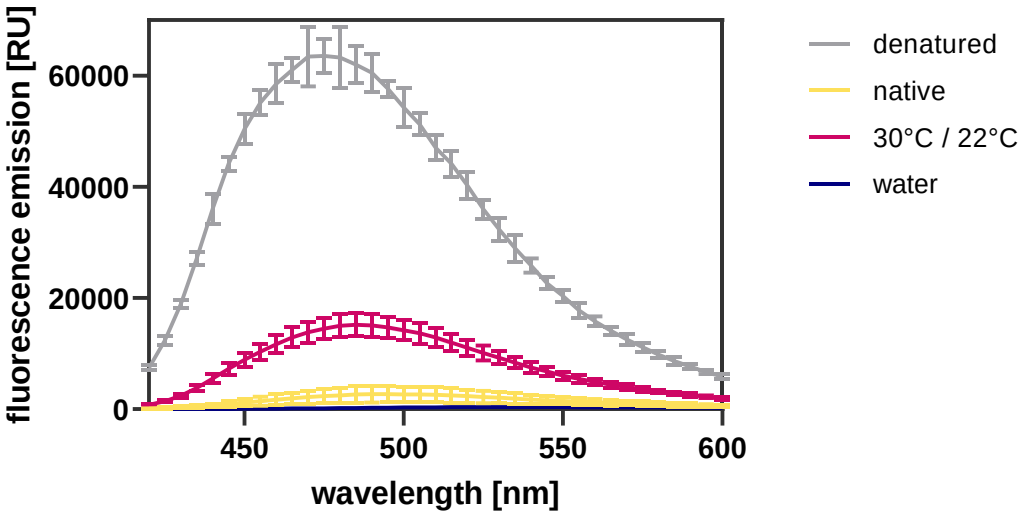

Figure 48: Influence of spray drying with 30°C inlet and 22°C outlet temperature on the tertiary structure of chymotrypsin; mean curve of n=3, error bars=CI

Here, the protein changed more than under previous spray drying conditions with a maxima ratio of 0.21 and a blueshift of 10 nm (Table 16). When comparing the maxima ratios of 150°C inlet temperature to 30°C inlet temperature a very significant change (p-value 0.0049) from 0.36 to 0.21 was found. This indicated a small change in protein structure. Spray drying at 50°C inlet led to an even larger maxima ratio decrease from 0.36 to 0.09 with a high significance (p-value $9.8*10^{-5}$). This temperature profile seemed to have the lowest influence on chymotrypsin´s tertiary structure. The lower protein degradation of 50°C inlet temperature compared to 30°C inlet temperature very likely relied on the aforementioned major technical problems during the spray drying process at 30°C inlet temperature. Even though spray drying at 50°C inlet temperature appeared to be less stressful for the protein than 150°C inlet temperature, it still changed the protein structure. The intensity change was very similar to the change of the samples atomised with the two-fluid nozzle (maxima ratio 0.08). The samples spray-dried at 50°C inlet additionally shifted 5 nm to blue which may indicate a larger structural change and an additional influence of the dehydration stress. The difference between the two samples, however, was too small for further interpretation.

7.3.2.2 Influences of Excipients

This part of the study investigated the influence of excipients on the stability of chymotrypsin during the different phases of spray drying with the two-fluid nozzle. The previous sections identified the drying phase with dehydration and thermal as crucial destabilising factors during spray drying. Sugars and sugar alcohols are generally known to be good stabilisers against these factors (see Section 3.5.1.3). Therefore, this section investigated mannitol as a stabilising excipient for chymotrypsin. Since also spray drying at very low temperatures

seemed to influence chymotrypsin, glycine was chosen as an amnio acid to generally stabilise the protein during the spray drying process (see Section 3.5.1.3). The section examined the influence of the addition of mannitol and glycine each in a concentration equal to the concentration of the protein (4.0 mg/mL). Also, the addition of the combination of mannitol and glycine each in in the concentration of 4.0 mg/mL was tested, to investigate if a synergistic stabilising effect of the two excipients exists.

The spectra of the pumped and atomised samples with additional excipients overlaid with the spectrum of the native chymotrypsin for all protein-excipient combinations (Figure 49, Figure 50, Figure 51).

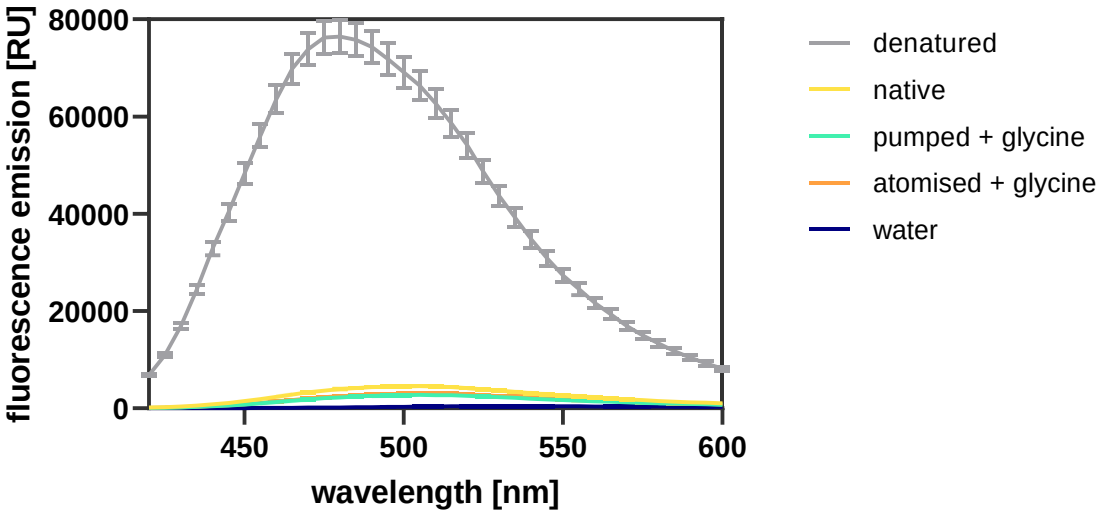

Figure 49: Influence of co-processing with glycine as stabilising excipient in the pumping and the atomising phase; the mean curve of n=3, error bars=CI

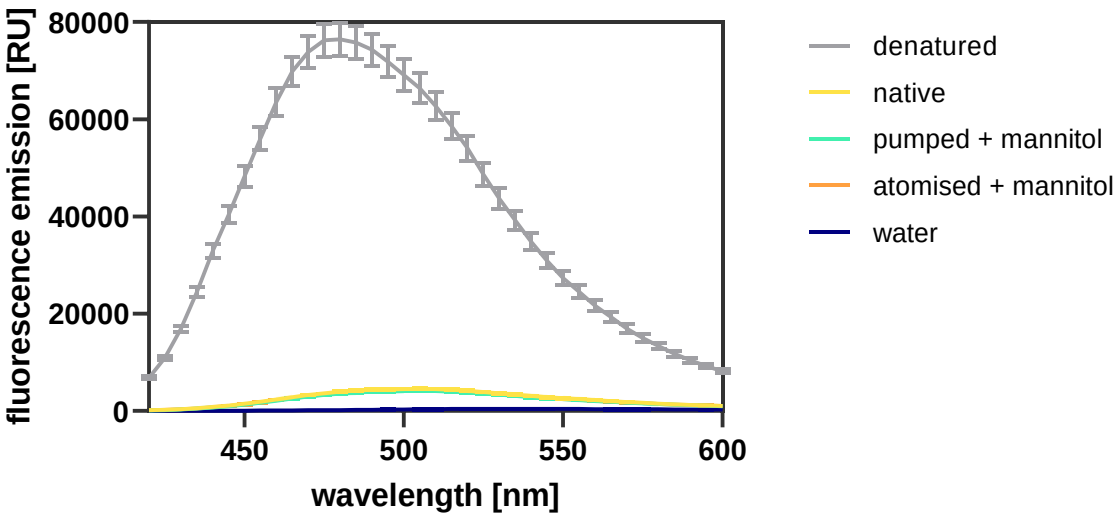

Figure 50: Influence of co-processing with mannitol as stabilising excipient in the pumping and the atomising phase; the mean curve of n=3, error bars=CI

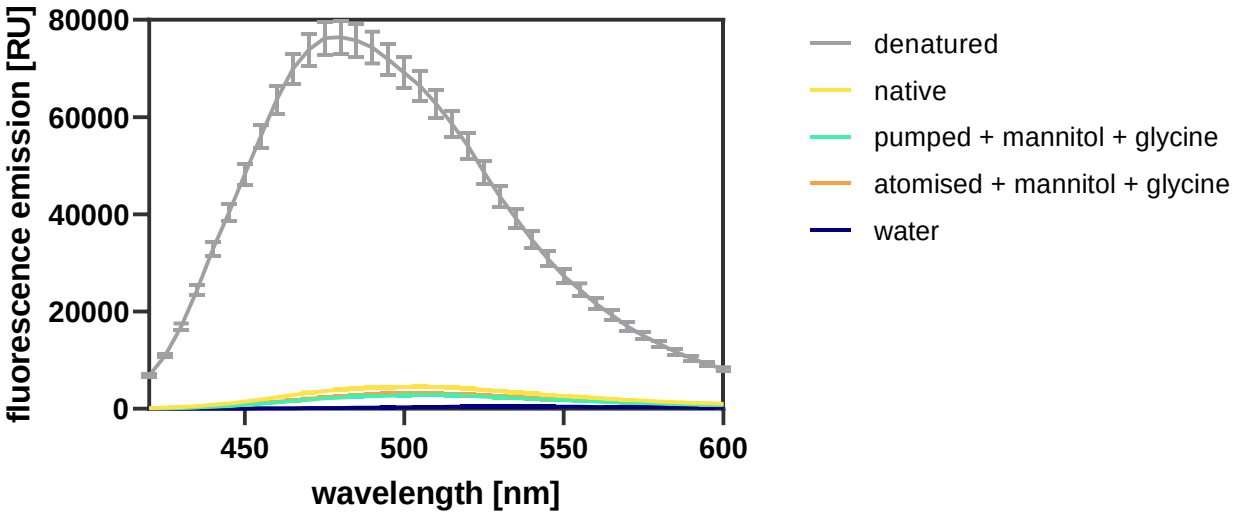

Figure 51: Influence of co-processing with glycine and mannitol as stabilising excipients in the pumping and the atomising phase; the mean curve of n=3, error bars=CI

This indicates no structural change. Since the pure chymotrypsin did not change detectably after the pumping phase, the results do not indicate any influences of the excipients. For the atomisation phase, however, a small change occurred for the pure protein. The excipients, therefore, might have a stabilising effect during this phase. The change of the pure protein was, however, very small making further interpretation very difficult.

For all spray-dried samples with additional excipients, the emission intensity increased less than the samples spray-dried without excipients (Figure 52, Figure 53, Figure 54).

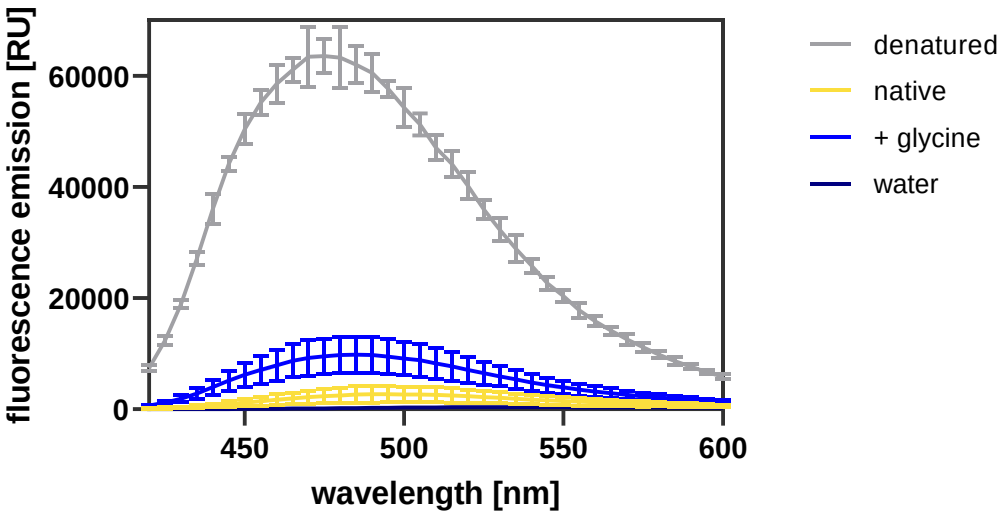

Figure 52: Influence of co-spray drying with glycine as stabilising excipient; the mean curve of n=3, error bars=CI

