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Identification of critical bulk protein properties with respect to subsequent manufacturing

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Abstract

The aim of this work was the investigation of protein powder properties which can have significant impact on subsequent process steps, such as dissolution and filtration. Therefore, a detailed literature review was compiled to get an overview about this particular topic.
It was found, that storage conditions have an immense impact on the water content of freeze-dried protein powders. Therefore, the bulk lyophilized fibrinogen powder, which was used as model substances in these studies, was stored at four different relative humidities. Subsequently a detailed powder characterization, including dissolution tests, particle size and shape, relative crystallinity and BET surface measurements as well as secondary structure determinations by spectroscopic methods was compiled. Differences in the specific inner surface area, crystallinity, particle size and shape were clearly identified, and correlated to the dissolution behaviour. It was found that a water content of 9% is best for a fast dissolution as the specific surface of the powders enhances and therefore, offers a larger area for the solvent.
To investigate the influence of residual water content on the secondary structure of the freeze dried fibrinogen ATR-FT-IR (attenuated total reflectance fourier transformed infrared spectroscopy) and Raman measurements were performed. Furthermore, a PLS (partial least square) with the generated crystallization data was performed. The results demonstrated that the combined ATR-FT-IR and Raman approach could be used to predict the crystalline state in freeze-dried fibrinogen products. Furthermore, the nano-structural properties were studied by small and wide angle X-ray scattering and related to the dissolution behavior. The results show a complex, biphasic behavior: above 9% water content the crystallinity increased, and the specific surface decreased with increasing water contents; at the lowest water contents (6%), however, where the WAXS patterns showed amorphous structure of the fibrinogen, the specific surface and dissolution rates diverged over a wide range of values. It was found that dissolution rates decreased with an increasing specific surface area, most notably in the amorphous form. Protein conformational changes and hydrophobic surface formation upon depletion of water could be possible causes.

Kurzfassung

Das Ziel dieser Arbeit war die Untersuchung des Einflusses von Proteinpulvereigenschaften auf deren weitere Prozessierbarkeit (z.B.: Auflöse- und Filtrationsprozesse).
Ein detaillierter Überblick über Protein- und Pulvereigenschaften wurde erstellt, welcher Methoden zur Pulver- und Proteincharakterisierung beinhaltet. Lagerungsbedingungen haben einen signifikanten Einfluss auf die Prozessierbarkeit von Proteinpulvern, daher wurden mehrere Chargen von gefriergetrockneten Fibrinogen Pulver in unterschiedlichen relativen Luftfeuchtigkeiten (14%, 52%, 75%, 92%) gelagert und analysiert. Im Speziellen wurde der Einfluss der sich veränderten Pulvereigenschaften auf deren Auflöseverhalten beobachtet. Unterschiede in der spezifischen Oberfläche, Kristallinität, Partikelgröße und –form wurde identifiziert und mit dem Auflöseverhalten korreliert.
Es wurde gezeigt, dass Pulver mit einem Wassergehalt von 9% das beste Auflöseverhalten zeigen, da bei diesen Pulvern die spezifische Oberfläche am größten ist und daher am meisten Angriffsfläche für das Medium gegeben ist. Um den Einfluss des Restwassergehaltes auf die Sekundärstruktur von gefriergetrockneten Fibrinogen zu untersuchen, wurden ATR-FT-IR und Raman Messungen durchgeführt. Des Weiteren wurde eine direkte Korrelation der Veränderung der Sekundärstruktur der Fibrinogen Pulver und deren Kristallinität identifiziert. Die Ergebnisse zeigen, dass kombinierte spektroskopische Methoden zur Vorhersage des kristallinen Zustands verwendet werden können.
Um die nano-strukturellen Eigenschaften des gefriergetrockneten Fibrinogenpulvers zu bestimmen wurden SAXS (Small angle X-ray scattering) und WAXS (wide angle X-ray scattering) Messungen durchgeführt und mit dem Auflöseverhalten korreliert. Die Ergebnisse zeigen folgendes Verhalten: bei einem Wassergehalt von über 9% steigt die Kristallinität in den Pulvern während die spezifische Oberfläche ab nimmt; bei einem Wassergehalt on 6% scheint das Fibrinogenpulver amorph, die spezifische Oberfläche und die Auflöseraten zeigen eine breite Verteilung. Die Ergebnisse zeigen, dass die Auflöseraten sinken, während die spezifische Oberfläche steigt. Dies kann durch Konformationsänderungen des Proteins und die Bildung hydrophobischer Oberflächen erklärt werden.

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1. Introduction

1.1 Lyophilization

During the last century, freeze-drying evolved to a well-established method for the preservation of biopharmaceutical products. During this development the spectrum of pharmaceutical applications has been spread from drying labile pharmaceutical products, i.e antibiotics and proteins, to new technical developments and better process understanding resulting in more complex freeze-dried products. Lyophilization is considered to be the safest, most reliable process for the dry storage of stable forms of sensitive drugs. Thus, freeze-drying requires a combined understanding of process science, formulation aspects and container effects [1]. Despite the advantages for long time storage the process also bears its risks to influence drug quality [2]. Most of the literature in the field of freeze-drying is dealing with pharmaceutical proteins.

Lyophilization or freeze-drying usually consists of three process steps. Initially, the aqueous sample is frozen to reach a vitreous state, where water is crystallized and the amorphous content remains in the interstitial region. The freezing of the product includes two steps, namely ice nucleation and ice crystal growth. Afterwards, the water is removed by sublimation (primary-drying) at a temperature below the glass transition or collapse temperatures. Any material which is going to be freeze-dried has a critical temperature, the so called collapse temperature (Tc). Above this temperature defects are likely to occur. Thus, the shelf temperature ramp has to be well designed to avoid product collapse and shrinkage as a result of overheating [2], [3], [4].

The freezing step is mainly controlled by the shelf temperature and the chamber pressure. Furthermore, factors such as container geometry, stopper design, container heat transfer coefficient, freezing behavior, freeze-dryer geometry, fill depth, formulation type and concentration can influence the sublimation.

As 10-20% water remains adsorbed after primary drying, non-frozen (sorbed) water is removed by diffusion and desorption during secondary drying at higher temperatures. Residual water content in typical freeze-dried materials ranges from a fraction of 1% to about 3%. A number of excellent reviews dealing with the description of the freeze-drying (lyophilization) process are available [3], [5]–[7].

Although freeze-drying is used to stabilize labile products, the process itself generates both freezing and drying stresses and might often be harmful for pharmaceutical proteins [8] and therefore, do not guarantee long – term stability.

Variation in product characteristics is a common phenomenon that can be caused either by inconsistencies in substrates or process variations. This can ultimately lead to problems during the reconstitution of the sample or further processing, e.g. during filtration due to filter fouling.

1.1.1 Bulk Lyophilization

Usually, freeze-drying is performed in vials to enhance the storage stability of active pharmaceutical ingredients (API). Furthermore, freeze-drying is useful for some stages of intermediate production, e.g. as API operations [9]. In general, open metal trays are used for bulk freeze-drying. Thus, problems related to contamination of the equipment due to product blowout can occur. Furthermore, high sublimation rate processes, results in loss of material and cleanup problems. According to the repeated use, trays may become warped, which results in a poor and variable heat transfer from the shelf to the tray [10].

1.2 Fibrinogen

Fibrinogen is a large, complex, fibrous glycoprotein with three pairs of polypeptide chains linked together by 29 disulfide bonds. It is 45nm in length, with globular domains at each end. It is connected by α-helical coiled-coil rods in the middle. Fibrinogen is normally present in human blood plasma at a concentration of about 2.5 g/L. It is essential for wound healing, hemostasis, inflammation, angiogenesis, and other biological functions. Although fibrinogen is a soluble macromolecule, it has the nature to form clots or insoluble gel on conversion on fibrin. This protease-resistant, mechanically stable clot is necessary to prevent blood loss and promote wound healing [11].

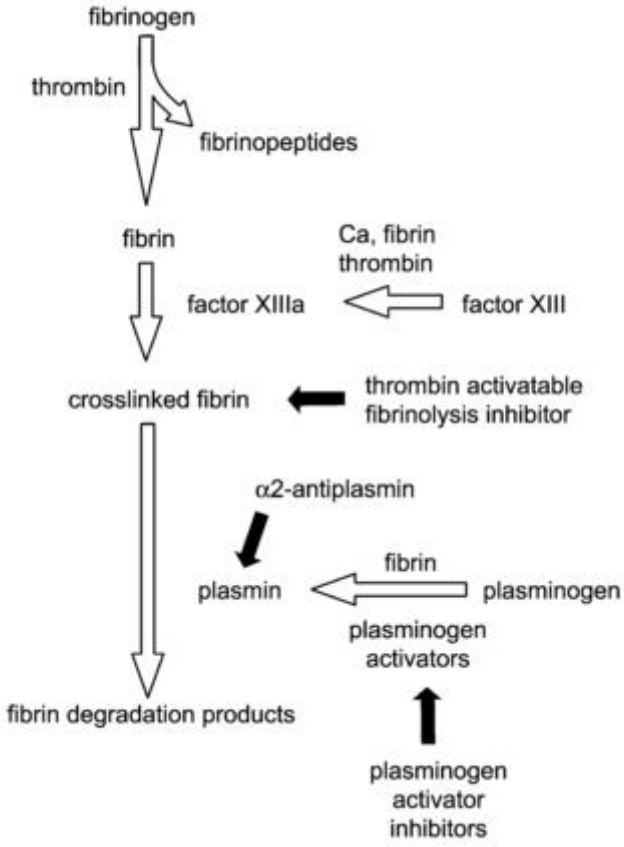

Figure 1: Scheme of fibrin polymerization and fibrinolysis [11]

Fibrin is formed when thrombin removes small peptides from the amino termini of R and β chains, exposing sets of “knobs” that interact noncovalently with ever-present “holes” on neighboring molecules to form oligomers termed protofibrils (Figure 1). As polymerization progresses, thrombin-activated factor XIII incorporates covalent cross-links, initially between the carboxy-terminal segments of γ-chains, but eventually including α-chains also [12].