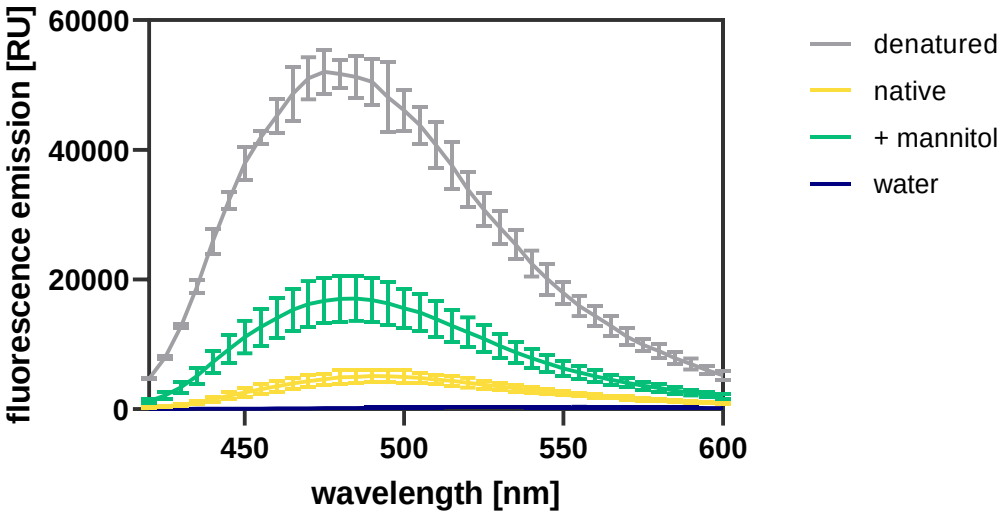

Figure 53: Influence of co-spray drying with mannitol as stabilising excipient; the mean curve of n=3, error bars=CI

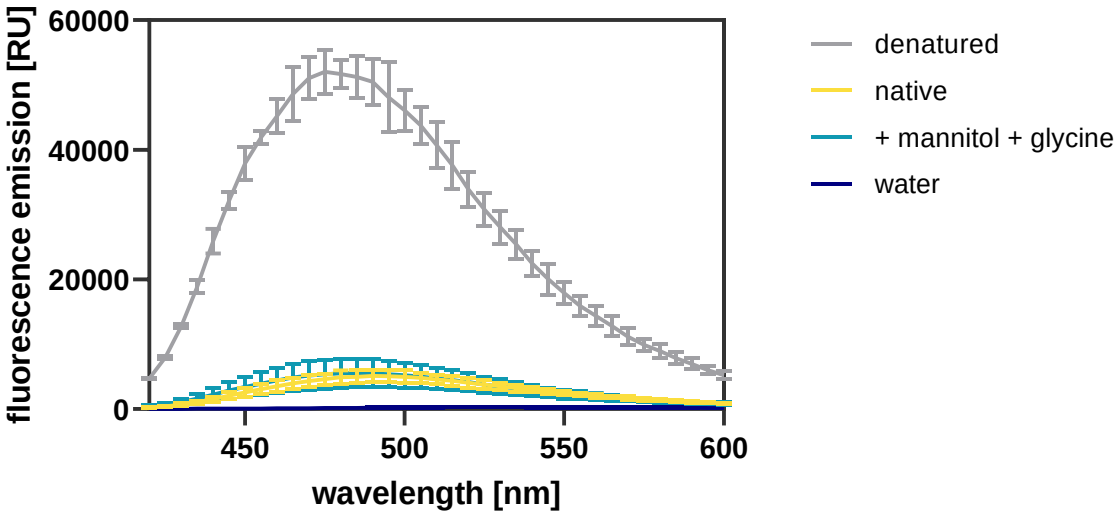

Figure 54: Influence of co-spray drying with mannitol and glycine as stabilising excipients; the mean curve of n=3, error bars=CI

The samples with additional mannitol showed a maxima ratio of 0.25 and a blueshift of 5 nm and the samples with glycine had an even lower maxima ratio of 0.12 and a blueshift of 10 nm (Table 17).

Table 17: Influence of spray drying with mannitol and glycine as stabilising excipients during spray drying; maxima ratio and blueshift of each mean curve

Sample	Maxima ratio	Blueshift
Chymotrypsin spray-dried	0.36	5 nm
Chymotrypsin spray-dried with mannitol	0.25	5 nm
Chymotrypsin spray-dried with glycine	0.12	10 nm
Chymotrypsin spray-dried with a combination of glycine and mannitol	0.01	5 nm

The combination of the excipients led to the lowest maxima ratio of 0.01 indicating the highest stabilising effect. Nevertheless, as well for these samples, the maximum shifted 5 nm to blue (Table 17).

The maxima ratio changed highly significantly after co-spray drying with glycine (p-value $9.6*10^{-5}$) and with the combination of glycine and mannitol (p-value $7.3*10^{-12}$). Indicating a large stabilising effect of glycine. The co-spray drying with mannitol, however, did not lead to a significant change (p-value 0.054). The pure mannitol might, therefore, not be able to stabilise chymotrypsin enough during spray drying. In combination with glycine, however, a high stabilising effect occurred.

7.3.3 Identification of Protein-Destabilising Factors During Spray Drying

7.3.3.1 Comparison of the Different Nozzles

Although the section aimed to discriminate all destabilising influences during spray drying, it is often not possible to investigate all different stress factors separated from each other. The main stress factors during the drying phase, thermal and dehydration stress, could not be separated from the stress factors caused by the other phases of spray drying. To dry a solution into small particles it is always necessary to atomise it into small droplets and thus, the sample will be exposed to shear-related and interfacial stress. Drying the solution in bulk would expose the protein to a different intensity of thermal and dehydration stress and would not be comparable. The technical differences between the two spray dryers and the three nozzles also caused different temperature profiles during the spray drying processes. This made it impossible to separate the different stress factors caused by the varying droplet sizes from the stress caused by the different temperature profiles.

Furthermore, the three nozzles showed major differences in their technical usability. The spray drying procedure with two-fluid nozzle caused no problems during the spray drying process. Only a small quantity of the spray-dried particles adhered to the walls of the spray dryer. The ultrasonic nozzle produced larger droplets which did not dry as well as the smaller particles that were produced with the two-fluid nozzle and therefore adhered to the walls more often. In the nano spray dryer, almost all products could be collected from the collection electrode. Collecting the product from the collection electrode of the nano spray dryer posed, however, more difficulties than collecting from the product collection beaker of the mini spray dryer. Furthermore, proteins that tend to form very large aggregates, like insulin, or foam intensively, like ovalbumin, interfered with the atomisation process in the spray head usually leading to an interruption of the spray drying process.

The findings demonstrate that the different stress factors during spray drying and the type of spray drying nozzles had different effects on the protein structure. For all three nozzles, the combination of all stress factors (spray-dried samples) influenced the tertiary structure the most. Furthermore, in all spray-dried samples, the enzyme activity decreased slightly after spray drying. Spray drying with each of the three nozzles caused a similar effect on the tertiary structure, even though the two-fluid nozzles had a slightly lower influence. There is no discernible difference between the spray head samples with the ultrasonic membrane and the ultrasonic nozzle samples according to the statistical evaluation (p-value of 0.57). The difference between the two-fluid nozzle samples and the other two samples was very significant with a p-value of 0.0045 (ultrasonic nozzle) and 0.0019 (spray head).

Pumping through the tubing system caused no detectable effect on the protein structure. Also, no change was observed when additionally pumped through the heated spray head during spray drying with the nano spray dryer. This indicates that this phase exposes the protein only to minor stress. Although pumping did not affect chymotrypsin in this study, Thomas et al observed an increased level of aggregation of virus-like particles with an increasing pumping rate [14]. The effect seemed, however, to depend on the tubing material [14]. Furthermore, Gomme et al. found an aggregation of human albumin after pumping through a lobe pump [104].

Atomisation with the spray head of the nano spray dryer affected the tertiary structure but not the enzyme activity. Atomisation with the other two nozzles, however, had little impact on the tertiary structure. This indicates that the combination of shear-related stress, the vibrating membrane, the ultrasonic frequencies and the high interfacial stress due to the small droplet size produced the most stress. The combination of shear-related stress, interfacial

stress and pressurised air or ultrasonic frequencies in the nozzles of the mini spray dryer produced less stress.

Nevertheless, spray drying with the two-fluid nozzle at low temperatures still influenced the tertiary structure. This was also found in other studies. Although the protein degradation seemed to be temperature-dependent, lower outlet temperatures still caused an activity loss in lysozyme [99, 100]. This can either be explained by the stress caused by dehydration on its own or the combination of stress factors caused by the other phases and dehydration stress. Major differences were found in the stability after spray drying at different temperatures. This, again, demonstrates that the stress on the protein during spray drying is multifactorial and is not only caused by increased temperatures.

Grasmeijer et al. also found differences in the destabilising influences of different nozzles [105]. This study compared different phases of spray drying with the two-fluid nozzle and the spray head using lactate dehydrogenase as a model protein. They also found a higher impact of spray drying with the spray head [105]. Ziaee et al. compared the influence of spray drying with the two-fluid nozzle and the ultrasonic nozzle on the activity of lysozyme [100]. They also found a greater degrading effect of the ultrasonic nozzle after the spray drying process. After atomisation without drying, they also found no or only minor effects on the protein. They additionally observed a temperature increase in the ultrasonic nozzle during atomisation.

The results of the present work and of studies of others demonstrated differences in the influence of the spray drying nozzles that did not seem to be specific to a certain protein. Of the investigated nozzles the two-fluid nozzle was found to have the lowest influence on the model proteins. When implementing a spray drying process correct choice of the nozzle can, therefore, be essential to produce a stabile protein formulation.

7.3.3.2 Spray Drying with Stabilising Excipients

The examined excipients influenced the protein stability during spray drying differently. Glycine on its own stabilised chymotrypsin during spray drying with the two-fluid nozzle. This effect was heightened for a combination of glycine and mannitol. Mannitol on its own did not significantly stabilise during spray drying with the two-fluid nozzle. During spray drying with the spray head, however, it had a very significant but small influence. The previous section also demonstrated a stabilising effect during storage in heat. This indicates another advantage of mannitol for the formulation during storage which might decrease the need for cold storage or prolong the self-live.

Both excipients are frequently used as stabilisers for spray drying studies on proteins. Also, others found a positive effect on protein stability. Ajmera et al. investigated the influence of different amino acids on the stability of catalase during spray drying [106]. They found that glycine was capable of stabilising catalase in a 1:1 mass ratio. The effect could be increased by increasing the glycine concentration to a mass ratio of 2:1. For both spray-dried glycine-catalase formulations, additionally, the storage stability increased compared to pure catalase.

The results of this work as well as that of others demonstrated that a combination of excipients may be needed to obtain the best stabilising effect. The combination of the stabilising effects of mannitol and glycine led to the lowest change in the tertiary structure after spray drying. This seems logical since the protein is exposed to various stress factors during the spray drying process against which different excipients can stabilise in different ways. While mannitol as a sugar alcohol mainly stabilises against thermal and dehydration stress, amino acids, like glycine, stabilise the protein during the whole spray drying process. Schüle et al. additionally found an increase in protein storage stability of a humanised monoclonal antibody after co-spray drying with a combination of these two excipients [107]. Furthermore, some studies found a negative effect on antibody aggregation of high mannitol concentrations due to mannitol´s tendency to crystallise during spray drying. The effect was found to be concentration-dependent and was not found for formulations containing 40% or less of mannitol [108]. This phenomenon is, however, discussed controversially since it does not seem to occur for all investigated proteins and peptides. Chan et al. found a lower aggregation tendency of salmon calcitonin when spray-dried with 70% mannitol compared to 30% mannitol [109]. They did, however, not investigate the crystallinity of the products. The effect also seems to rely on the total formulation and can be avoided by the addition of other excipients, as e. g. in Exubera® [65]. In this work, only a positive effect of mannitol on the protein stability occurred. Even formulations with 50% mannitol stabilised chymotrypsin. Nevertheless, the lower mannitol concentration (ca. 33%) in the samples with excipient combination with glycine may be favourable for proteins or antibodies more sensitive to the crystallinity of the excipients.

7.4 Influence of the Excipients on the Particle Morphology and Size

This section investigates the influence of the protein-excipient combinations on the characteristics of the particles spray-dried with the two-fluid nozzle of the mini spray dryer. The differences in the interface activity of the proteins and the interface active excipients can lead to different droplet sizes and shapes and therefore different particles. As the particle characteristics define the aerodynamic behaviour, it is important to investigate them to asses a formulation´s suitability for dry powder inhalation. The following samples (Table 18), all containing a protein with or without the addition of excipients, were used.

Table 18: Sample composition for the evaluation of the particle characteristics

	Glycine concentration (mg/mL)	Mannitol concentration (mg/mL)	Polysorbate 20 concentration (mg/mL)	Total solute concentration (mg/mL)
BSA	-	-	-	4.0
BSA + glycine + mannitol	4.0	4.0	-	12.0
BSA + glycine + mannitol + polysorbate 20	4.0	4.0	1.0	13.0
Chymotrypsin	-	-	-	4.0
Chymotrypsin + glycine + mannitol	4.0	4.0	-	12.0
Chymotrypsin + glycine + mannitol + polysorbate 20	4.0	4.0	1.0	13.0
Lysozyme	-	-	-	4.0
Lysozyme + glycine + mannitol	4.0	4.0	-	12.0
Lysozyme + glycine + mannitol + polysorbate 20	4.0	4.0	1.0	13.0
Ovalbumin	-	-	-	4.0
Ovalbumin + glycine + mannitol	4.0	4.0	-	12.0
Ovalbumin + glycine + mannitol + polysorbate 20	4.0	4.0	1.0	13.0

7.4.1 Particle Size

The geometric diameters of the spray-dried particles, measured with laser diffraction, depended on the protein and the excipients. After the addition of the excipients to the protein solutions, the particle size increased. The solute concentration is generally known to influence the particle size. Also in this study, the samples with the lowest solute concentration containing solely the protein had the smallest particle size for BSA, lysozyme and ovalbumin (Table 19). For chymotrypsin, on the contrary, these were the largest particles.

Table 19: Particle sizes and their distribution of the particles spray-dried with the two-fluid nozzle of the mini spray dryer; n=3, ± CI

	X_{50} (µm)	Span
BSA	1.32 (± 0.01)	1.46 (± 0.01)
BSA + glycine + mannitol	2.62 (± 0.05)	1.91 (± 0.03)
BSA + glycine + mannitol + polysorbate 20	5.37 (± 0.05)	1.60 (± 0.03)
Chymotrypsin	5.69 (± 1.71)	19.96 (± 4.14)
Chymotrypsin + glycine + mannitol	5.59 (± 0.08)	1.67 (± 0.05)
Chymotrypsin + glycine + mannitol + polysorbate 20	4.61 (± 0.19)	2.68 (± 0.18)
Lysozyme	3.69 (± 0.07)	2.02 (± 0.07)
Lysozyme + glycine + mannitol	4.86 (± 0.38)	1.88 (± 0.12)
Lysozyme + glycine + mannitol + polysorbate 20	6.93 (± 0.31)	2.91 (± 0.03)
Ovalbumin	1.23 (± 0.05)	5.31 (± 0.39)
Ovalbumin + glycine + mannitol	2.26 (± 0.04)	1.83 (± 0.01)
Ovalbumin + glycine + mannitol + polysorbate 20	6.14 (± 0.08)	1.52 (± 0.02)

For the protein samples co-spray-dried with mannitol and glycine, the average particle sizes increased by around 1 µm for BSA, lysozyme and ovalbumin (Table 18). This particle size increase seems reasonable since the sample solutions with additional mannitol and glycine

had a concentration three times as high as the pure protein solutions (Table 17). The samples containing additional polysorbate 20 had the largest particle sizes (Table 19). Generally, surfactants were found to decrease droplet sizes with increasing concentrations due to the decrease in surface tension [110]. Salade et al., however, found a size increase for high concentrations of surfactants [111]. They explained this effect by the increasing viscosity of the solutions. In the current work, the particle sizes were compared to the particle size of samples containing proteins. Surface active substances, like proteins, define the characteristics of a droplet´s surface. Since the polysorbate 20 expels the proteins from the surface it defines the droplet's surface characteristics in the samples containing it. The droplets of the samples containing only protein and the samples containing additional polysorbate 20 may be very different in their surface characteristics making direct a comparison regarding the expected particle characteristics difficult. In addition, the polysorbate 20 sample solutions contained the highest total solute concentration (Table 18). The only exception was the samples with chymotrypsin. For these samples, the particles of the pure protein and the protein co-spray-dried with mannitol and glycine were of almost the same size. The particles containing additional polysorbate were slightly smaller. Chymotrypsin also was the protein with the highest interfacial activity and was only partly influenced by the surfactant. It is, therefore, possible that the droplets' surface is not influenced by the surfactant in the same way as for the other proteins. All samples had a similar small span value indicating a narrow size distribution (Table 19). Only the span value of the pure ovalbumin and chymotrypsin samples was larger. This was very likely caused by aggregated particles (see Section 7.4.2). Furthermore, for most formulations, the average geometric particle size is below the maximum inhalable particle size of 5 µm (Table 19). Only for the samples containing polysorbate 20, pure chymotrypsin and chymotrypsin with mannitol and glycine, the x_{50} is slightly above 5 µm.

7.4.2 Particle Morphology

Spray drying usually produces particles with a hollow spherical shape. These particles can, however, collapse during the process and solvent evaporation can cause holes in the shell of the particle. The particles of the pure BSA (Figure 55 A) and the BSA with glycine and mannitol (Figure 56 A) collapsed completely leading to a deeply wrinkled shell.

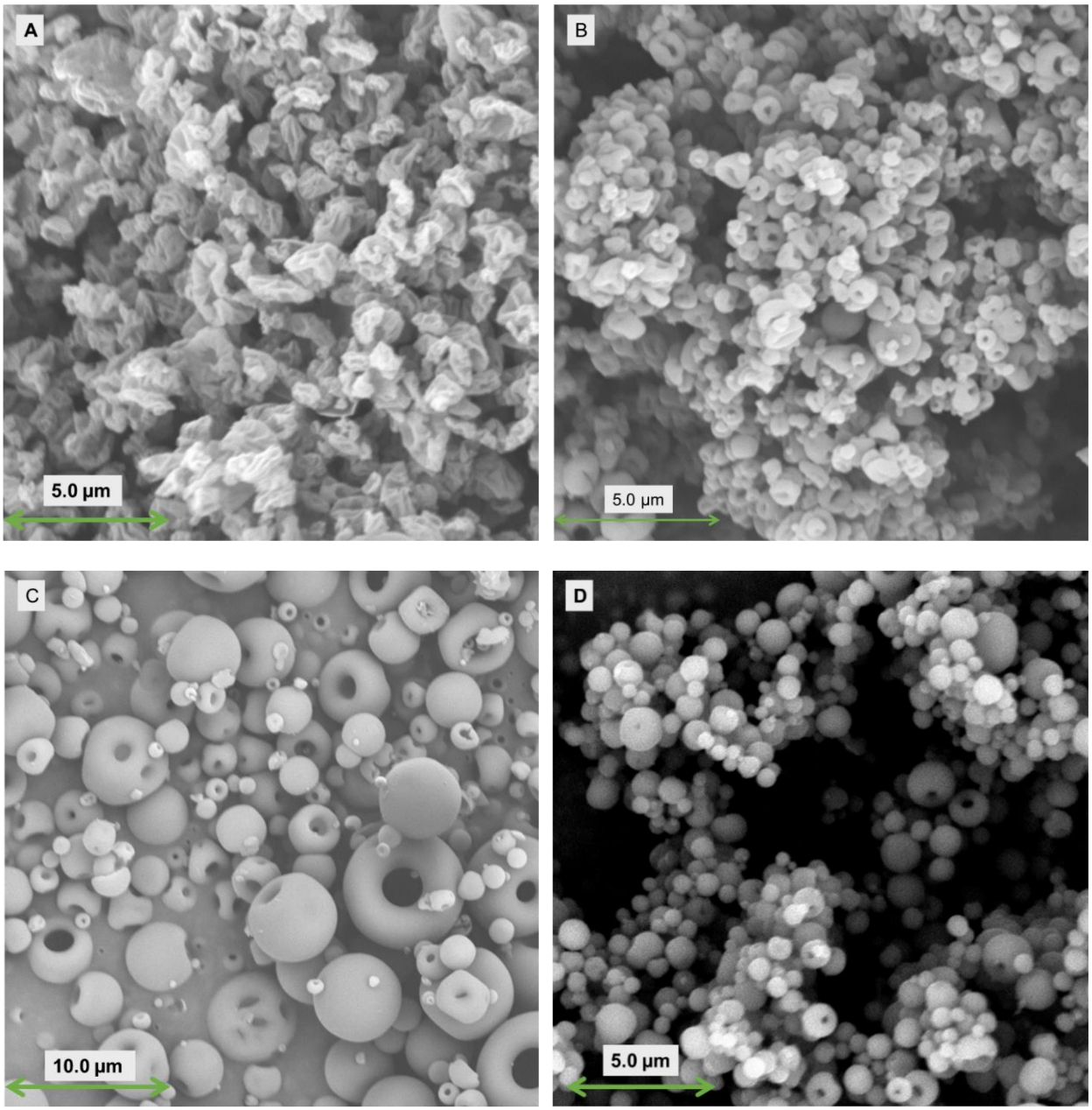

Figure 55: Spray-dried particles with BSA (A), chymotrypsin (B), lysozyme (C) and ovalbumin (D)

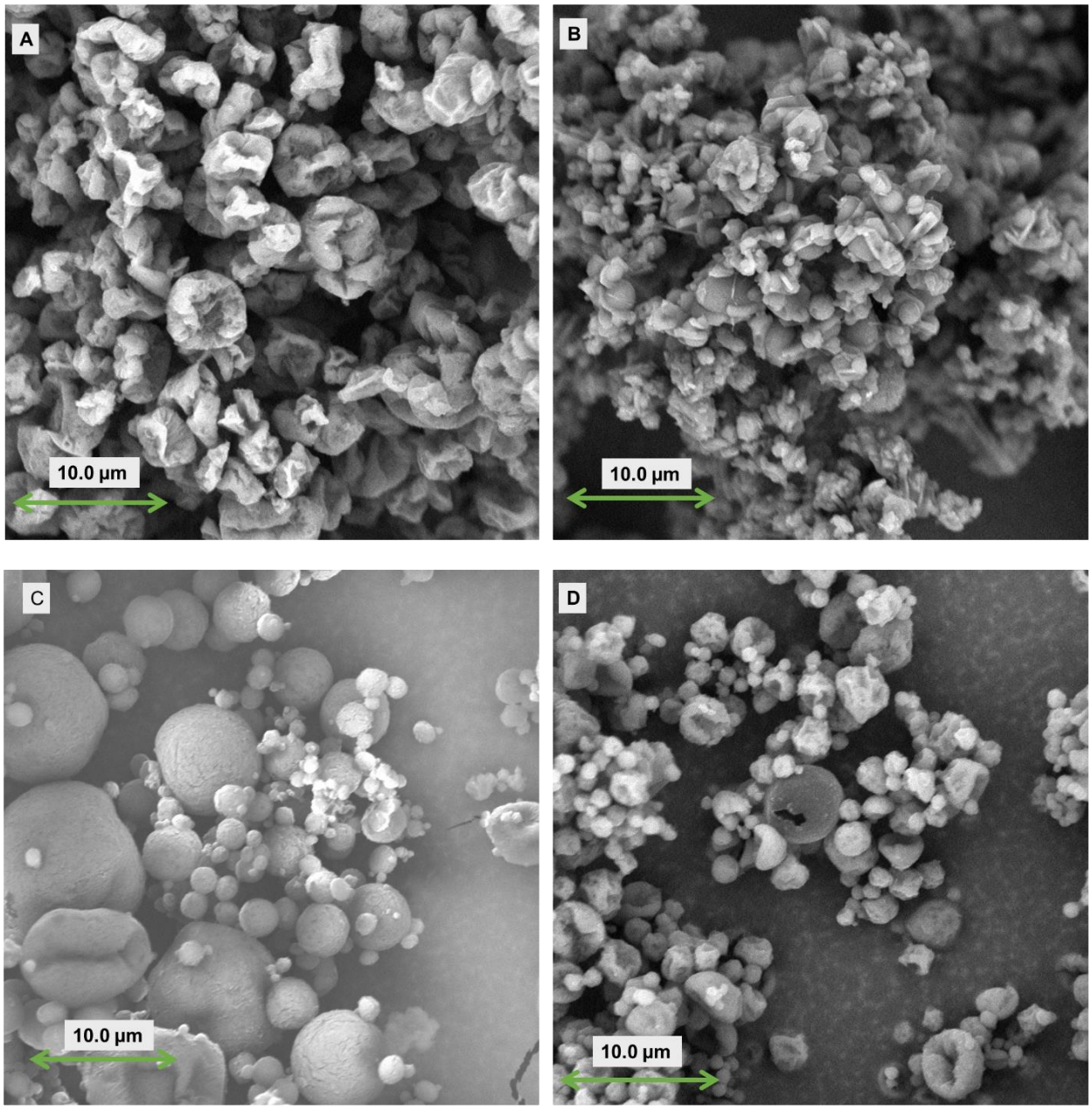

Figure 56: Spray-dried particles with glycine and mannitol and BSA (A), chymotrypsin (B), lysozyme (C) or ovalbumin (D)

The ovalbumin particles containing glycine and mannitol partly collapsed and wrinkled (Figure 56 D). The pure chymotrypsin, lysozyme and ovalbumin particles were spherical with holes in the shell (Figure 55 B, C, D). The other particles were completely spherical (Figure 56 B, C, Figure 57 A, B, C, D).