1.3 Motivation of the thesis

Physico-chemical attributes of freeze-dried protein powders have a significant impact on subsequent process steps, e.g. dissolution/reconstitution, filtration. The complex coherences of the protein powder properties and their processing remain largely unclear (Figure 2). This results in unstable processing and significant loss of raw-product.

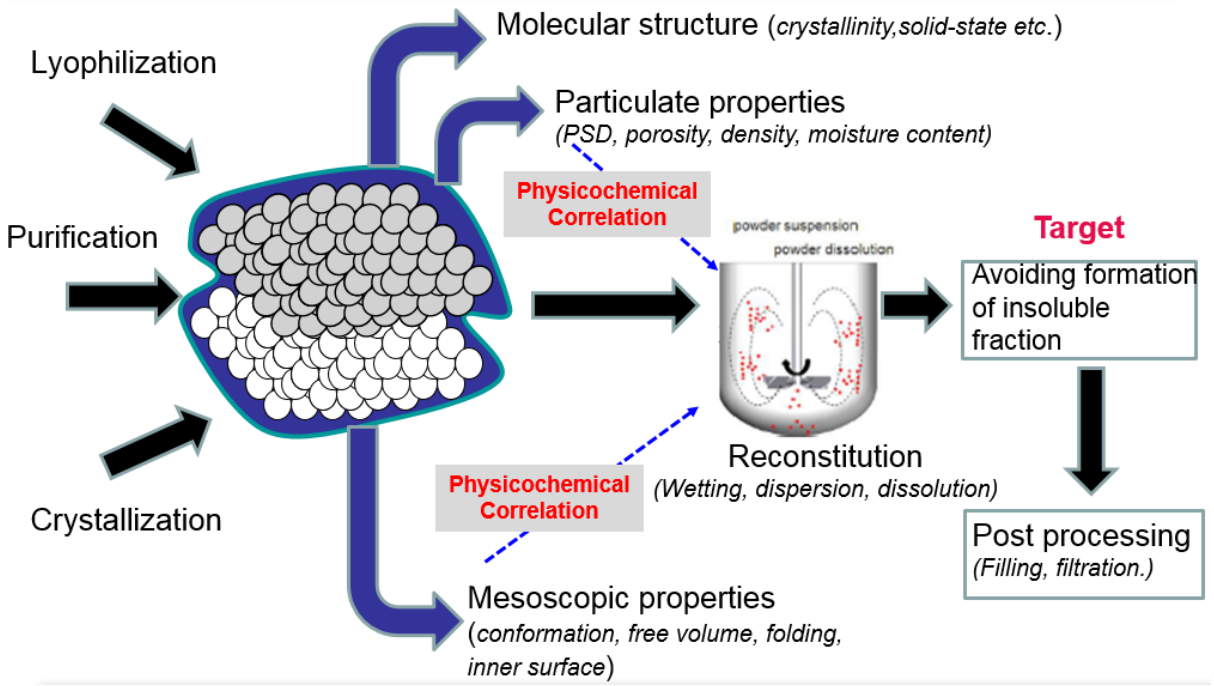

Figure 2: Overview–Coherence of protein powder attributes and subsequent process steps.

Long-term stability of freeze dried protein powders before subsequent processing is known to be a main limiting factor for a successful development of biopharmaceuticals, although, a lot of research has been conducted in the field of the storage stability of freeze-dried proteins. Stability during storage and transport is important for manufacturers as well as for the life scientist. Proteins can undergo conformational changes as well as other structural changes, which can lead to protein aggregation. Therefore, freeze-dried proteins can lose their activity in solution after reconstitution [7].

Less has been published in the field of investigating the influence of lot-to-lot differences on subsequent processes, e.g. dissolution and filtration. Exposure to various relative humidities and temperatures can lead to altered protein powder properties, i.e. aggregation and inactivation, which may have a negative influence on further processing.

The aim of this work was to investigate the influence of moisture sorption and therefore, the influence of various water contents on freeze dried fibrinogen bulks as well as subsequent process steps. The work is divided in 4 main chapters: the first one is reviewing various powder and protein properties, which influence the reconstitution behavior of freeze dried proteins and how to measure and quantify them. Further, the influence of certain properties on the reconstitution behavior of six different fibrinogen bulks was determined. This work was advanced with further investigations of the relationship of inner surface and crystallinity of the powders on the reconstitution behavior. Moreover, the influence of the secondary structure on crystallinity and thus, on the dissolution behavior of freeze dried fibrinogen was evaluated.

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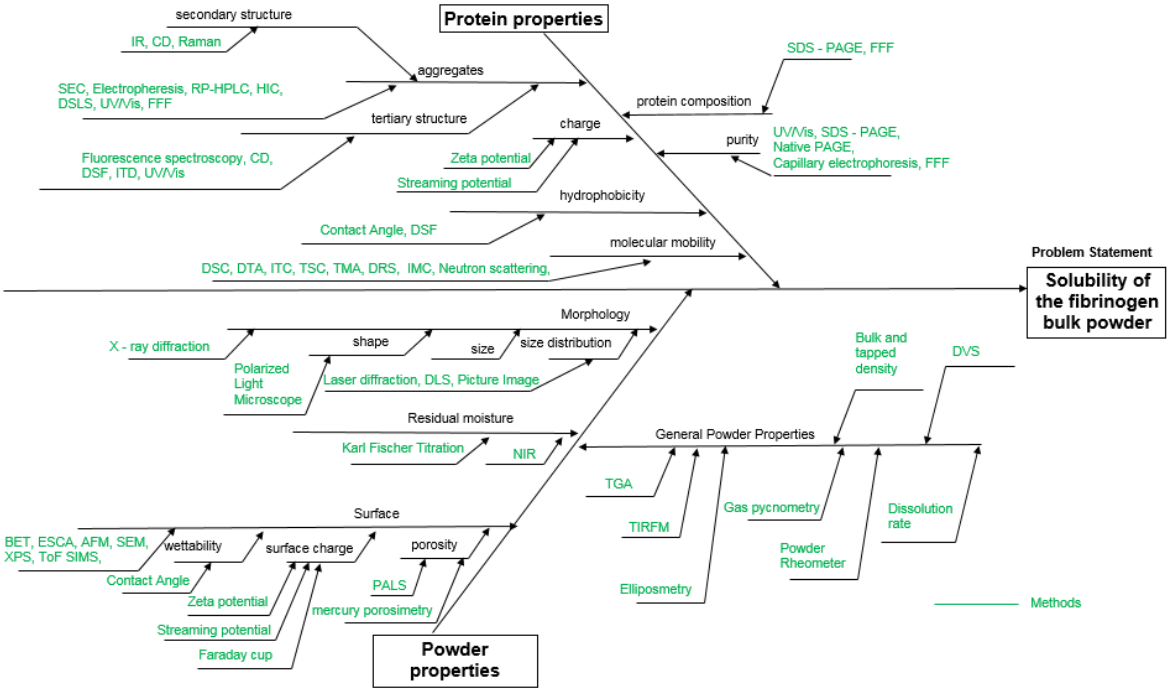
2. Chapter 1: Physico-chemical aspects of lot-to-lot variabilites of lyophilized protein powders in subsequent processing-a Review



Abbreviations:

AFM	Atomic Force Microscopy
BET	Brunnauer, Emmet, Teller
CD	Circular Dichroism
DLS	Dynamic Light Scattering
DRS	Dieelectric Relxation Spectroscopy
DSC	Differential Scanning Calorimetrie
DSF	Differential Scanning Fluorimetry
DSLS	Differential Scanning Light Scattering
DTA	Differential Thermal Analysis
DVS	Dynamic Vapor Sorption
ESCA	Electron Spectroscopy for chemical analysis
FFF	Field Flow Fractionation
HIC	Hydrophobic interaction chromatography
IMC	Isothermal microcalorimetry
ITC	Isothermal titration calorimetry
ITD	Isothermal denaturation
NIR	Near Infrared Spectroscopy
NMR	Nuclear magnetic resonance
PALS	Positron anhilition life-time spectroscopy
RP - HPLC	Reversed Phase High pressure liquid chromatography
SEC	Size exclusion chromtography
SEM	Scanning electron microscopy
TSCS	Thermally stimulated current spectroscopy
TGA	Thermogravimetric Analysis
TIRFM	Total internal refelctance microscopy
TMA	Thermal mechanical analysis
ToF SIMS	Time of flight secondary ion mass spectroscopy
UV/Vis	Ultraviolett/visible spectroscropy
XPS	X-ray photoelectron spectroscopy

2.1 Abstract

Lyophilization of proteins can introduce lot-to-lot variabilities in different product properties that are crucial in terms of product performance and stability. As these variations can raise several issues related to the product quality, the characterization of the physical and chemical properties of protein lots is required to identify critical deviations between the lots and rationally control the process to yield the acceptable product. The critical concerns are potential quality issues of drug product and (negative) influences on the reconstitution behavior of the solid-state lyophilized proteins. This review provides an overview of powder properties and physiochemical attributes of solid protein formulations manufactured by lyophilization that are potentially associated to lot-to-lot variabilites. Brief descriptions of various conventional as well as novel analytical techniques which are amenable for the characterization of the pharmaceutical protein powders properties are discussed.