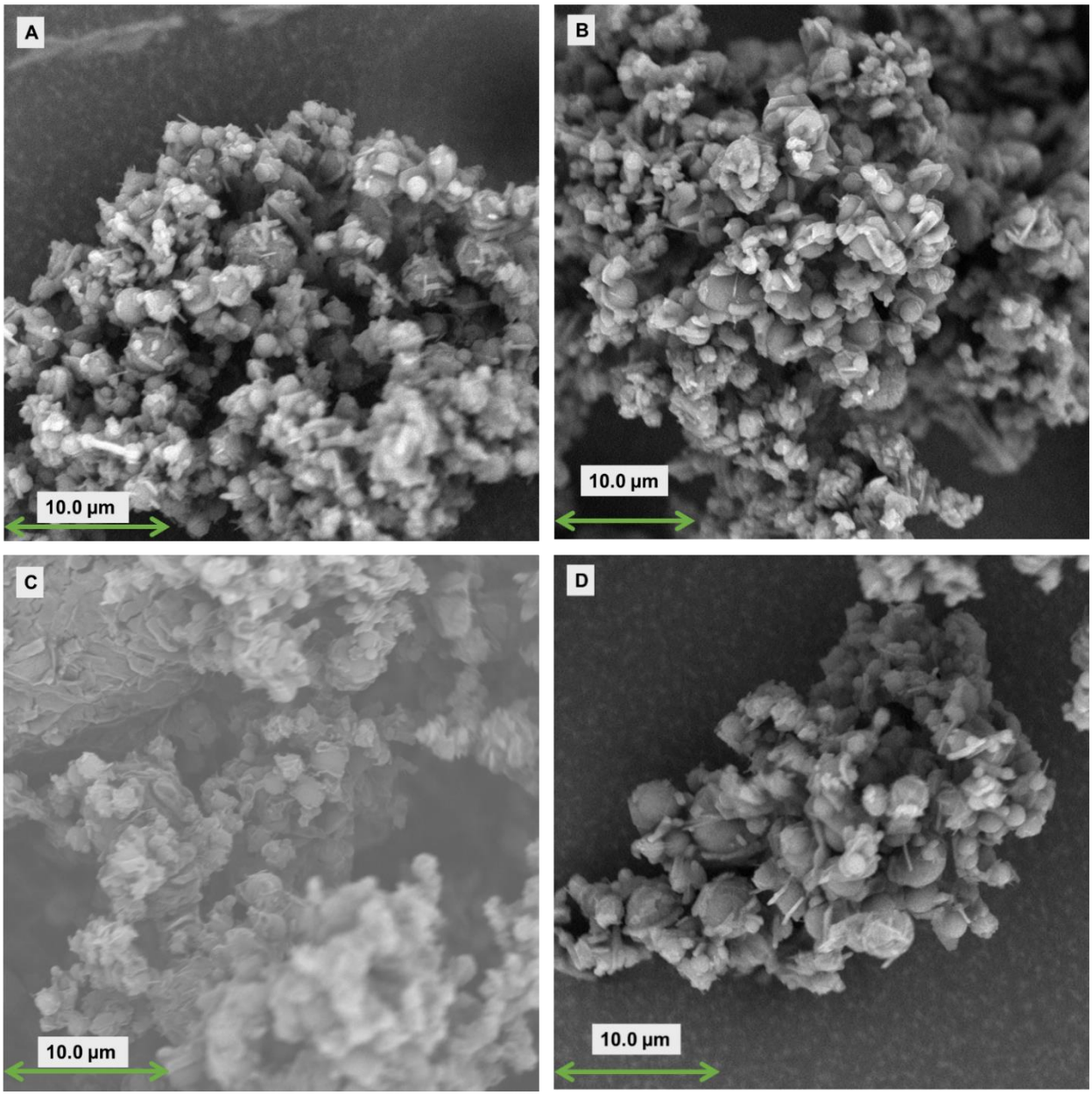

Figure 57: Spray-dried particles with glycine, mannitol and polysorbate 20 and BSA (A), chymotrypsin (B), lysozyme (C) or ovalbumin (D)

The morphology of the particles analysed in this section seemed to be influenced by the proteins and the excipients. The surface activity of solutes can generally influence particle morphology [110]. Small surfactant molecules accumulate fast at the droplet´s interface and tend to form more spherical and more hollow particles [110]. Adler et al. performed an electron spectroscopy for chemical analysis to investigate the surface composition of spray-dried BSA particles [62]. They found that polysorbate 80 was able to impede the surface adsorption of BSA. This demonstrated that surfactants can expel the proteins from the surface to the inside of the droplets and then remain the main component in the particle shell. Surfactants should, therefore, define the surface properties of the particles. This was also found

by Maa et al. where the addition of polysorbate to BSA solution altered the particle morphology after spray drying [112]. Also, the particles in the present section containing additional polysorbate 20, had a spherical shape. Nuzzo et al. found a decreasing ability to stabilise droplets during spray drying with increasing molecular size of surface active polymers [113]. Also in the present work, the smaller proteins formed the more spherical particles. Nevertheless, the number of different model proteins in the present work was too small for systematic evaluation. The SEM pictures also illustrated differences in the particle sizes which were already found in the measurement of the geometric particle sizes (Section 7.4.1). For pure chymotrypsin and ovalbumin, the span value indicated particle aggregation. The SEM pictures might show aggregated particles, also this is hard to distinguish. The single particles of chymotrypsin, however, seem to be smaller than the found x_{50} value and seem to be equally sized as the BSA particles.

7.4.3 Aerodynamic Performance

The aerodynamic performance assessed with the FSI showed the impact of the formulation. For this, the two different samples co-spray-dried with excipients were analysed as possible DPI formulations. Ovalbumin was chosen as a model protein for this experiment due to its good quantification properties. Furthermore, the measured average geometric diameter of 2.26 ± 0.04 µm for the ovalbumin samples with additional mannitol and glycine was well below the required maximum of 5 µm. This was also the lowest geometric particle size of the formulated proteins. For the samples containing additional polysorbate 20, the average geometric diameter (6.14 ± 0.08 µm) was slightly above the inhalable maximum particle size.

The emitted dose was similar for the samples with and without polysorbate 20 (p-value 0.051) (Table 20).

Table 20: Aerodynamic performance of ovalbumin with different excipient combinations; n=3, +/- CI

	Ovalbumin + glycine + mannitol	**Ovalbumin+ glycine + mannitol + polysorbate 20**
Emitted dose	86.31% (± 2.20%)	90.62% (± 2.13%)
Fine particle fraction	63.51% (± 7.68%)	12.38% (± 2.53%)

The majority of the particles followed the airflow out of the inhaler, which is favourable for a DPI formulation. The fine particle fraction was much smaller for the samples containing additional polysorbate 20 compared to the samples without polysorbate 20 (Table 20). The difference between the two samples was highly significant (p-value 0.0002). Therefore, a

large amount of the samples without polysorbate 20 would be inhalable. The formulation with polysorbate 20, on the contrary, would not be favourable for inhalation since only a very small fraction of the drug would reach the deeper parts of the lung. These findings seem reasonable when comparing the geometric diameters of the two samples (see Section 7.4.1). For the formulation with only mannitol and glycine, a smaller aerodynamic diameter was expected since the average geometric diameter is below 5 µm, whereas for the formulation with additional polysorbate 20, a larger aerodynamic diameter was expected due to the much larger geometric diameter.

7.4.4 Evaluation of the Particle Size and Shape

This section demonstrated that it was possible to produce spray-dried particles in a size range and shape suitable for dry powder inhalation of the model proteins. The use of excipients in the samples influences not only the protein stability but also the shape and size of the spray-dried particles. With the addition of the excipients the solute concentration and therefore the particle size of the spray-dried product increased. The effect was found for the geometric particle size for all protein and excipient combinations besides the chymotrypsin samples. For the ovalbumin samples this, as expected, also affected the aerodynamic performance. Furthermore, the surfactant led to an increase in particle size and influenced the particle shape of some of the samples. It is most likely that the most surface-active molecules define the characteristics of the droplet´s interface and therefore of the dried particle´s shell. In the solutions without polysorbate 20, the protein was the only surface-active substance and adsorbed at the surface. Polysorbate 20 seemed to influence the surface activity of all used model proteins (see Section 7.1.2). It therefore probably dominated the surface of the droplet and defined the morphology of the dried particle. Since particle size and morphology highly influence the usability of a formulation for inhalation, the whole formulation has to be considered to evaluate it.

7.5 Dissolution Behaviour Changes

During the spray drying experiments changes in the dissolution behaviour of the dried products compared to the starting material were observed. This section investigates methods to characterise the dissolution behaviour of the lyophilisate (commercial product used as starting material) compared to the spray-dried protein using ovalbumin as a model protein. It investigates the influence of the dissolution velocity using the Franz dissolution cell and the influence on the solubility in an ammonium sulphate solution. Furthermore, it aims to find a structural explanation for these dissolution behaviour changes. It examined the tertiary structure using the fluorescence emission spectra assay, the crystallinity using XRPD and the wettability using contact angle measurements.

For chymotrypsin, the other of the model proteins showing visual dissolution behaviour changes after spray drying, the dissolution behaviour could not be investigated. Chymotrypsin generally tends to form irreversibly insoluble gel-like solid pieces when exposed to dissolution medium in higher protein concentrations. The performed dissolution experiments mainly exposed the dry powder to small amounts of dissolution media which caused a very high protein concentration. Therefore, performing these experiments with chymotrypsin was technically not feasible.

7.5.1 Solubility

The measurement of the solubility in water is in general an easy-to-perform method to compare the hydrophily of dry powders. The solubility of ovalbumin in pure water, however, was too high to be determined. Detection was limited by the extremely high viscosity of the highly concentrated protein solution. Around a concentration of 200 mg/mL, the viscosity was so high it was impossible to tell whether further protein did completely dissolve. Also, filtration was not possible anymore. The solubility could only be determined by precipitation by the addition of 33.3% ammonium sulphate.

The solubility of ovalbumin in water in the presence of 33.3% ammonium sulphate changed after spray drying with both nozzles. The lyophilisate had a solubility of 37.45 mg/mL (± 0.13 mg/mL). The solubility decreased significantly (p-value 0.024) after spray drying with the ultrasonic nozzle samples (28.28 mg/mL ± 2.90 mg/mL) and very significantly (p-value 0.006) after spray drying with the two-fluid nozzle samples (28.30 mg/mL ± 1.45 mg/mL). Between the two nozzles, no difference in solubility occurred. Spray drying, therefore, appears to affect protein solubility, which could be caused by structural changes. Since in proteins in the native state usually the hydrophobic amino acids face the core and hydrophilic the outside, native proteins tend to be more hydrophilic and have a higher solubility in water.

A decrease in solubility might, therefore, indicate structural changes with more hydrophobic amino acids facing the outside of the molecule.

7.5.2 Dissolution Velocity

Commonly used dissolution test set-ups expose samples directly to high amounts of dissolution media. For the protein dry powder samples, this would lead to a dissolution velocity that would be too fast to measure. The Franz dissolution cell set-up was chosen for the measurements since it only exposes the samples to the small amount of dissolution medium that penetrates through the membrane. It, therefore, slows down the dissolution process making measuring and comparing the dissolution velocity of the ovalbumin samples possible. The measurements found different dissolution velocities for the lyophilisate (starting material) and the spray-died samples. While undissolved residues of the spray-dried samples were found to remain on the membrane after the 90-minute measurement, the lyophilisate samples were visually fully dissolved. However, not a 100% of the protein could be found in the acceptor medium which might have been caused by protein molecules adsorbing to the donor compartment or the membrane. Furthermore, a longer measurement time might have led to a higher recovery. Nevertheless, the dissolution profiles of the lyophilisate and the spray-dried samples were different (Figure 58).

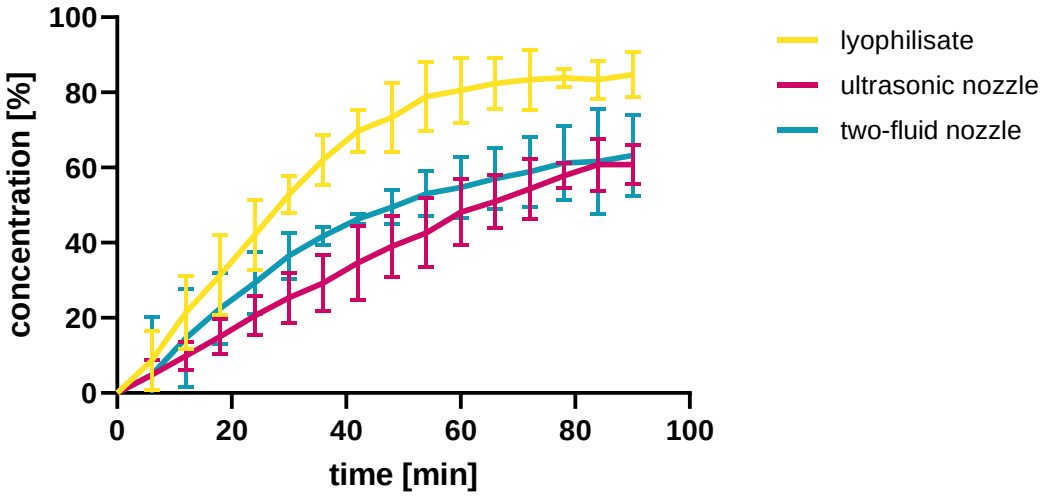

Figure 58: Franz diffusion cell measurements of ovalbumin before (lyophilisate) and after spray drying with the two-fluid and the ultrasonic nozzle; the mean curve of n=3, error bars=CI

84.70% of the lyophilisate could be detected in the acceptor medium after 90 min. The two spray-dried samples dissolved more slowly. 63.24% of the two-fluid nozzle samples and 60.80% of the ultrasound nozzle samples were detected after 90 min. This trend also occurred for all other time points. The dissolution of the lyophilisate consistently exceeded that of both spray-dried samples.

Furthermore, a smaller percentage of the ultrasonic nozzle samples dissolved compared to the two-fluid nozzle samples at all time points (except the first). However, the difference was not as large as the difference between the lyophilisate and the spray-dried samples. The similarity factor for the dissolution profile comparison confirmed these impressions. With the lyophilisate as a reference, f_2 was lower than 50 for both products (two-fluid nozzle 34.7 and ultrasonic nozzle 28.3), indicating no similarity. The spray-dried samples do not significantly differ between each other (f_2 = 55.3). Spray drying, therefore, seems to influence not only the solubility but also the dissolution velocity of the protein which might be caused by the structural changes described above.

It, however, has to be kept in mind that spray drying also changes the physical properties of the powder which might influence the dissolution velocity as well. For the evaluation of physical changes in the protein molecules in the powder, a combination with other methods, like the solubility measurement, might be necessary.

7.5.3 Crystallinity

Since spray drying with both nozzles led to a very similar dissolution behaviour change the XRPD diffractograms were recorded for the lyophilisate and the two-fluid nozzle samples as an example. The sample spray-dried with the two-fluid nozzle as well as the commercial product are mostly amorphous indicated by the lack of clear peaks in the diffractograms (Figure 59). Both diffractograms almost overlaid.

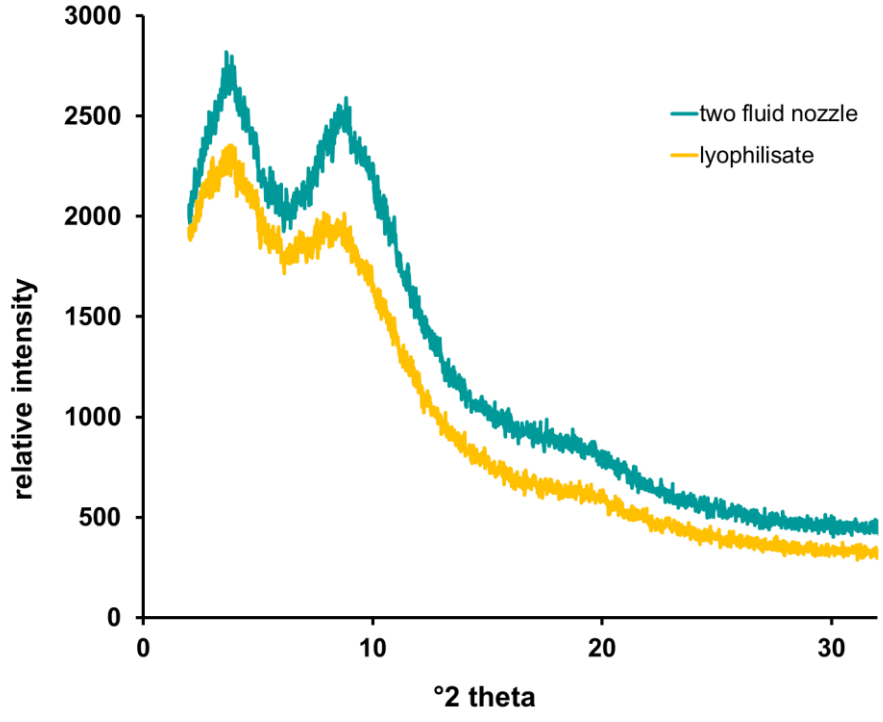

Figure 59: XRPD diffractograms of ovalbumin before and after spray drying; n=1

Also, others recorded XR(P)D diffractograms of ovalbumin in the range from 2° to 35° theta. Yu et al. and Savadkoohi et al. prepared ovalbumin forms hen eggs on their own [95, 114]. While the ovalbumin of the first group seemed to be crystalline, the ovalbumin of the second group appeared to be amorphous. Niu et al. purchased their ovalbumin as a lyophilised powder from Amresco Chemical Co. (Boise, USA) which appeared to be amorphous [115]. Although different results in terms of crystallinity were found the main events all occurred in the range analysed in the present work. Therefore, the present diffractograms should have recorded all important events which indicated no changes in the crystallinity of ovalbumin after spray drying. Crystallinity is generally known to influence the solubility [116]. Processing methods can influence the crystallinity. Lyophilisation as well as spray drying can change crystalline structures to a more amorphous structure [117]. An amorphous solid state usually leads to an increased apparent solubility compared to a crystalline solid state [118].

Commercially available proteins in dry powder forms are usually already supplied as lyophilised, amorphous powders, as in this section. During spray drying, this structure, however, may again change to a more crystalline structure. For ovalbumin both the lyophilisate as well as the spray-dried sample were amorphous. A change in crystallinity, therefore, does not seem to be a likely explanation for the dissolution behaviour changes.

7.5.4 Wettability

As spray drying with both nozzles resulted in a comparable change in dissolution behaviour, the contact angle measurement was carried out on the lyophilisate and the two-fluid nozzle spray-dried product as an example. The contact angle measurement indicated a small but very significant (p-value 0.003) difference between the lyophilisate and the spray-dried ovalbumin with 34.6° ± 1.04° and 41.3° ± 2.86°. The contact angle of both samples is below 90° which indicates a favourable wettability for water. For a hydrophilic protein, like ovalbumin, this seems reasonable. The increased contact angle after spray drying indicated a decreasing hydrophilicity which might indicate structural changes (see Section 7.5.1). Nevertheless, the measurement was problematic. First, the comprimates produced with the hydraulic press were fragile and broke easily. This problem especially occurred during the compaction of the spray-dried protein. Second, the measured value of the contact angle highly depended on the droplet's position on the pellet. Furthermore, some pellets started dissolving immediately after the addition of the water drop. The wettability measurements on their own may, therefore, not be robust enough to evaluate the hydrophilicity of a protein powder. A combination with other analytical methods e. g. the measurement of the solubility might be necessary to get reliable results.

7.5.5 Tertiary Structure and Protein Aggregation

The intrinsic as well as the more sensitive extrinsic fluorescence emission spectra assay demonstrated no change in the fluorescence emission of ovalbumin after spray drying (see Section 7.1.4.5, Figure 31, Figure 32). In both assays, the spectra of the samples produced with both nozzles overlaid with the spectrum of the native protein. They, therefore, did not indicate changes in the proteins´ tertiary structure or aggregation. Both assays detect changes in the dissolved samples. It is, therefore, possible that structural changes in the dry powder samples are reversible after dissolution are not detected with the assay. Although the measurement principle of the extrinsic fluorescence assay relies on changes in hydrophobicity (see Section 6.5.4), the assay's detection of very small changes may be limited. Even these very small changes may, however, still influence the dissolution behaviour.

The visual inspection in the laboratory of the spray-dried samples and the lyophilisate after dissolving found no aggregates in any of the solutions. This method is, however, very limited as it can only detect aggregates in sizes and amounts large enough to be visible to the human eye.

7.5.6 Identifying Physical Changes in Protein Dry Powders

As one of the major protein components of egg white, ovalbumin is supposed to function in a hydrophilic environment. In the native state, it is highly water-soluble. However, a change in dissolution behaviour in aqueous solutions was observable for ovalbumin after spray drying. The dissolution velocity, the solubility and the wettability changed. The protein became less water soluble and less hydrophilic.

The most likely explanation for these changes would be a change in tertiary structure [119]. The majority of the proteins in the native state are folded in the same pattern: the hydrophilic amino acids face the outside while the hydrophobic amino acids face the protein´s core. This also applies to ovalbumin. During denaturation or aggregation, the protein´s folding can change, leading to a structure where more hydrophobic amino acids face the outside. The degraded protein becomes less hydrophilic and less water-soluble making it more susceptible to aggregation. Another possibility would be the formation of insoluble aggregates which can occur during the degradation process [119]. For the samples investigated in this section, however, no insoluble aggregates were visible after dissolution. The fluorescence emission assay detected no change in fluorescence emission and therefore no aggregation or changes in tertiary structure. It is, however, possible that structural changes in dry powders were reversible after dissolution.

The detection of protein structure changes is a highly complex process that cannot be fully covered by one detection method. A relatively certain elimination of possible structural changes, therefore, requires several different analytical methods (see Section 3.2.1). Although already two different kinds of fluorescence emission assays were used in this work, other methods detecting tertiary structure changes, like CD, the detection of chemical changes via protein MS or methods to detect aggregates, like DLS or SEC might have found structural changes. Due to limited time and resources, using that many methods is not always possible or practical (as in this work). The determination of the dissolution behaviour might be an easy-to-perform additional method to evidence structural changes in protein dry powder formulations. Furthermore, it seemed to be a sensitive method even for small structural changes, that could not be detected with other methods used in this work.

The change in dissolution behaviour might also influence the protein formulation's functionality. Especially, if a further redispersion of the DPI formulation is necessary for the application with the nebuliser, it needs to dissolve completely and fast.

7.6 Influence of Nebulisation on Protein Stability

This part of the work investigated the influence of nebulisation on the model protein chymotrypsin. Furthermore, it examined the possibility of redispersing a protein DPI formulation and applying it with a nebuliser. It compared the influence of three different nebulisers on the commercial starting material (lyophilisate) and a spray-dried formulation containing chymotrypsin, mannitol and glycine in equal parts. All samples were dissolved to a chymotrypsin concentration of 4.0 mg/mL. For all nebulisers used in this section, a collection of the aerosol in a simple collection device (e. g. a centrifuge tube) was not possible due to the lack of airflow. Furthermore, the collection in an outlet filter using a breathing simulator, as described in the Ph. EUR 2.9.44 [91], was not suitable for the experiment as the aim was to collect the aerosol with as less changes as possible. First, this set-up requires washing the sample out of the filter, which requires a few additional mL of dissolution media. The resulting solution may have a concentration too low for the fluorescence assay. Furthermore, the protein might interact with the filter and thereby change its structure. Therefore, the samples were collected in the NGI. Depending on the nebuliser different amounts of the various droplet sizes were produced. This led to a variable distribution in the stages of the NGI. In some stages, only a small amount of solution was collected and those solutions could not be analysed for protein stability or concentration. During the nebulisation and aerosol collection, however, a change in protein concentration can occur due to evaporation. Before the stability analysis in the fluorescence emission spectra assay, the samples were diluted to a concentration of 4.0 mg/mL in case of a concentration increase after nebulisation.

7.6.1 Comparison of the Nebulisers

7.6.1.1 Pari eFlow®*rapid*

The membrane nebuliser eFlow®*rapid* produced droplets collectable in stages 2-5 in the NGI. In all collected samples protein concentration did change compared to the starting solution. With decreasing droplet size an increased concentration of the collected solution was measurable (Table 21).

Table 21: Protein concentration in the different NGI stages after nebulisation of the pure chymotrypsin solution with the eFlow® rapid; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	4.72 (± 0.06)	18.56 (± 1.44)
Stage 3	4.79 (± 0.02)	19.47 (± 0.47)
Stage 4	5.00 (± 0.02)	25.21 (± 0.50)
Stage 5	6.11 (± 0.05)	53.84 (± 1.27)
Nebuliser	4.30 (± 0.04)	7.65 (± 0.92)

Furthermore, a small increase in protein concentration occurred in the solution remaining in the nebuliser after the collection time (Table 21). The concentration increase in the collected samples can most likely be explained by evaporation which increases with increasing surfaces of the droplets and therefore with decreasing droplet size.

The fluorescence emission spectra of the different protein samples collected in NGI stages 2-5 and the protein samples remaining in the nebuliser (almost) overlaid with the native protein (Figure 60). They, therefore, indicated no structural changes in chymotrypsin caused by nebulisation.

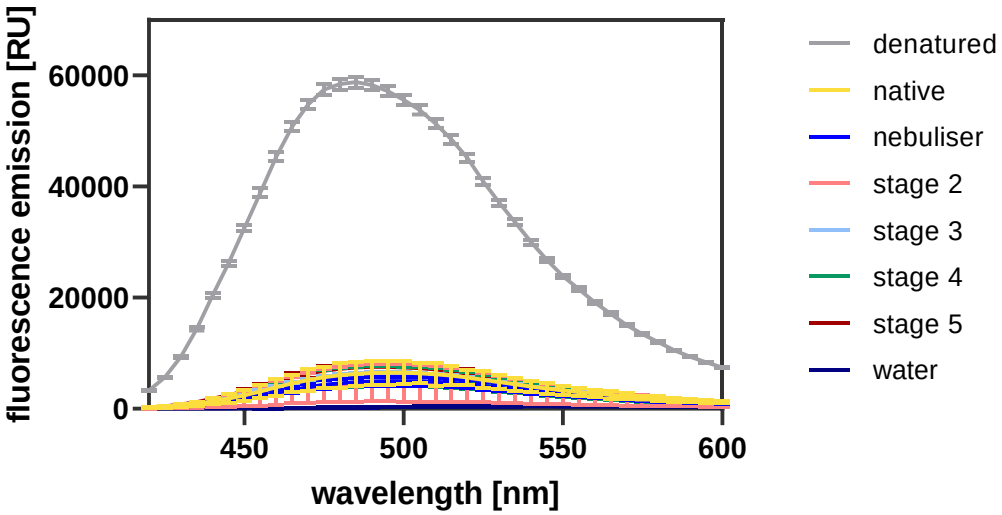

Figure 60: Influence of nebulisation with the nebuliser eFlow® rapid on the commercial product (lyophilisate); the mean curve of n=3, error bars=CI

7.6.1.2 Pari TurboBoy®

The jet nebuliser Pari TurboBoy® produced droplets collectable in stages 2-5 in the NGI for the protein solutions. In stage 2, however, not enough solution was collected for performing

structural analysis. Here, as for the eFlow®*rapid*, a decreasing droplet size led to an increased concentration of the solution. Additionally, a small increase in protein concentration occurred in the solution remaining in the nebuliser (Table 22).