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2.2 Introduction

During the manufacturing process, pharmaceutical proteins are exposed to different stresses and conditions that may alter the physical to molecular properties of protein and hence may interfere quality and stability [1]. Especially for biopharmaceutical drugs one critical factor is the long-term stability of proteins as the molecule needs to stay in its native state over a certain period of time [2]. Long term stability is an important issue as physical or chemical alterations may influence immunogenicity, toxicity and efficacy [3]. Proteins are usually stored in the liquid, solid or frozen state [4]. To achieve long shelf lives of protein products, strategies applied are for example engineering of molecules for aggregation resistance or the inclusion of additives that inhibit the formation of aggregates [5], [6]. Whereas the liquid products are more conveniently applied, the liquid environment allows higher mobility and therefore can lead to stability issues. Thus, proteins are often dried to reduce the rate and the extent of unwanted physical transformations and chemical degradation [6]. The most commonly used method for preparing solid protein pharmaceuticals is freeze-drying with alternatives being spray drying, spray-freeze-drying and freeze-thawing [7]–[9]. Two physical criteria have been proposed to be important for long term stability of dried proteins. In the first place, the solid protein is generally present as a glassy amorphous phase that includes other amorphous additives (e.g. sucrose). It may be necessary to include multiple excipients in a given formulation to protect against the thermal, chemical, mechanical stresses involved in freezing and drying [10]. Secondly, long term stability depends on retaining the native protein structure in the dried solid [11]. As the lyophilization step itself is a complex interplay of dynamic and non-equilibrium processes, it can be very challenging to consistently achieve a stable and aesthetic final product [7], [12], [13]. Differences between various batches of the freeze-dried protein formulations are often difficult to characterize as the variations (that can lead to the altered product quality) are small and indistinct. Analytical methods to identify and control the quality are necessary, since variations in chemical or physical state among production batches often rise quality issues during pharmaceutical development [14]–[16].

Physical and chemical manifestations triggered by changes of conditions/parameters or different stress factors during freeze-drying can lead to – along with above mentioned quality issues - prolonged reconstitution times due to dissolution problems as well as issues in filtration and subsequent process steps [7], [14], [17], [18]. This can influence the techno-economics of the process for bulk lyophilization as well as of the downstream processes as fill and finish operations and eventually can have impact on patient compliance of final drug product.

Possible damages of the product related to freezing and drying stresses, e.g. solute concentration, formations of ice crystals, pH changes [19], [20], etc. as well as storage conditions, can be divided into (a) denaturation, like dissociation or aggregation, which can lead to changes in secondary, tertiary and quarterny structure [7], [13], [21], [22] and (b)

chemical degradation via proteolytic activity, acid or base catalysed hydrolysis, deamidation, radiolytic degradation, oxidation, formation and exchange of disulphide bonds as well as Maillard reactions [6], [7], [13], [21]

Most literature for chemical degradation of biopharmaceuticals is based on investigations in the liquid state, both because the drug product is finally administered as a liquid and because of the capability of most of the existing analytical methods. Therefore characterization of degradation pathways in the solid-state of protein powders still remains largely unexplored [6]. The amorphous form is generally the intrinsically favored physical state for formulations containing proteins as macromolecular structure of favors its disordered molecular organization on solid-state. Thus, the physical state of the freeze-dried product formulation is mostly amorphous. Additionally the partially amorphous state and alternative polymorphic modification of protein API and/or excipients can emerge via lyophilization process [23]. The amorphous state exhibits several advantages in comparison to the crystalline one, e.g. molecules in the amorphous state exist in a disordered state thus represents higher energy state and show faster dissolution rates compared to the crystalline counterpart [23]. The resulting physical states of solid protein and peptide formulations are kinetically controlled by the temperature, composition (API and excipient), moisture, and the time the protein sample is exposed to the environment. Therefore, instabilities due to alteration of these parameters are able to occur [24]–[26].

Residual water in amorphous formulations plays a critical role for stability and subsequent processing. In general, lyophilized protein formulations show higher stability against physical changes and chemical degradation (eg hydrolysis) at lower water contents [27]. Various studies are available on the effect of increased residual water amounts on physical and chemical instabilities [27]–[30]. Furthermore, several publications about moisture-induced aggregation of freeze-dried proteins have been published periodically [22], [31]–[34]. However, residual water can have various influences on the other physicochemical properties lyophilized protein powders, as water molecules bound at the surface to charged and polar groups and to the peptide backbone groups can cause the protein to be unstable and degrade [35]. Increased water content also favors a higher conformational mobility of the protein molecule, the process referred to as plasticization [29] making it more susceptible for chemical modifications. Besides the impact of occupant water outside protein structure, the structural fraction of water is essential for some protein to maintain its structural integrity in solid-state [36].

Furthermore, the increase in water content due to the absorption of moisture by solid protein during storage can lead to the issues related to protein aggregation[34]. Bulk lyophilized

pharmaceutical proteins will be exposed to various levels of humidity during storage and processing [31].

In recent years, a plethora of literature with focus on improving the lyophilization process itself and the optimization of the protein formulation has been published. Therefore, a wide range of literature focusing on e.g. thermal denaturation, role of excipients during freezing and storage exists [17], [18], [37]–[39]. Excipients have an important role during the lyophilization process, as they stabilize the product against degradation due to the thermal stress introduced via processing. Furthermore interactions of product and excipients as well as phase separation can be observed [40]–[43].

The focus of this review is to provide an overview about critical physicochemical parameters of solid-state proteins. This includes various micromeritic powder properties or protein structure-related molecular properties, with a special focus on analytical methods employed for the characterization of these properties and the identification of lot-to-lot variability of lyophilized protein formulations.

2.3 Physicochemical parameters originating lot-to-lot product variability

Physical and chemical parameters of solid-state biopharmaceuticals which could influence variability in bulk processing, product quality related to the pharmaceutical performance, stability and patient compliance can be broadly categorized in generic powder properties and protein properties (Figure 1). The following section will describe factors that are critical during the life-time of a protein based drug.

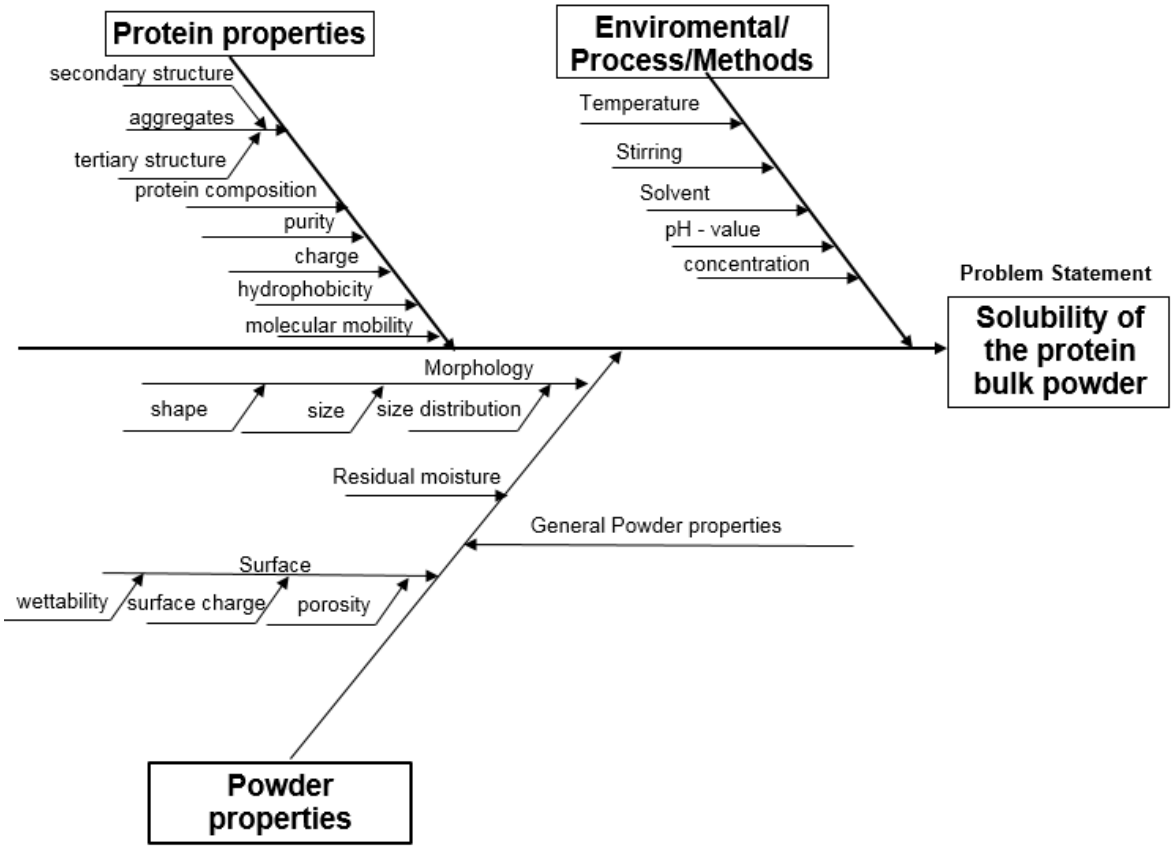

Figure 3: Fishbone diagram of parameters influencing the reconstitution of solid-state proteins.