Table 22: Protein concentration after nebulisation with the TurboBoy®; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	5.05 (± 0.35)	26.37 (± 8.78)
Stage 3	4.98 (± 0.09)	24.76 (± 1.88)
Stage 4	5.18 (± 0.23)	29.58 (± 5.82)
Stage 5	6.48 (± 0.52)	62.04 (± 12.90)
Nebuliser	4.56 (± 0.05)	14.05 (± 1.12)

The fluorescence emission assay also exhibited an (almost) overlay of the spectra of the protein samples collected in stages 3-5 of the NGI and remaining in the nebuliser with the native protein (Figure 61). It, therefore, indicated no structural changes caused by nebulisation.

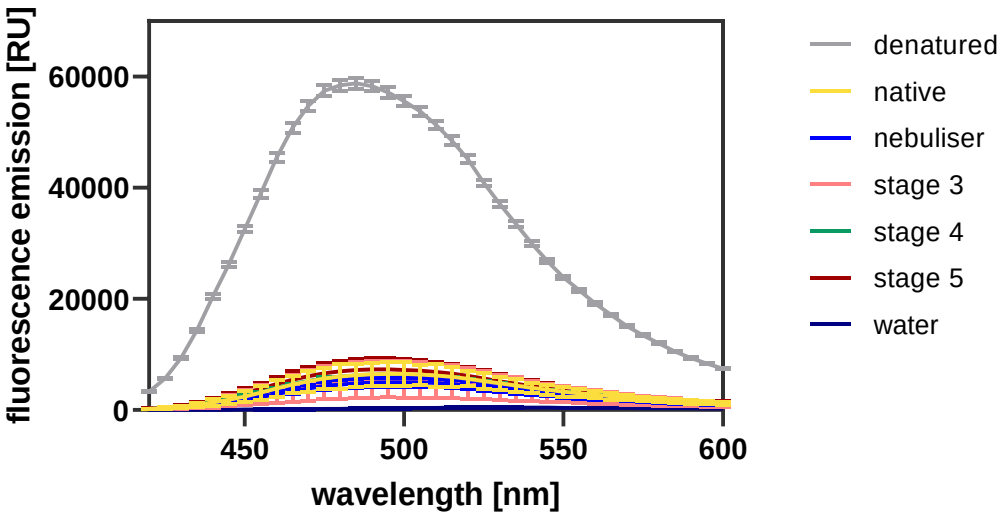

Figure 61: Influence of nebulisation with the nebuliser TurboBoy® on the commercial product (lyophilisate); the mean curve of n=3, error bars=CI

7.6.1.3 Multisonic®

Also, for the samples nebulised with the ultrasonic nebuliser Multisonic® a change in the concentration of the solution occurred. Samples were collected in the stage 1-5. With decreasing droplet size an increasing protein concentration of the collected solution was measurable (Table 23).

Table 23: Protein concentration after nebulisation of the spray-dried formulation with the Multisonic®; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 1	4.25 (± 0.90)	13.08 (± 16.03)
Stage 2	5.53 (± 1.30)	38.30 (± 32.47)
Stage 3	5.88 (± 1.23)	46.93 (± 30.71)
Stage 4	6.22 (± 0.95)	55.50 (± 23.71)
Stage 5	8.99 (± 3.60)	124.71 (± 89.95)
Nebuliser	5.95 (± 0.74)	48.81 (± 18.59)

Furthermore, this increase in protein concentration occurred also in the solution remaining in the nebuliser after the collection time. The described trend occurred for all samples. Nevertheless, the measured concentrations of the Multisonic® samples showed large differences between the triplicates compared to the samples of the other two nebulisers. This indicates that the used Multisonic® is less consistent in the produced droplet. Since this work only used one Multisonic®, the phenomenon could be caused by the specific device and might not generally occur in Multisonic® nebulisers.

The fluorescence emission spectrum indicated an influence on the protein structure of nebulising with the Multisonic® (Figure 62).

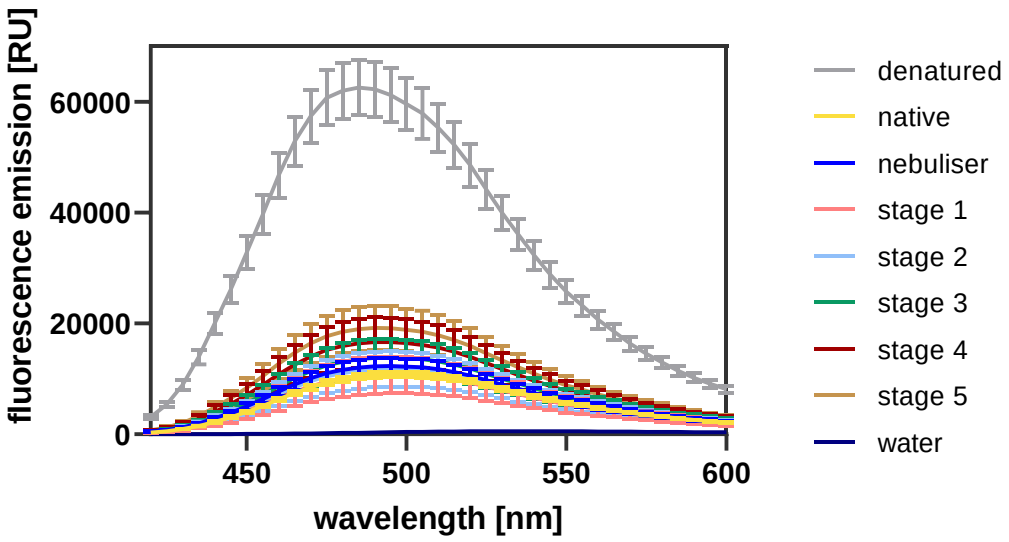

Figure 62: Influence of nebulisation with the nebuliser Multisonic® on the commercial product (lyophilisate); the mean curve of n=3, error bars=CI

This change depended on the droplet size (Table 24).

Table 24: Influence of nebulisation with the nebuliser Multisonic® on the commercial product (lyophilisate); n=3, +/- CI; maxima ratio and blueshift of each mean curve

NGI stage	Blueshift	Maxima ratio
Stage 1	0 nm	0
Stage 2	0 nm	0.02
Stage 3	0 nm	0.06
Stage 4	5 nm	0.11
Stage 4	5 nm	0.16
Nebuliser	0 nm	0.03

No structural changes occurred for the samples collected in stage 1. With decreasing droplet size, however, the fluorescence intensity increased and for stages 4 and 5 a blueshift occurred. The emission intensity of the samples collected in the nebuliser increased only slightly.

The results show tertiary structure changes caused by nebulisation with the Multisonic®. They seemed to rely on the droplet size and indicated that the smaller droplets exposed the protein to a higher intensity of stress. The nebuliser does not seem to be suitable for nebulising chymotrypsin in this set-up and was not used for investigating the DPI formulation.

7.6.1.4 Evaluation of the Concentration Changes

To identify if the concentration change is chymotrypsin specific, this section investigated if the concentration of other substances being nebulised with the eFlow® rapid (as an example) and collected with the NGI changed. It investigated brilliant blue, as an example of a surface-active substance, and mannitol, as an example of a non-surface-active substance in an aqueous solution. For brilliant blue a concentration of 0.0172 mg/mL and for mannitol a concentration of 0.0157 mg/mL was used. For both substances samples could be collected in stages 2-4 and the nebuliser.

Also, for the brilliant blue samples the concentration increased with decreasing droplet size in the NGI (Table 25).

Table 25: Brilliant blue concentration after nebulisation with the eFlow® rapid; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	0.0191 (± 0.0003)	11.28 (± 1.63)
Stage 3	0.0198 (± 0.0011)	15.11 (± 6.27)
Stage 4	0.0218 (± 0.0030)	26.69 (± 17.35)
Nebuliser	0.0152 (± 0.0005)	-11.64 (± 2.81)

In the solution remaining in the nebuliser, however, the concentration decreased a little (Table 25).

For the mannitol samples, as well, concentration increased with decreasing droplet size in the NGI (Table 26).

Table 26: Mannitol concentration after nebulisation with the eFlow® rapid; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	0.0179 (± 0.0005)	13.50 (± 2.80)
Stage 3	0.0196 (± 0.0009)	24.62 (± 5.45)
Stage 4	0.0243 (± 0.0021)	54.79 (± 13.48)
Nebuliser	0.0161 (± 0.0004)	2.38 (± 2.33)

The solution remaining in the nebuliser only showed a minor concentration increase (Table 26).

The results demonstrate that the concentration increase is not protein-specific but occurs for mannitol and brilliant blue as well. The effect is likely caused by evaporation in the NGI. The concentration of the solution remaining in the nebuliser did also change but only slightly as in protein samples. It was not large and consistent enough for further interpretation.

7.6.2 Nebulisation of the DPI Formulation

This part of the study investigated the possibility of redispersing and nebulising the DPI formulation that was found most stable in the spray drying study. The nebulisation was only performed in the two nebulisers the eFlow® *rapid* and the TurboBoy® that did not affect the tertiary structure of chymotrypsin in the previous part of the section.

For the samples nebulised with the eFlow® *rapid* the concentration increased with decreasing droplet size. Only a small increase in protein concentration occurred in the solution remaining in the nebuliser after the collection time (Table 27).

Table 27: Protein concentration in the different NGI stages after nebulisation of a solution of chymotrypsin spray-dried with mannitol and glycine with the eFlow rapid; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	4.43 (± 0.27)	10.34 (± 6.26)
Stage 3	4.62 (± 0.25)	15.40 (± 6.38)
Stage 4	5.21 (± 0.29)	30.17 (± 7.35)
Nebuliser	4.43 (± 0.12)	10.53 (± 3.16)

After nebulisation of the spray-dried formulation, the spectra of the solution in the nebuliser and stage 3 overlaid with the native protein (Figure 63).

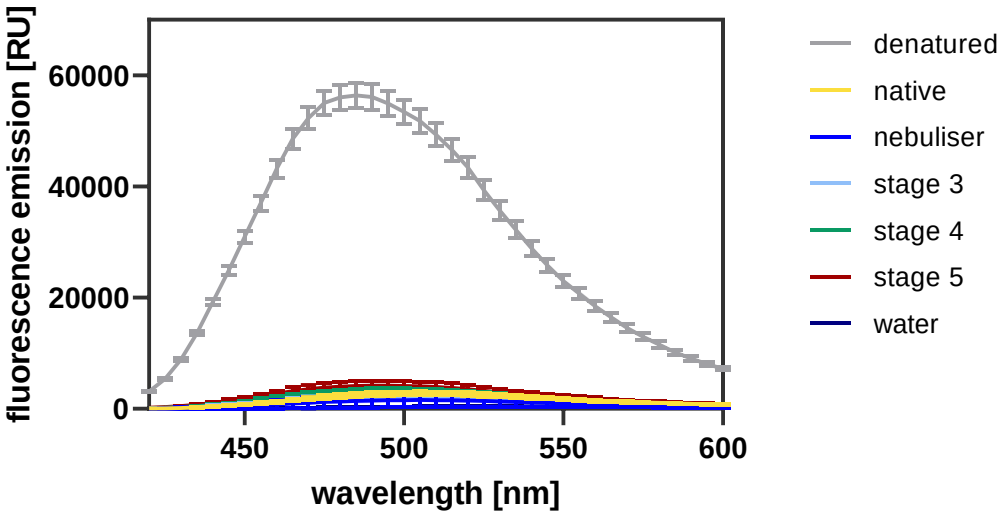

Figure 63: Influence of nebulisation with the nebuliser eFlow® rapid on the spray-dried formulation; the mean curve of n=3, error bars=CI

The maximum wavelength of stages 4 and 5, however, shifted 10 nm blue and the maximum did not increase (Figure 63). This indicated a small change in tertiary structure.

The samples nebulised with the TurboBoy® also showed an increase in concentration as the droplet size decreased (Table 28).

Table 28: Protein concentration after nebulisation of the spray-dried formulation with the TurboBoy®; n=3, +/- CI

NGI stage	Concentration [mg/ml]	Concentration change [%]
Stage 2	4.54 (± 1.00)	13.49 (± 25.08)
Stage 3	5.07 (± 0.35)	24.50 (± 13.05)
Stage 4	5.16 (± 0.90)	29.16 (± 22.56)
Stage 5	7.26 (± 0.64)	81.50 (± 15.93)
Nebuliser	4.46 (± 0.21)	11.39 (± 5.25)

The solution remaining in the nebuliser increased only slightly in concentration.

The fluorescence emission spectra of the solutions in stage 3 and the nebuliser overlaid with the native protein (Figure 64).

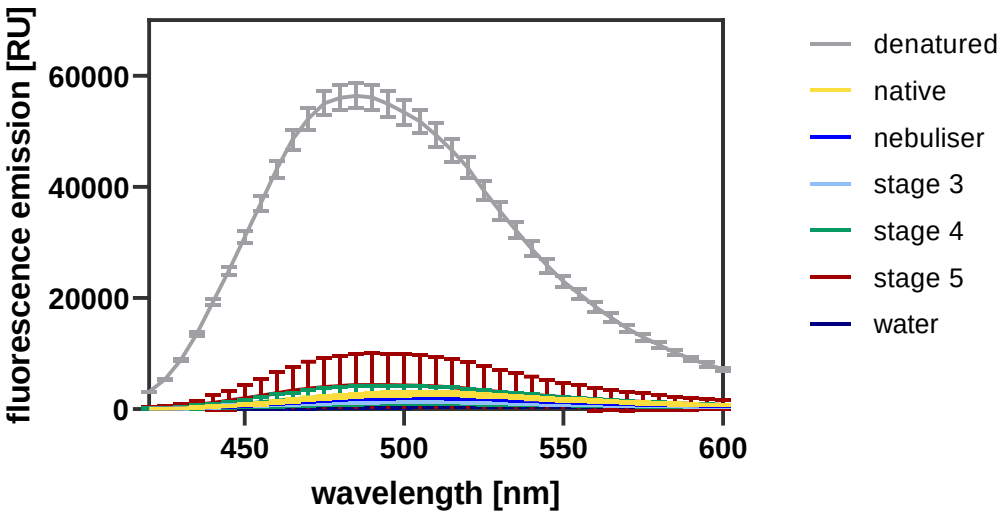

Figure 64: Influence of nebulisation with the nebuliser TurboBoy® on the spray-dried formulation; the mean curve of n=3, error bars=CI

The solutions collected in stages 4 and 5, however, shifted 10 nm to blue but the emission intensity did not increase (Figure 64). This indicated a small change in protein structure.

The overall results were very similar for the eFlow® *rapid* and the TurboBoy®. Redispersion and nebulisation of the spray-dried formulation worked without any technical difficulties in both nebulisers. The protein concentration of the redissolved spray-dried formulation changed similarly to that of the lyophilisate. The tertiary structure changed only slightly and was comparable to the structural change in the DPI formulation directly after spray drying.

7.6.3 Evaluation of the Suitability of the Nebulisers for DPI-Formulation

Nebulisation and sample collection with the NGI did influence the concentration of the nebulised solutions. This occurred for the protein as well as for mannitol and brilliant blue. Although the NGI was cooled before the measurement, evaporation seems to be the most likely explanation for the concentration increase as it occurred independently of the substance's characteristics. This phenomenon can affect the protein´s stability since generally higher protein concentrations can trigger aggregation and denaturation also during nebulisation [16]. The analysis of nebuliser aerosols in cascade impactors is known to be difficult since unexpected and ambient condition-dependent alterations in the aerosol may occur [70]. This is not likely to happen in vivo, since evaporation does not occur at 100% humidity in the lung. A found destabilising effect might, therefore, not occur in vivo. An ex vivo experiment in 100% humidity might verify destabilising results found in the NGI. An influence of the aerosol collection step also occurred in the study of Bodier-Montagutelli et al. [120]. This study identified distinct protein stabilities of Ig1 using the same nebuliser but collected with six different collection devices, including the NGI. Furthermore, they compared the protein stability of Ig2 and Ig3 after nebulisation collected in two of the collection devices. Depending on the protein and the parameter of protein stability they found different results on which collection device influences the protein the least. They concluded that although no general recommendation on the collection device choice is possible, they all can influence protein stability.

Nebulisation of the chymotrypsin lyophilisate solution with the three nebulisers in the present work resulted in different effects. None of the examined samples showed visible signs of aggregation after nebulisation. No change in the tertiary structure occurred after nebulising with the membrane nebuliser eFlow® *rapid* and the jet nebuliser TurboBoy®. Both nebulisers exposed the protein to shear-related and interfacial stress. In the samples produced with these nebulisers, these stress factors as well as the protein concentration increase did not seem to interfere with the structural integrity of chymotrypsin. The ultrasonic nebuliser Multisonic® did influence the tertiary structure of chymotrypsin. The influence did, however, only occur with decreasing droplet size. This might be caused by the combination of the ultrasonic frequencies with the increasing interfacial stress. The effect of this kind of stress not only depends on the certain protein but also on the available surface area [11]. Especially, partially unfolded proteins are prone to surface adsorption [11]. It is possible that the Multisonic® degraded a minor number of protein molecules which then accumulated more and more at the increasing surface. This might have caused increasing amounts of degraded protein molecules in the bulk which could then be detected at a certain point.

An effect on protein stability was found for all types of nebulisers in other studies. Respaud et al. found that nebulisation with different membrane nebulisers increased the occurrence of protein aggregates of the mAb IgG1 and IgG4 [90]. They also found a difference in the amount of aggregation between the two nebulisers. Furthermore, they could reduce aggregation by the addition of different surfactants. A negative effect of nebulisation with a membrane nebuliser was also found on the enzyme activity of lactate dehydrogenase for the eFlow® *rapid* by Hertel [121]. Niven et al. found a negative effect of nebulisation with an ultrasonic nebuliser on this protein [122]. They found a complete activity loss after 20 min of nebulisation with the Aeorosonic® nebuliser. They also detected heating during the process and were able to decrease the degradation by cooling the nebuliser. Furthermore, the addition of polysorbate 80 stabilised lactate dehydrogenase during nebulisation. Khatri et al. compared two jet nebulisers and two ultrasonic nebulisers and found that the decrease in enzyme activity depended on the nebuliser [123]. The nebulisers used caused different levels of aggregation. They also reported that the addition of surfactants led to a decrease in protein aggregation.

The results of the present section as well as the results of other studies demonstrate that different nebulisers cause different effects on model protein´s structural integrity during nebulisation. Therefore, choosing the nebuliser individually for each protein is necessary to minimise degradation. Furthermore, stabilising excipients, like surfactants, or stabilising conditions, like cooling, should be considered.

The present section evidenced that the nebulisation of the chosen spray-dried formulation was possible. The examined samples visually all dissolved completely and could then be nebulised without technical difficulties. A comparable technical feasibility is likely for other spray-dried formulations. The tertiary structure of the spray-dried formulation already changed a little before nebulisation (see Section 7.3.3.2). For both nebulisers, a blueshift of the samples in stages 4 and 5 occurred. No blueshift was found for the other stages. It is possible that the combination of different stress factors and the increased air-liquid-interface stress led to structural changes in the pre-stressed protein molecules in the smaller droplets (as in the samples of the Multisonic®, see above). In the larger droplets, the total degradation stress may not be high enough to cause these changes. The structural change was, however, comparable to that directly after spray drying and rather small, making further interpretation difficult.

In this section, the change in protein structure was too small for further investigations or interpretation. The effect, however, also occurred in another study [120] where Bodier-Montagutelli et al. found different amounts of protein aggregates in different NGI stages. Therefore, the influence of the collection method and different droplet sizes may be necessary to consider.

8 Conclusion and Outlook

This thesis focuses on DPI formulations for therapeutic proteins and methods to characterise the influence of production and application. The overall results demonstrate that a deeper look into the stress factors during the different spray drying phases is essential to produce a stable DPI formulation of a certain protein. The amount of protein degradation highly depended on the phase of spray drying and the spray drying system. This emphasised the importance of choosing stabilising excipients meeting the individual needs of each spray drying process. Furthermore, different application methods were found to be suitable for protein DPI formulations. This shows that it is possible to consider the need of multiple patient groups during formulation development.

The identification of suitable model proteins was highly complex. Contrary to the fact that proteins in general are highly sensitive molecules, not all of the examined proteins showed detectable changes after spray drying at high temperatures. While the molecular structure of chymotrypsin underwent major changes, lysozyme changed only slightly. Ovalbumin and BSA exhibited no changes detectable with the extrinsic fluorescence assay. For the model proteins chymotrypsin, lysozyme and ovalbumin only some of the analytical methods detected structural or physical changes in the protein. First, this relies on the sensitivity of the methods which may vary for different proteins. Second, structural changes in proteins can be of various shapes and therefore change different properties of the proteins. The proteins had different melting temperatures and different interface activities. A prediction of the sensitivity during spray drying was, however, not possible based on these properties. In this work, chymotrypsin was found to be a highly suitable model protein for stability studies, while the other model proteins were found less suitable. It was sensitive enough to be influenced by various stress factors tested, like thermal and interface stress, but stable enough to only degrade partly. This section demonstrated the essential need for specific experimental data on individual proteins to evaluate the influence of complex production methods, such as spray drying, on protein stability.

This thesis investigated several different methods to evaluate protein stability. For the model protein chymotrypsin, some of the detected structural changes led to no or only minor activity changes but still to a large change in fluorescence emission. Therefore, the extrinsic fluorescence spectra assay was found to be a sensitive and easy-to-perform fast screening method for the structural analysis of chymotrypsin´s tertiary structure changes. Nevertheless, discriminating between small structural changes remained impossible and the method did not work for BSA and insulin. For these proteins, the emission spectra changed only

slightly after denaturation. The assay was also not able to detect structural changes in ovalbumin after spray drying. A novel approach found in this work was the comparison of the dissolution behaviour and the wettability of the different protein powders to identify small structural changes that cannot be detected otherwise. The analysis of the dissolution behaviour was easy to perform, highly sensitive and was found to be a very suitable method for the characterisation of protein powders. It might, therefore, be a helpful analytical tool when evaluating structural changes in dry powder protein formulations.

A discrimination between the destabilising influence of the different phases of spray drying and of the different spray drying systems was possible. The stress factors during the drying phase could be identified as the major destabilising factors for chymotrypsin. The chosen spray drying temperature also highly influenced the protein structure. A lower spray drying temperature might be more favourable for the protein stability. The choice of the spray drying nozzle also had a great impact on the protein. The two-fluid nozzle thereby caused the lowest destabilisation of the nozzle examined in the present work and other studies. It, therefore, seems to be the nozzle most suitable for the production of a stabile protein DPI formulation. Others additionally found further destabilisation caused by decreasing droplet sizes [99, 100]. For inhalation, however, the particles and therefore the droplet have to be very small and this parameter cannot be chosen in favour of a high protein stability. To avoid protein destabilising effects that cannot be met by the adjustment of spray drying parameters, stabilising excipients need to be added.

The excipient combination of glycine and mannitol showed very promising results as stabilising excipients for a spray-dried protein formulation. Both are approved for inhalation and were already used as stabilisers in Exubera®. In this work, they almost completely stabilised the protein against degradation during production and others also found a stabilising effect during storage. Although the drying phase appeared to have the highest impact on the protein structure, mannitol, as a well-established stabiliser against thermal and dehydration stress, was only partially able to stabilise chymotrypsin on its own. Only in combination with glycine, as a suitable stabiliser for the whole spray drying process, an almost complete stabilisation was possible. This demonstrated that although stabilisers chosen on basis of specifically identified stress factors stabilise the protein, a single excipient meeting these stress factors might not be effective enough. Especially, the combination of excipients with different stabilisation methods could be promising.

For protein DPI formulations two application ways could be identified. First, the aerodynamic behaviour of the DPI formulation was found to be suitable for the inhalation as a dry powder.

Second, redispersing the DPI formulation and using the liquid as a formulation for nebulisation was technically possible. Of the investigated nebulisers, only the ultrasonic nebuliser influenced the protein structure. The protein in the redispersed DPI formulation showed comparable very small structural changes before and after nebulisation. The DPI formulation would, therefore, be a suitable storage formulation and the mesh as well as the jet nebuliser a suitable application method for patients who are not able to use a DPI device.

This thesis presented a set-up for the systematic evaluation of influence of stress factors during protein DPI production and application on the chosen model proteins. The next steps would require a transfer of this set-up to other model proteins or drug proteins. Nevertheless, as proteins vary greatly in their molecular structure and their sensitivity to certain stress factors an individual adjustment for each protein may be necessary. When implementing a protein DPI formulation several findings of this thesis and other studies have to be kept in mind. The complexity of the proteins´ structure can require a combination of multiple analytical methods relying on different principles. Not all occurring structural changes interfere with the proteins´ activity or other therapeutic functions. Furthermore, a correlation between structural changes and therapeutic function might be difficult. For therapeutic proteins, the implementation of a functional assay may, therefore, be essential as early as the formulation development process. Furthermore, different spray drying systems and parameters might expose the proteins to different stress factors and intensities. A correct choice of these parameters might, therefore, minimise the process´ negative influence on the protein. Various excipients can stabilise the protein during spray drying. Their combination and concentration should be chosen individually for each protein and each spray drying process. Last, for protein drugs that are supposed to target locally considering additionally the application via nebuliser might be interesting for patients with poor lung function.