Molecular, particulate and bulk properties of protein powders

To investigate lot-to-lot variabilites between pharmaceutical freeze-dried powders, it is important to consider the physical and chemical attributes of the lyophilized powders. Thus, various powder properties like particle size and shape, density and specific surface area are required to be thoroughly elucidated. Moreover, one essential property is the dissolution/solubilization behavior of the solid-state proteins intended for parenteral preparations. *In vitro* dissolution testing is important for correlating in vitro and in vivo bioavailability, determining batch-to-batch equivalence in early and late pharmaceutical development stages [14], [44], [45]. Typically, rapid dissolution is required from lyophilized products, as they are often reconstituted by adding into an aqueous medium and administered as injection, e.g. in an emergency room [46]. The physical process governing lyophilized powder reconstitution involves multiple competitive steps.

Wetting is a primary step in the reconstitution process that is followed by the submergence, dispersion, and dissolution/solubilization of the particles [47]. Wettability is an interfacial properties that is a measure of the balance between the cohesion and adhesion forces working at solid-liquid interface and thus reflects the ability of a bulk powder to imbibe the liquid under the influence of capillary forces of wettability of a solid powder is a function of various surface and powder properties and solid-state such as particle size, density, porosity, surface charge, surface area, and surface activity etc. Furthermore, reconstitution is influenced by the morphology of the lyophile cake, the presence of cake collapse or meltback, hydrophobic coatings, the homogeneity of the dry matrix, and the formation of channels between pores and the solid-states [48]. Wettability is mainly characterized by the contact angle between the powder surface and the penetrating diluents [49]. Additional factors such as surface roughness, chemical heterogeneity, sorption layers, molecular orientation, swelling, and partial dissolution of the solid in the liquid directly impact the contact angle measurements of pharmaceutical powders [50].

As powder wetting and dissolution begins at the powder surface, one major influencing parameter is the particle size distribution and thereby the available surface area. Noyes-Whitney equation is a classical expression to describe the relation of dissolution rate with particle size, surface area and solubility of a solid material [51].

$$\frac{dm}{dt} == \frac{DA}{h}(c_s - c) \text{.....Eq. 1}$$

Where *dm/dt* is the dissolution rate, c_s is the equilibrium solubility and *c* is the drug concentration in the bulk solution at time t. D is the diffusion coefficient, A is the surface area and h is the thickness of the diffusion boundary layer.

Specific surface areas and porosity

A high specific surface area reduces the dissolution time and increases the hydration rate [52], [53]. A direct correlation of particle size and specific surface area is only valid for single globular particles. For pharmaceutical powders, which include a wide range of size and shapes, the relationship is much more complex. The smaller the particles or the more the particles are non-spherical, the higher is the tendency to agglomeration as cohesivity increases for smaller particles as well as for the non-spherical particles [54]. Furthermore, particle shape and size influences the powder packing which in turn has an impact on interstitial spaces for penetrating liquids [55]. Penetration of imbibing medium in the powder bed is essential step of powder dissolution. In general, larger particles can be more irregular-shaped than smaller ones and therefore can provide more interstitial space [56]. Another parameter affecting the dissolution rate and hence, the penetration of the powder bed, is the porosity of the lyophilized protein powder. Capillaries, porosities and cavities near surfaces initiate the rehydration process, whereas the presence of trapped air bubbles and closed pore structures pose a major obstacle to the ingress of fluid [57], [58]. Haeger et al. showed that an increasing porosity and low bulk density of a lyophilized parenteral formulation promoted rapid reconstitution [59].The structure of pharmaceutical solids at the nanoscale, (between ca. 1 and 100nm) is a further critical for reconstitution of a freeze-dried compound [53]. The specific inner surface denotes the interfacial area (solid-solid, solid-liquid, solid-gas) between two phases per unit volume of a particular material [60]. Overall, alteration in one or various particulate and molecular properties in different lots of lyophilized proteins can result a significant variability in the reconstitution performance of final formulations [61]–[64].

Surface energetics

Another important factor in terms of pharmaceutical performance, post-processability and stability is surface or interfacial free energies and their heterogeneity for the lyophilized powders [65]. It is the energy required to produce a unit area of surface. The total surface free energy consists of a number of different physical force components. Solid-state protein has non-polar (dispersive) forces as well as polar forces such as hydrogen bonding or acid-base interactions. The magnitude of this interfacial free energy can correlate to that of wetting energy and spreading coefficient of a liquid over a solid surface [66]. Relative humidity of an environment surrounding a powder product can influence the surface energy. The presence of water changes the surface energy of powders. Detailed information on the models associated with the surface energy related parameters can be found elsewhere [67].The cohesive energy of a material is a parameter which quantifies the attraction that atoms or molecules have for one another. Thus, it is directly related to the stability and other physical properties of the material. A common method of quantification is the solubility parameter or the square root of

cohesion energy density (the ratio of enthalpy of vaporization to the molar volume of a material). Solubility parameters can sometimes predict the absorption of simple solvents or complex drug molecules across a variety of substrates [68], [69]. The specific interaction of a solid with another solid or liquid can be described in terms of acid–base properties. Gutmann and Drago numbers are often used to describe interactions at the interface [70]–[73]. The bulk absorption/adsorption behavior has a considerable impact on stability and manufacturing [69]. Surface energy variability and heterogeneity originating from manufacturing processes has been reported for various small molecular weight APIs and excipients, while the same for biopharmaceuticals is yet to be explored [62]. [61].

Surface charge and dielectric properties of biopharmaceuticals

Furthermore, surface charge variations among different batches of powder products can impact product qualities that are associated with electrostatics and tribo-electrifications. During the handling of powders, differences in electrostatic charge can occur as the protein powder particles are in contact with each other and different devices surrounding the powder. These contacts cause that electrons may be donated or accepted during various processes. Electron exchange occurs because of surface potential differences between the contacting bodies. These potential differences are mainly caused by different contact materials but may also be influenced by different particles size, surface roughness, etc. The difference of surface potential is compensated by exchanging charges. If the separation of the bodies occurs fast enough, the charge backflow is disabled and the contacting bodies remain charged. This charging mechanisms are influenced by RH, mixing processes, amorphicity, particle shape and size as well as surface roughness [74].

Other properties of lyophilized proteins which have to be considered are dielectric parameters. It express the ability of a material to get polarized when placed in an electromagnetic field. Polarization is a dynamic process that depends on the structure and molecular properties of the material. Therefore, dielectric analysis can provide information about particle size and porosity and different types of molecular mobility [13]. As an electric field can penetrate deep into a sample, it is possible to characterize bulk properties of the material. Five different polarization mechanisms contribute to the dielectric response of materials: ionic polarization (e.g. surface conductance), interfacial polarization (or face charge) dipolar reorientation (e.g reorientation of water molecules), atomic polarization (e.g. bond stretching) and electronic polarization (deformation of electronic orbitals) [13].

Mechanical properties

A further attribute determining the physical structure of the lyophilized protein cake is their mechanical performance, namely compressive strength, elastic moduli and toughness etc. Thus, mechanical characterization using compressive indentation stress-strain curves can

generate information on the apparent strength of lyophilized cakes. Furthermore, mechanical tests can be an effective way for studying pore properties of freeze-dried products. Note that residual moisture content can pose a remarkable influence on these mechanical parameters [75]. This mechanical properties of lyophilized pharmaceutical proteins are less explored hitherto, however very important factor in terms of downstream and powder handling.

Powder flow properties

The powder flow of is of great importance in the pharmaceutical industry in terms of handling and processability characteristics. In case of pharmaceutical proteins, powder flow becomes crucial when handling with bulk freeze-dried powders. Powder flow can be determined by powder flow meter. Additional derived attributes, such as bulk density, compressibility, adhesion and permeability data can be generated during shear testing [76]. Segregation of powders can occur because of particle size and shape as well as composition and structure of the powders. Furthermore, powder segregation can be influenced by density differences and particle interactions [77].

Solid-state properties

As powder properties are influenced by the physical state of freeze-dried pharmaceutical proteins, the physical state influences the reconstitution of freeze-dried protein products enormously. The dissolution rate of crystalline powders is intrinsically slower in comparison with the amorphous structures [78]. On the other hand, the high energy amorphous states of solids are less stable physically and chemically compared to the corresponding crystals. Amorphous forms can crystallize or undergo structural relaxation towards the equilibrium-state [79], [80]. The residual water remaining in protein lyophile can induce the physical transformation of solid-states eg. crystallization, anhydrate-hydrate transition. On the one hand, it acts as a plasticizer and enhance molecular mobility, [79], [81]. Furthermore, phase separation (protein/excipient) in composite protein formulations can be a challenge. Three main types of phase separation during the freeze-drying process have been reported: crystallization of amorphous solids, separation into different amorphous phases, and amorphization from crystalline solids [82]. The tendency of protein-protein interactions in the protein rich phase of the phase separated product is prominent leading to the aggressive formation of aggregates [83]–[85]. Moreover, (naked) protein molecules located on the surface of freeze-dried proteins have significantly faster degradation rates owing to the higher surface molecular mobility [86]. In addition to phase heterogeneity, the composition or chemical heterogeneity can be one of the major issues of freeze-dried products that can originate from formulation and/or process alteration [83], [86]–[89]. For example, composition heterogeneity can arise from the surface activity of the protein (thermodynamic tendency to migrate towards

the air- water interface). The detection and characterization of multiple amorphous phases are keys to better understanding of how phase separation affects product stability [90].