9 Abstract

The pulmonary application of protein drugs broadens the therapeutical option for several severe lung diseases. The local therapy decreases the needed dose and the risk for systemic side effects dramatically. The systemic therapy could be a non-invasive and easier-to-formulate alternative for the parenteral application. Nevertheless, proteins are large and highly complex molecules. They are very sensitive to various types of physical or chemical degradation caused by several stress factors. Each protein has an individual vulnerability to different stress factors. Although several methods exist to analyse changes in the protein structure, they are rarely able to detect all possible changes for every protein on their own. The formulation as an inhalable dry powder increases the storage stability, is easy to formulate and highly environmentally friendly. For proteins these dry powders are best prepared by spray drying. The spray drying process, however, exposes the protein to various stress factors during the different phases of the process.

This thesis, therefore, aimed to identify and characterise destabilising factors that occur during the production and application of inhalable protein drugs. Spray dryers using different techniques and atomisation nozzles were compared. Each exposed the protein to varying levels and types of stress factors and ultimately affected the structure of the protein in different ways. The degradation was also depending on the stage of spray drying and the spray drying temperature. As stabilising excipients glycine and mannitol were able to protect the structure of the model protein chymotrypsin during the spray drying process to a high extent. Small structural changes, however, still occurred. Furthermore, spray drying changed the dissolution behaviour of the model protein ovalbumin in terms of dissolution velocity, solubility and wettability. Dissolution behaviour analysis was found as a fast, easy to perform and sensitive method to detect changes in dry powder protein formulation. Last, the investigation of application opportunities revealed two possibilities. First, the inhalable dry powder (DPI) formulation exhibited an aerodynamic performance suitable for dry powder inhalation. Second, this work found that the formulation could be redispersed and applied using a nebuliser. This would be an option to deliver the protein drug to the lung of patients that are not able to use a DPI device, due to e. g. poor lung function.

All in all, this thesis provided a framework for systematic investigation of the influence of the stress factors during protein DPI production and application on protein stability. This might be a useful tool for the implementation of DPI formulation of certain therapeutic proteins.

10 Zusammenfassung

Die inhalative Gabe von Proteinarzneimitteln erweitert die therapeutischen Möglichkeiten bei verschiedenen schweren Lungenerkrankungen. Die lokale Therapie reduziert die erforderliche Dosis und das Risiko systemischer Nebenwirkungen stark. Die systemische Therapie könnte eine nicht-invasive und einfacher zu formulierende Alternative zur parenteralen Anwendung darstellen. Allerdings sind Proteine große und sehr komplexe Moleküle, welche sehr empfindlich gegenüber verschiedenen Arten von physikalischem oder chemischem Abbau sind, welcher durch verschiedenste Stressfaktoren verursacht wird. Jedes Protein zeigt eine individuelle Empfindlichkeit gegenüber den verschiedenen Stressfaktoren. Es gibt zwar verschiedene Methoden zur Analyse von Veränderungen in der Proteinstruktur, diese sind jedoch selten alleine in der Lage, alle möglichen Veränderungen für jedes Protein zu erfassen. Die Formulierung als trockenes Pulver erhöht die Lagerstabilität, ist einfach zu formulieren und sehr umweltfreundlich. Sprühtrocknung ist die beste Formulierungsstrategie um Proteine als solche inhalierbaren Trockenpulverformulierungen (DPI) herzustellen, dabei wird das Protein jedoch verschiedenen Stressfaktoren ausgesetzt.

Der Fokus dieser Arbeit liegt auf der Identifizierung und Charakterisierung von Stressfaktoren, die während der Herstellung und Anwendung von Proteinarzneimitteln auftreten können. Sprühtrockner mit unterschiedlichen Bauweisen und Zerstäubungsdüsen wurden untersucht. Jeder dieser Sprühtrockner setzte das Protein unterschiedlichen Stressfaktoren aus, die die Struktur des Proteins unterschiedlich beeinflussten. Neben der Düse hing das Ausmaß der Zerstörung der Proteinstruktur von Sprühtrocknungstemperatur und der jeweiligen Prozessstufe ab. Glycin und Mannitol konnten als stabilisierende Hilfsstoffe die Struktur von Chymotrypsin als Modellprotein während der Sprühtrocknung weitgehend schützen. Es traten jedoch immer noch geringfügige Strukturveränderungen auf. Darüber hinaus veränderte die Sprühtrocknung das Lösungsverhalten von Ovalbumin, als weiteres Modellprotein, hinsichtlich Lösungsgeschwindigkeit, Löslichkeit und Benetzbarkeit. Die Analyse des Auflösungsverhaltens erwies sich als eine schnelle, einfach durchzuführende und empfindliche Methode zur Erfassung von Veränderungen von verschiedenen Trockenpulver-Proteinformulierung. Als letztes wurden Applikationsmöglichkeiten für Protein DPI Formulierungen untersucht. Einerseits eigneten sich die Formulierungen auf Grund ihres aerodynamischen Verhaltens zur Inhalation als trockenes Pulver. Andererseits war es möglich die DPI Formulierung zu redispergieren und einen Vernebler für die Verabreichung der flüssigen Formulierung zu verwenden. Dies könnte eine Alternative für Patient*innen sein die auf

Grund ihres Gesundheitszustandes, z. B. wegen sehr schlechter Lungenfunktion, nicht dazu in der Lage sind einen Trockenpulverinhalator zu benutzen.

Insgesamt hat diese Arbeit ein Vorgehen für die systematische Beurteilung von Stressfaktoren, die während der Herstellung und Anwendung von Protein-DPI Formulierungen auftreten können und deren Einfluss auf die Proteinstruktur entwickelt. Dieses Vorgehen kann bei der Entwicklung von DPI Formulierungen spezifischer Proteinarzneistoffe genutzt werden.

11 Annex

11.1 Abbreviations

Table 29: Abbreviations used in this work

ANS	8-Anilino-1-Naphthalene Sulfonic acid
BLA	Biological License Applications
BSA	Bovine Serum Albumin
BTEE	Benzoyl-L-Tyrosine Ethyl Ester
CD	Circular Dichromism
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease 2019
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DPI	Dry Powders for Inhalation
EtOH	Ethanol
FDA	Food and Drug Administration
FDKP	Fumaryl DiKetoPiperazine
FSI	Fast Screening Impactor
FTIR	Fourier-Transform-Infrared spectrometer
HPLC	High-Performance Liquid Chromatography
iGC	inverse Gas Chromatography
MALLS	Multiangle laser light scattering
Mini spray dryer	Mini Spray Dryer B-290
MMAD	Mass Median Aerodynamic Diameter
MOC	Micro-Orifice Collector
Nano DSF	Nano Differential Scanning Fluorimetry
Nano spray dryer	Nano Spray Dryer B-90 HP

NGI	Next Generation Impactor
PEG	Polyethylene Glycol
Ph. Eur	European Pharmacopoeia
SDS-PAGE	Sodium Dodecyl Sulphonate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SLS	Static Light Scattering
UV	Ultra Violet
VIS	Visible
x_{10}, x_{50}, x_{90}	10%, 50% or 90% of the particles have a smaller diameter than the value x

11.2 Substances

Table 30: Substances used in this work

8-Anilino-1-naphthalene sulfonic acid (ANS)	Merck KGaA, Darmstadt, Germany; Batch: 011M1243
Bovine Serum Albumin (BSA)	Merck KGaA, Darmstadt, Germany; Batch: 9048-46-8
Calcium chloride	Merck KGaA, Darmstadt, Germany; Batch: F1726592248
Chymotrypsin	Merck KGaA, Darmstadt, Germany; Batches: SLBV2540, SLCH1926
Ethanol 99%	AppliChem GmbH, Darmstadt, Germany; Batch: 7U013801
Glycine	Merck KGaA, Darmstadt, Germany; Batch: 631K2467190
HCl 25%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany; Batch: 14148889
Lactose (InhaLac® 230)	Meggle Group, Wasserburg am Inn, Germany; Batch: L1019470392316A538
Lysozyme	Merck KGaA, Darmstadt, Germany; Batch: SLCC4285
Mannitol (Parteck® M DPI)	Merck KGaA, Darmstadt, Germany; Batch: HO2-1
Methanol	J.T. Baker, Phillipsburg, New Jersey, United States; Batch: 1409112008
mQ water	In house production
N-Benzoyl-L-tyrosin-ethylester (BTEE)	Merck KGaA, Darmstadt, Germany; Batch: BCCC4805
Ovalbumin	Merck KGaA, Darmstadt, Germany; Batch: SLCK7421
Polysorbate 20	Croda International, Snaith, United Kingdom; Batch: 1912NP3853
Polysorbate 80	Croda International, Snaith, United Kingdom; Batch: 0000239071
Tris (hydroxymethyl) aminomethane	Merck KGaA, Darmstadt, Germany; Batch: 1475359

11.3 Summary of Samples Produced with the Spray Dryers

All described pumped and atomised samples were aqueous solutions in mQ water. All described spray-dried samples were aqueous solutions in mQ water before spray drying.

Table 31: Summary of pumped samples

Pumping system of	Composition	Described in Section
Nano spray dryer	4.0 mg/mL chymotrypsin	7.3.1
Nano spray dryer	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	7.3.1
Mini spray dryer	4.0 mg/mL chymotrypsin	7.3.2
Mini spray dryer	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine	7.3.2.2
Mini spray dryer	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	7.3.2.2
Mini spray dryer	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine, 4.0 mg/mL mannitol	7.3.2.2

Table 32: Summary of atomised samples

Spray drying nozzle	Composition	Described in Section
Spray head	4.0 mg/mL chymotrypsin	7.3.1
Spray head	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	7.3.1
Two-fluid nozzle	4.0 mg/mL chymotrypsin	7.3.2
Ultrasonic nozzle	4.0 mg/mL chymotrypsin	7.3.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine	7.3.2.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	7.3.2.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine, 4.0 mg/mL mannitol	7.3.2.2

Table 33: Summary of spray-dried samples

Spray drying nozzle	Composition	Inlet-/outlet temperature (°C)	Described in Section
Two-fluid nozzle	4.0 mg/mL BSA	150/80-90	7.1.4.1
Ultrasonic nozzle	4.0 mg/mL BSA	150/65	7.1.4.1
Two-fluid nozzle	4.0 mg/mL chymotrypsin	150/80-90	7.1.4.2
Ultrasonic nozzle	4.0 mg/mL chymotrypsin	150/65	7.1.4.2
Two-fluid nozzle	4.0 mg/mL insulin, 4.0 mg/mL glycine, 4.0 mg/mL mannitol	150/80-90	7.1.4.3
Ultrasonic nozzle	4.0 mg/mL insulin, 4.0 mg/mL glycine, 4.0 mg/mL mannitol	150/65	7.1.4.3
Two-fluid nozzle	4.0 mg/mL lysozyme	150/80-90	7.1.4.4
Ultrasonic nozzle	4.0 mg/mL lysozyme	150/65	7.1.4.4
Two-fluid nozzle	4.0 mg/mL ovalbumin	150/80-90	7.1.4.5

Ultrasonic nozzle	4.0 mg/mL ovalbumin	150/65	7.1.4.5
Spray head	4.0 mg/mL chymotrypsin	120/45	7.3.1
Spray head	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	120/45	7.3.1
Spray head	4.0 mg/mL lysozyme	120/45	7.1.4.4
Two-fluid nozzle	4.0 mg/mL chymotrypsin	150/80-90	7.3.2
Ultrasonic nozzle	4.0 mg/mL chymotrypsin	150/65	7.3.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin	50/35	7.3.2.1
Two-fluid nozzle	4.0 mg/mL chymotrypsin	30/22	7.3.2.1
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine	150/80-90	7.3.2.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL mannitol	150/80-90	7.3.2.2
Two-fluid nozzle	4.0 mg/mL chymotrypsin, 4.0 mg/mL glycine, 4.0 mg/mL mannitol	150/80-90	7.3.2.2

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13 Erklärung nach § 9 der Promotionsordnung

Hiermit erkläre ich gemäß § 9 der Promotionsordnung der mathematisch-naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel, dass ich die vorliegende Abhandlung, abgesehen von der Beratung durch meine Betreuerin, nach Inhalt und Form eigenständig und ohne fremde Hilfe verfasst habe. Weiterhin habe ich keine anderen als die angegebenen Quellen oder Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht. Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde weder ganz noch in Teilen an einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Weiterhin wurde mir weder ein akademischer Grad entzogen, noch habe ich an dieser oder einer anderen Fakultät einen früheren Promotionsversuch unternommen.

Friederike Johanna Elisabeth Roth

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