Molecular mobility of solid protein and relation to stability

Primary and secondary molecular mobilities can the relevant descriptors for determining/predicting physical and chemical stability of an amorphous lyophilized drugs [91], [92]. Primary molecular or global mobility involves translational and rotational motions of entire molecules, often referred to as α process occurring predominantly from and beyond glass transition temperature (T_g) while secondary or local motional processes constitute segmental molecular motions of macromolecules (β, γ, δ, processes and so on) [35], [82]. Embedding proteins in glassy matrices that are rigid and exhibit slow molecular motions generally improve protein stability by slowing down dynamics in glassy matrices [18], [85], [93]. Three physical parameters can mainly be used to determine the extent and kinetics of protein degradation process: the T_g region, a structural relaxation time (τ) for α process [94], [95] and the mean square displacement of hydrogen atoms, a measure of fast, local β dynamics [96]. [97]. Below T_g, rates of crystallization and chemical reactivity overall decrease and largely governed by local molecular motions (21). Therefore, local motions such as rotation of the side chains and small amplitude vibrations may also be important to storage stability [98].

Aggregated and unfolded proteins exhibit “polymer like glass behavior” [98]. It is assumed that aggregation involves partial unfolding and the thus the rate of protein aggregation generally depend on the rate of translational mobility of protein molecules. Correlations between aggregation rates and structural relaxation have been shown in various protein systems including lyophilized powders, such as enhancement of aggregation associated with an decrease in T_g and changes in the temperature dependence of aggregation rates around T_g [35], [99]–[105]. It has been suggested that aggregation of protein in the solid-state occurs among nearest neighboring protein molecules [106], [107]. Therefore, some studies have suggested that molecular mobility at a length scale shorter than that of structural relaxation, such as β-relaxation, rather than α-process is critical to protein aggregation [29], [106], [107]. Recently, it was shown that protein stability is directly linked to β-relaxation processes of the sugar matrix as these β-relaxations are also directly linked to diffusion of small molecular reactive species in the glass [96]. It is suggested that structural relaxation time may be a better indicator of global dynamics [108]–[110]. Reaction rate constants increase proportionally with structural relaxation time [111]. Water, which is bound to protein surfaces may cause protein surface groups to relax more rapidly as compared to the bulk. When water molecules bind to protein molecules, their motion is restricted resulting in decrease in the overall mobility of the water while that of protein molecules increase leading to the decrease of T_g, process known as plasticization [35].

Collapse properties of lyophilized powders

Collapse of freeze-dried pharmaceutical matrices can occur during the freeze-drying process or during the subsequent storage [112]–[114]. It may adversely affect properties of the freeze-dried materials. The rate of collapse increases above T_g [115]. Collapsed systems usually show poor rehydration propensity [116]–[118]. Several factors influences the characteristic collapse temperatures (T_c), e.g moisture content, temperature and molecular weight of the excipient matrix [119], [120]. In Figure 5 a non-collapsed and collapsed sample is imaged. A time-temperature relationship as a function of matrix properties can aid in understanding the collapse kinetics and associated thermal regions thus can help in predicting Tc for a particular formulation. Thereby, the control space and optimization of storage conditions could be established to possible avoid agglomeration [116]. The stability of amorphous protein solids is dependent on “thermal history” of a production process. Thus, the drying processes with different thermal histories may impact the stability of pharmaceuticals [9], [105], [121], [122]. Therefore, the drying process need to be duly controlled to yield the desired product stability [39].

2.4 Structural properties and chemical stability of protein pharmaceuticals

Critical point during biopharmaceutical development is evidently the stability of drug substance and drug product. Most methods for the analysis of physicochemical stability and thus degradation of proteins are well applicable in the liquid-state. Nevertheless, techniques for monitoring protein quality in the solid-state are emerging in recent years. Attributes such as secondary structure, solid-state aggregation and thermal and mechanical properties can be investigated in addition to degradation events taking place in the solid-state.

Chemical degradation via secondary and tertiary structural alteration

Freeze-drying can potentially alter the secondary structure of the protein. Maintaining the native structure of a protein during freeze-drying is necessary as changes in such structural features can often be irreversible thus can lead to permanent protein deactivation [124]. Usually proteins become more structured via dehydration during the lyophilization process and increasing β-sheet content with decreasing α- helices can be observed [125]. It was found that dehydration induces spectral alterations in the conformationally sensitive amide I region due to protein unfolding [126]. Changes in secondary structure occuring during freeze-drying can sometimes be reverted after re-hydration, resulting in a fully recovered, native and biologically competent protein. [127], [128]. Usually, changes in secondary structure are accompanied by the loss of tertiary structure of different proteins. However, secondary structural change is not necessarily the precursor for the tertiary structural changes for all protein products [129], [130]. Subsequent downstream processing of protein lyophiles may affect the tertiary structure

leading to the opening of the hydrophobic cavity and enhances protein aggregation [131]. Overall, these structural changes in protein at different spatial scales can affect the long-term stability. Intensive research data exisit on in-line/ real-time monitoring the freeze- drying by NIR and Raman spectroscopy to control secondary structure during the process [132], [133]. Protein chain unfolding in the dried state during storage can be observed using IR spectroscopy [40], [124]. Furthermore, the secondary structure is sometimes qualitatively correlated to aggregation and chemical degradation in the solid-state [85]. The extent of denaturation can be measured by the extent of loss of secondary structure.

Chemical degradation via covalent modifications

Covalent bonds modifications governing chemical degradation of protein generally involve the modification of the primary structure via bond cleavage or formation. In contrast, the changes in secondary, tertiary and quaternary structure are mediated by the loss of conformational integrity of the protein structure leading to aggregation and precipitation. It is challenging to distinguish between conformational and chemical instability as most of them are not always mutually exclusive [17], [134].

Different routes of protein denaturation are possible to occur during storage of solid protein pharmaceuticals. Unfolding of protein chains exposes many hydrophobic residues enveloped in the native structure. Intermolecular hydrophobic interactions between such residues are the major trigger for protein aggregation. Typical inactivation mechanisms, either of intermolecular or intramolecular nature, are deamidation, oxidation and aggregation are commonly observed phenomena during storage [135]. Deamidation of asparagine and glutamine residues, and racemization of amino acid residues have been published [24], [136]. In case of glycoproteins or the presence of free reducing sugars in protein formulation, the Maillard reaction can potentially takes place [137]. For the tetanus toxoid, it was found that aggregates were formed via covalent non-disulfide bond. These bonds were Schiff–base intermediates and the side chains of lysine, tyrosine and histidine residues in the toxoid molecule [138]. Furthermore, conformational processes can trigger solid-state aggregation [31]. Deamidation was detected for hGH (human growth hormone), recombinant interleukin 1 receptor antagonist, recombinant bovine somatropin and insulin [25], [139]–[142]. Additionally, it was indicated that various chemical degradations can occur simultaneously in the solid-state. For example methionine oxidation, asparagines deamidation and irreversible aggregation was detected during storage of freeze-dried hGH [7], [36]. Proteins containing disulfide bonds and free thiols are able to aggregate via thiol-disulfide interchange during processing. The intermolecular cross linking is often triggered by moisture [143]. For a more detailed insight into solid-state reactions of proteins, we refer the interested reader to the excellent works published periodically [24].

Moisture-induced chemical instability of solid-state proteins

Moisture-induced aggregation of lyophilized biopharmaceuticals has been considerably covered in literature [21], [27], [53], [128], [144]. It is well established that water vapour from the environment can be absorbed into the bulk powder of amorphous protein, in addition to being adsorbed in the surface. The amount of water taken up depends on the environmental conditions, such as RH and temperature, as well as the relative polarity of the solid and hygroscopicity [145]. This aggregation can lead to reduced bioactivity, increased immunogenicity and poor solubility [146]. In the presence of water vapour at 37°C, it was found that lyophilized recombinant human albumin (rHA) undergoes thiol-disulfide interchange, therefore forming water–insoluble aggregates. It was found for some protein formulations that excipient-water interactions rather than excipient protein interactions play the dominant role in stabilization [147]. In case of freeze-dried insulin stored at 50°C and 96% RH, aggregation via β-elimination occurred that is followed by thiol-catalysed disulfide exchange [21]. As already discussed, the residual water content of lyophilized protein formulations can have a tremendous impact on protein stability [27]. Breen et al.studied the effect of increased residual water amounts on physical and chemical instabilities (79). Water can act as reactant and therefore promote e.g hydrolysis [81]. Residual water can evolve as product in chemical reactions. The remaining water decreases the T_g and thus increased molecular mobility [23], [37], [81], [111]. Flores-Fernandez et al. investigated the influence of moisture on secondary structure in freeze-dried insulin [22]. With increasing moisture, changes in secondary structure were increasing and therefore, promoted irreversible aggregation [22], [34], [64].

While residual water often increases chemical degradation rates, it can as well suppress chemical reactions [25]. In a few cases, a certain amount of water must be present to ensure structural integrity of protein in solid-state and thus necessary for the chemical stability [81]. Thus, the exact relationship between solid-state instability and water content has yet been difficult to delineate and is dependent on the type of formulation and degradation reactions [24]. The residual water can induce solid-state chemical degradation in amorphous protein via different mechanism compared to the more ordered structures [81], [148]. Moisture content includes both an immobilized part (e.g. water of hydration) and an active part. Water activity or equilibrium relative humidity (ERH) indicates the degree of freedom of the water absorbed in a material and shows dimensions, structure, cohesion, agglomeration properties as well as electrical and chemical properties sometimes better than moisture content does. Water activity is a measure of the energetic status of the free water in a system. If half of the water in a protein system is so tightly bound to a protein molecule thus not available for chemical reaction such as hydrolysis, the overall water activity would be reduced [149]. Therefore, batch-to-batch differences in water activity can have an impact on further processing.

2.5 Analytical methods to determine batch-to-batch variability in lyophiles

Extensive explorations have been accomplished so far in applying several analytical techniques working in diverse measurement principles for the characterization of lyophilized protein structure and various associated properties. Some of them are especially modified for freeze-drying processes, others are applied as for conventional solid-state and protein characterization. Various reports about solid-state characterization can be found be found in the literature [53], [79], [82], [150], [151]

In this section, the techniques for characterizing bulk powder and structural properties of protein are briefly described. The direct investigation of lyophilized protein lots is advantageous since no further sample preparation is necessary which results in faster processing, less sources of variation and potential use as PAT (process analytical technology) tool. In table 2, an overview about the different methods is presented.

Table 1: Overview of different analytical techniques applicable for the structural analysis of protein and the information decipherable from them

Analytical techniques	Obtained information
Atomic force microscopy	Particle size, shape, surface structure, aggregation
Contact angle **Sessile drop method,** **Wilhelmy plate method**	Wettability, spreading coefficient, surface free energies
Dielectric relaxation spectroscopy (DRS)	Dielectric permittivity; Tg, primary and secondary molecular mobilities
Differential scanning calorimetry (DSC)	Tg, molecular mobility, crystallization kinetics, degree of crystallinity, denaturation
Dynamic mechanical analysis (DMA)	Molecular mobility, mechanical modulus, Tg
Dynamic vapor sorption (DVS)	Powder structure (amorphous/crystalline), sorption/desorption isotherms
Electron spectroscopy for chemical analysis (ESCA)	Protein surface concentration
Faraday pail	Electrostatics, net charge
Fluorescence spectroscopy	Tertiary structure
Freeze- drying microscopy	Cake collapse, transition temperatures
Gas adsorption	Specific surface area, porosity
Helium pycnometry	True density
Hydrogen/deuterium exchange-mass spectroscopy (H/D X-MS)	Exchangeable protons, protein structure, interactions between protein and excipients
Infrared spectroscopy (IR)	Secondary structure, molecular structure
Inverse gas chromatographie (iGC)	Solubility Parameters, Surface energy
Isothermal microcalorimetry	Glass transition temperature, degree of crystallinity, crystallization kinectics, solvations
Karl Fischer Titration	Residual water content
Laser diffraction analysis	Particle size distribution, Particle shape
Mechanical analysis	Strength of lyophilized cake
Mercury porosimeter	Porosity (inter- and intraparticulate)

Micro - X – ray - computed tomography (micro-CT)	Microstructure, porosity
Neutron scattering	Molecular mobility, molecular interaction
Nuclear magnetic resonance (NMR)	Molecular mobility, tertiary structure, interactions
Optical coherence tomography – freeze drying microscope (OCT – FDM)	Collapse temperature, Tg
Powder rheometer	Bulk density, permeability, shear testing
Polarized light microscopy	Particle size, particle shape, transitions
Raman spectroscopy and microscopy	Secondary structure, distinguishing between protein particles and excipients
Scanning electronic microscopy (SEM) and Crogenic-SEM	Particle size, Particle shape, roughness, size range, morphology
Sorption measurements	Wettability
Thermal mechanical analysis (TMA)	Glass transition temperature
Thermally stimulated depolarized or polarized current (TSDC, TSPC) spectroscopy	Dielectric relaxation, Glass transition temperature, microheterogeinity
Thermogravimetric analysis (TGA)	Moisture content, hydration level, decomposition temperature
Transmission electron microscopy (TEM)	Particle size, Particle shape; crystallinity
Water activity measurements	Water activity, Sorption behavior
X – Ray diffraction	Powder structure (amorph/crystalline), inner surface

Particulate analysis

Particle size, shape, density and flow properties

Some characteristics powder properties important in terms of processability and the final product quality are particle size and particle shape. Various other derived powder properties such as bulk density and surface area of powder bed depend upon particle size and shape. Since the absolute values generated during the particle size/shape measurements depend highly on the working principle of a particular method, it becomes important to distinguish between different methods [152]. Detailed literature about particle size measurements can be found elsewhere [153], [154]. Laser diffraction is one of the most common techniques to determine particle size distributions. During the measurement, the scattering pattern and intensity of monochromatic laser light after incident onto the dispersed sample particles are recorded [153].The optimal dispersion pressure during the measurements is important to obtain the data on the native particles without the breakage of the particle agglomerates during the measurement for fragile samples. [152]. Since the size of the particle is estimated based on the projection to the size, area or volume of equivalent perfect sphere, the accuracy of particle size measured depends on the shape of the particle as well. Details about methods for characterization as well as classification of shape can be found in the literature [155]. The most accurate way to determine particle shape is microscopy, as it enables the visualization of the particles. However, as microscopy is a very time consuming method and often lacks of

resolution for smaller particles. Alternative methods such as dynamic image capturing and analysis exist wherein statistically signification population of particles in a dispersed samples oriented with various possible shapes are temporally imaged using high speed camera thus providing higher shape resolution for the heterogeneous powder sample [156].

Density of a powder bed is a very crucial property that can help in choosing right processing and downstream. A common method for true density measurement is gas pycnometry, helium being the most used gas. Provided the sufficient sample homogeneity and reproducible measurement, true density can help in distinguishing the amorphous versus partially or fully crystalline states of some materials. As helium penetrates into smallest pores and crevices, it permits to approach the real volume of the sample. Helium pycnometry has its limitation when used for channel crystalline hydrates and other systems containing loosely bound solvents because the volatile water or solvents will alter the helium pressure and introduce measurement errors. Therefore, the samples have to be completely dry for the measurement [157]–[160]. In contrast to true density, the bulk and tapped density of a powder can be investigated by looking at powdered drugs under loose and tapped packing conditions, respectively. The bulk density of a powder can be defined by measuring the volume of a known mass of powder sample that may have been passed through a sieve into a graduated cylinder or by measuring the mass of a known volume of. The tapped density is characterized by mechanically tapping a graduated measuring cylinder or vessel containing the sample. After observing the initial powder volume or mass, the measuring cylinder or vessel is mechanically tapped, and volume or mass readings are taken until little further volume or mass change is noticed [161]. The method has its limitations in the amount of powder, as a relatively high amount of material is needed. Thus, only bulk freeze-dried powders can be used. Using the parameters bulk and tapped density, it is possible to calculate different powder flow indices such as the Hausner ratio and the compressibility index.

Furthermore, evaluation of flow properties of powder is an inevitable task for the product development. The methods based on the measurement of the angle of repose (AoR) and shear cell based measurement are the common powder flow evaluation techniques. The angle of repose represents characteristics related to inter-particulate friction. The measurements are very operator dependent. Furthermore, segregation and consolidation of the material is likely to occur while performing the experiments [162]. Using a shear cell can enable wide range of parameters, including the angle of internal friction, the unconfined yield stress, the tensile strength, and a variety of other parameters such as flow factor and other flowability indices. Detailed information can be found elsewhere [76], [163]–[165]

Surface and porosity measurements

A critical parameter of particulates and powder beds is the specific surface area, which can be determined by gas adsorption measurements using N_2 or Krypton as the adsorbate gad. Briefly, this method employs the gradual introduction ofa known volume of adsorbate gas to the dried sample and the pressure is allowed to equilibrate. A relative pressure range (p/p_0) of 0.05-0.3 is normalized to the saturation pressure of the adsorbate. As the volume of the sample chamber is known, the total amount of adsorbed gas can be calculated. The addition of adsorbate gas followed by equilibration in the sample chamber can be repeated multiple times for each sample measured. Since samples need pre-process of evacuating the residual moisture, it can be disadvantageous for vacuum-sensitive samples. As some lyophilized samples often show low surface areas, krypton gas is necessary for the analysis of the specific surface area [113].. Usually, the Brunauer, Emmet and Teller (BET) adsorption theory is used to analyze the data [87], [166]. While the use of the Barrett-Joyner-Halenda (BJH) or Kelvin equations allows the determination of the pore size distribution [167]. A further technique for measuring the specific surface is mercury porosimetry. This method also provides information about pore size distribution, the total porosity, the skeletal and apparent density of a sample [168]. Therefore, mercury porosimetry is a useful technique to quantify the microstructure of powdered samples. During the measurement, mercury is intruded in the sample at high pressure, as it is a non – wetting liquid and porosity is estimated using [169].

$$L^2 = \frac{\gamma D t}{4\eta} \quad \text{Eq. 2}$$

where t is the time, η the viscosity of the mercury and γ the surface tension; D describes the average pore diameter und L the penetration depth. A major disadvantage of this technique is the measuring of the largest entrance towards the pore, but not the actual inner size of a pore. Detailed information about the measurements can be found elsewhere [168].

A further approach to obtain the specific inner surface non-invasively is by using SAXS (small angle X-ray scattering). SAXS detects all interfaces between zones of different electronic density, irrespective of whether they are externally accessible or closed pores [63], [170]. Two important parameters are generated from the scattering curve, The integral intensity, the so-called ‘Invariant’ Q and the intensity decay, measured by the decay coefficient, according to Porod’s law [170].

Topographic methods

Polarized light microscopy is a simple, quick and sensitive technique to detect the crystallinity in freeze-dried pharmaceutical proteins through the birefringence in the sample [171]. Using freeze-drying microscopy (FDM) microstructures of the formulation solution sample under conditions of freezing can be determined. Thereby, a cryo-stage in connection with a

controllable cooling system and a vacuum system is mounted. Freeze-drying microscopy is regarded to be the best method to measure the collapse temperature of the freeze-dried product as well as determining the influence of formulation composition and freezing history on the morphology of ice [172], [173]. A recently evolving method for characterizing the collapse temperature T_c is optical coherence tomography based freeze drying microscope (OCT-FDM). OCT is a high resolution optical imaging technique, which measures the back reflection of light [174].

Scanning electron spectroscopy (SEM)

Using SEM structures less than 1nm can be viewed. A focused electron beam instead of light is used and therefore, is more powerful yielding better resolution compared to optical microscopes, than optical microscopy [152]. It scans a tiny spot of electrons across the specimen [175]. Changes in structure of the sample can occur due to sputter coating and the vacuum during the measurement. Only milligram quantities of material can be used to determine particle size, shape and texture, therefore the information is limited to a small imaged area. It is not possible to distinguish between crystalline and non–crystalline materials. Used in combination with other techniques such as laser diffraction, SEM provides valuable additional information on particle texture, which may help to explain aggregation events [152]. Environmental SEM (ESEM) applies lower vacuum, that is, higher pressures, thereby enabling analysis of hydrated samples, which is one of the advantages of ESEM. Further, no sample preparation is necessary. The main disadvantage of typical ESEM instrumentation is that the electron beam spreads in the high-pressure environmental chamber and excites fluorescent X-rays from the entire specimen, not just from under the electron beam. The fluorescent X-rays generated outside the area of interest are detected by the detector and reduce the image contrast [175].

Transmission electron microscopy (TEM)

TEM is a further electron microscopy method. Using this technique, no sample preparation is necessary, but the electron beam itself can change the original sample structure. TEM illuminates the specimen area under examination with a single, large spot [176]. An alternative option is cryo-TEM. [175]. Here, the sample can be analyzed in the frozen state, but again changes of the sample according to the electron beam are possible. By using TEM detailed information about the particle structure of monoclonal antibody aggregates and HSA (human serum albumin) aggregates in solution were found [177], [178].

Atomic force microscopy (AFM)

Using AFM the sample surface is scanned mechanically with a cantilever. The principle of AFM is presented in the literature [179], [180]. AFM provides three dimensional images down to 0.1

nm resolution under ambient conditions without sample preparation. Particle size, shape and surface structure as well as protein aggregation can be determined [181]. As the image area of AFM is limited to micrometer dimension the information about particle characteristics is limited [179], [181]. A technical difficulty of this technique is the need to find conditions wherein the cantilever tip does not draw the analyzed material with it as it moves across the surface [182].

Micro-X-ray computed tomography (micro-CT)

A powerful method to resolve structural properties of freeze-dried protein powders is micro-CT. With this method it is possible to investigate the microstructure of a lyophilized powder cake in 3D at any observation angle. Additionally, micro-CT enables the quantification of loss of connectivity (fragmentation index) for dried cakes [183]. Thus, rendered 3D models can be sliced at any level, and at any angle to show the inner structure of the material while no physical slicing is required [183], [184] . The technique has its limitations since the sample size is in the micrometer dimensions and the measurements are time consuming. Detailed information about micro-CT can be found elsewhere [184]–[186].

Thermo-physical techniques

Using thermal analysis techniques, physical properties of freeze-dried protein powders are monitored as a function of temperature or time while the sample is heated or cooled under controlled conditions. Processing and aging conditions may be simulated additionally to the generation of thermodynamic data. These kind of measurements are concurrent or complementary to other analytical techniques such as spectroscopy [187].
Glass transitional behavior can be detected by changes in volume/density, heat capacity, viscoelasctic moduli, electrical permittivity and refractive index as T_g is connected to changes in molecular mobility. Various thermo physical techniques for determination of glass transition temperature are available e.g. Differential scanning calorimetry (DSC), thermomechanical analysis (TMA) and dynamic mechanical analysis (DMA).

Differential scanning calorimetry (DSC) and isothermal calorimetry

DSC is according to published literature the most commonly used technique in characterizing freeze-dried protein products. The method involves the heating or cooling of a sample and reference and the measurement of the differential heat flow (power) between them with respect to maintaining an identical temperature in the sample and reference cell [187]. This technique allows the determination of glass transition temperature, eutectic temperature T_e, crystallization temperature, degree of crystallization, un-freezable water content and melting point in the lyophilisate [188], [189]. Within modulated DSC (mDSC) the separation of the relaxation endotherm from the glass transition is possible [190]. Thus, this technique is

especially used if difficulties in detecting T_g due to overlapping thermal events are observable. Furthermore, it can be used to determine the enthalpy recovery, recrystallization temperature and kinetics [191]. Detailed information about differential scanning calorimetry can be found in the literature [192]. Isothermal microcalorimetry works on the principle that all physical and chemical processes are accompanied by a heat exchange with their surroundings. The sample is maintained under isothermal conditions within the microcalorimeter, During an thermal event, a temperature gradient is formed between the sample and its surroundings. The resulting heat flow between the sample and its surroundings is measured as a function of time. The determination of amorphous amounts in samples is feasible using isothermal microcalorimetry [193]. Furthermore, the molecular mobility, especially global mobility can be measured [39], [109]. Isothermal microcalorimetry is a valuable technique for probing differences in wettability of a powder surface that could occur due to process changes [16].

Mechanical analysis

The deformation of the sample under stress (e.g. compression, penetration, tension) can be determined using thermomechanical analysis (TMA). The generated data is plotted against time or temperature while the temperature increases or decreases proportionally to the time. Changes are detected by mechanical, optical or electrical transducers. Additionally the determination of phase transformations such as glass transitions is observable.

Alterations in the viscoelastic properties can be measured by dynamic mechanical analysis (DMA). An oscillatory stress is applied to the sample and both the magnitude and phase relationship of the stress and strain values are measured as a function of either frequency or temperature. DMA, however is less suitable because the sample has to be prepared in form of a film or a compact. DMA is a further development to TMA measurements. When the sample is heated through the T_g the shear modulus decreases, often by several orders of magnitude. A further oscillatory technique is dielectric analysis (DEA). As opposed to DMA a sinusoidal oscillatory electric field is applied to the sample [194], [195]. The complex dielectric permittivity is measured as a function of temperature and may change abruptly through the glass transition and thereby allowing identification of the T_g [196]. Some reviews present the principle and applications of dielectric analysis in pharmaceuticals [13], [195]. The Tc can be determined using this method; furthermore the molecular mobility can be characterized. El Mozine et al. found significant differences for different freeze-dried formulation mixtures of sugar and drug in dielectric relaxation kinetics and activation energy [194], [197].

Recently a novel mechanical compression test was developed for measuring the mechanical properties of freeze-dried cakes. Using the mechanical compression test, the strength of lyophilized powder cakes can be determined by determining the stress (σ) and strain (ε). From these values the elasticity can be calculated [74].

Thermoelectric techniques

Thermally stimulated current (TSC) detects the mobility of dipoles and other permanent or induced electric charges. Two basic TSC techniques exist: thermally stimulated polarization current (TSPC) and thermally stimulated depolarization current (TSDC). In TSDC a sample is polarized by a permanent electric field at a relatively high temperature where there is significant molecular mobility to allow the attainment to equilibrium. The polarization is frozen by quenching the sample to a low temperature, and then the electric field is removed. When the dipoles become mobile, an electric current is induced by the spontaneous depolarization, and this current is measured by the electrometer. In the polarization method, an electrical field is applied at a low temperature where dipoles are immobile, and polarization of the dipoles by the electric field is detected during heating of the sample. As in TSPC measurements the samples are not exposed to high temperatures and the potential of chemical degradation is therefore minimized [92]. With TSDC the relationship between molecular mobility and temperature can be exploited. As proteins consist of polar repeating units in peptide bonds, they are sensitive to dielectric polarization. The micro-heterogeneity of amorphous systems can be detected using this kind of measurement. While glass transition temperatures can be determined [92], TSDC is also able to detect phase transitions which cannot be observed with differential scanning calorimetry (DSC), including α- and β- relaxations [198], [199]. Powders have to be compressed to tablets before starting a measurement [14], [79], [82]. Hirakura et al. published a study about detecting slight lot-to-lot differences in recombinant human Interleukin – 11 using TSDC [14].

Dielectric relaxation spectroscopy (DRS) determines the time dependency and the magnitude of electric polarization processes by measuring the rate and extent of polarizability of a material placed in a weak electromagnetic electric field. As this technique covers a broad band frequency window (from 10^{-5} – 10^{11} Hz) the investigation of slow and hindered macromolecular vibrations and restricted charge transfer processes to the relatively fast reorientations of small molecules or side chain groups is possible. The dielectric response provides information on structural characteristics of proteins, water content and states of water. Secondary relaxation (β – relaxation) of amorphous solids can be gathered. Further literature can be found elsewhere [79], [197], [200], [201].

Electro kinetic analysis, such as thermoelectric analysis (TEA), allows determining the electric resistance as a function of temperature. As the mobility of charge carrying species is limited, the resistance to electric current is relative high in freeze-dried products. Therefore, the characterization of the thermal properties is possible. TEA is not used commonly. Ma et al. determined glass transition temperature of a monoclonal antibody as well as the eutectic crystallization temperature and the ice melting temperature [202].

Thermo-gravimetric analysis (TGA)

During thermos-gravimetric analysis (TGA) the change in sample mass is determined as a function of temperature and /or time. The instrument is a thermos-balance that permits the continuous weighing of a sample as a function of time [187]. Solvents entrapped or bound as solvate is easily determined. This method is routinely used to determine the sample moisture content, hydration level and decomposition temperature. Furthermore, water sorption and desorption isotherms can be determined by using thermos-balances [203], [204].

Vapor sorption and surface analysis

Dynamic vapor sorption

Gravimetric dynamic vapor sorption can be used to measure the sorption of water vapor on a powder. Using a dynamic flow of a humidified gas a moisture sorption isotherm can be measured at a certain temperature range. The moisture sorption analysis may be performed using the Guggenheim Anderson deBoer (GAB) equation, which is an extension to the BET equation [205], [206]. A variation of dynamic vapor sorption is organic vapor sorption that can be used to determine the degree of crystallinity. Usually, calibration curves are needed for these measurements with a 100% amorphous and a 100% crystalline component as the relative absorption of the molecule on the solid-state of the protein will vary [207]. Dynamic vapor sorption is commonly used in characterizing freeze-dried powder formulations to define the sorption and desorption behavior [208], [209].

Contact angle measurements

Several techniques are available to measure the interfacial free energy. The contact angle of a drop placed onto a solid surface can be measured. As a powder does not present a smooth flat surface on which to place a drop of liquid, powder samples are very susceptible to transformation if manipulated during testing. Therefore, they have to be compacted [16]. The sessile drop technique is by far the most cited method to determine the contact angle of compacted powder material, saturate it with a saturated solution of the test liquid and then place the drop on the surface. The angle is measured directly using either photography, image analysis or eye piece protractor. An additional method to characterize the contact angle of a compacted powder is the Wilhelmy plate. When using the Wilhelmy plate method a rectangular compact of the powder is compressed and this form is suspended on a balance. The liquid is then raised to contact the plate by use of a motorized platform. From an extrapolated buoyancy slope to yield a force at zero depth of immersion, it is possible to obtain a contact angle [16]. Furthermore, the contact angle can be determined gravimetrically, as well as calorimetrically and chromatographically [16]. Beyond static contact angle measurements dynamic techniques are also available. With a dynamic contact angle, the immersion load is measured for the penetration of a solid in the given liquid. The results obtained from these techniques can be

affected by the compression of the powder into a disk or plate that can lead to changes in the surface properties, surface roughness and plate perimeter. The method of liquid penetration through a powder bed and applying the Washburn equation, does not require compression of the solid, but it has been shown that particle size, packing time and pore geometry can all affect the measurement [210]–[213].

Inverse gaschromatography (iGC)

With inverse gas chromatography (iGC) the investigation of physical properties, such as diffusion kinetics, powder energies and acid/base/polar properties of solid materials as well as solubility parameters, surface heterogeneity and phase transition temperatures/humidities is feasible. The powder is packed in a column without any pretreatment as the stationary phase and investigated by vaporized organic solvents. From the retention volume $V_{N,}$ which is a measure of the magnitude of the interaction between the probe and the stationary phase, it is possible to calculate a variety of thermodynamic and kinetic parameters. It is a very sensitive method to detect slight differences between solid surfaces. A lot of work has been done in the pharmaceutical research to examine batch-to-batch variations with iGC [14], [61], [62], [68], [69]. iGC offers its applicability where it is difficult and even impossible to characterize the surface of some forms of solids by means of other popular techniques as wetting method or FTIR [67].

A further important powder attribute is its charge. The electrical charge of a powder can be measured using a Faraday pail. Charge is indicated by an electrometer connected to the pail into which the sample is poured. Detailed information about the measurement principles can be found in the literature [74].

Spectroscopic characterization

Spectroscopic methods have the advantage to be fast and they can be well integrated as Process analytical technology (PAT) applications. For routine analytics the monitoring of deviations from the target product profile may be sufficient. Biophysical techniques such as Fourier transformed infrared spectroscopy (FTIR), RAMAN and fluorescence spectroscopy are used to asses structural changes [214].

Infrared spectroscopy

Infrared spectroscopy (IR) is a widely used method to monitor the secondary structure of proteins as it can be employed to solids and liquid formulations. It measures the absorption of light due to vibrations of the molecule from wavenumbers 125000cm^{-1} – 10cm^{-1}. Usually middle IR (4000 – 400 cm^{-1}) is used to analyze protein secondary structure as vibrations of functional groups such as amide groups are observed in this region [215]. Detailed information using IR spectroscopy for analyzing proteins is reviewed elsewhere [127], [216]. The most common

method is attenuated total reflectance (ATR)-FTIR spectroscopy, where the incoming light is reflected several times at the interface between an IR transparent crystal and the sample. Therefore an evanescent wave is generated at the reflection point, which reduces the reflected light reaching the detector and provides IR spectroscopic information of the sample [215], [217].

The analysis of these spectra can provide structural information and data on inter-and intramolecular interactions. Nine characteristics group frequencies arise from peptide linkages: amide A (≈33300cm^{-1}), amide B (≈3100cm^{-1}), amide I (≈1650cm^{-1}), amide II (≈1550cm^{-1}); amide III (≈1300cm^{-1}), amide V (735cm^{-1}), amide IV (≈635cm^{-1}); amide VI (≈600cm^{-1}) and amide VII (≈200cm^{-1}). These amide vibrational bands can be described in terms of five in – plane (C=0 stretching; C-N stretching, N-H stretching; OCN bending and CNH bending) and three out of plane (C-N torsions and C=O and N-H bendings) displacement coordinates [218]. Usually second derivative spectra are calculated and the amide I region (1600 – 1700cm^{-1}) is chosen to interpret the collected data. Various publications about denaturation processes occurring in solid-state protein derived from FTIR spectra can be found in the literature. [125], [126], [219]–[221]. Recently, a study about a combination of local mobility measurements and spectroscopic analysis of protein structural retention was published. It was shown that this combination can be used to predict rates of protein degradation in glassy solid matrices [93]. Since in the region of ≈1650 cm^{-1} (amide I) the water absorption is very high, amide III region region (≈1300cm^{-1}) is the spectral area to determine protein secondary structure, when higher water content is present in the sample, as the water absorption is neglect able in this area. Treating the generated data with Gaussian deconvolution of the amide bands of freeze-dried proteins, percentages of the individual elements of secondary structure (α–helices, β–sheets) can be determined [125].

Raman spectroscopy

Raman spectroscopy is based on inelastic Raman scattering. When illuminated by a laser, molecules absorb energy and emit it as a photon of lower energy/frequency than the absorbed photon. A detailed review about application of Raman on biopharmaceutics can be found in the literature [222]. It is possible to identify the chemical composition of a sample based on the presences and position of bands in the fingerprint region (2000 cm^{-1} – 400cm^{-1}) when compared with a database of Raman spectra. Various regions in the Raman spectrum can be attributed to interactions of the laser light with protein backbone amides. The latter are indicators for the presence of secondary structural elements in proteins. The most import ones are the amide I band (1600-1700cm^{-1}), the amide III band (1230-1340 cm^{-}1) and C-C stretching bands (890-1060 cm^{-1}) [223], [224]. Raman spectroscopy is also used as an inline process control during the freeze-drying to monitor changes during the process [132], [133], [225], [226]. The exact wavenumber of the distinct bands gives information about the environment of

the peptide bond and therefore the secondary structure of the protein, or aromatic side chains and disulfide bonds, providing hints about changes in tertiary structure [227]. Samples can be characterized in any physical state, but fluorescence is often disturbing Raman spectroscopy, as the fluorescence signal is clearly stronger than the Raman signal [80]. Usually Raman signal intensities are lower than IR signals and therefore Raman requires sufficient protein quantities and very sensitive detectors [228]. Aggregation processes of proteins were determined using Raman spectroscopy and reported elsewhere [229]–[231]. The principle of inelastic Raman scattering can be used for Raman microscopy as well [232]. Using Raman microscopy, which combines Raman spectroscopy and optical microscopy, it is possible to analyze proteinaceous and nonproteinaceous particles [222]. Lately a confocal Raman microscopic technique was optimized to detect amorphous – amorphous phase separation in lyophilized freeze-dried protein formulations [90]. Detailed information about Raman microscopy can be found in the literature [232], [233].

Solid-state nuclear magnetic resonance spectroscopy (ssNMR)

An additional powerful spectroscopic method is solid-state nuclear magnetic resonance spectroscopy (ssNMR). ssNMR probes atomic environment based on the different resonance frequencies exhibited by nuclei in a strong magnetic field. Detailed information about the principles and characterization of proteins by ssNMR can be found elsewhere [204], [206], [234]–[236]. ssNMR is an established method for determining mobility. Molecular mobility can be measured by spin-spin lattice (T1) and spin-spin (T2) relaxation times. ssNMR allows identification of the origin of molecular motion, and thus, determination of molecular mobility of the drug and excipient in freeze-dried protein formulations. Possible correlations between stability and mobility of proteins can be characterized. Separovic et al. found that relaxation times (T1) were correlated with changes in aggregation and activity of the protein and hydration increased the rates [35], [209]. Extensive research was carried out by Yoshioka and co – workers. They determined that aggregation rates of freeze-dried proteins can be correlated to relaxation time or critical mobility temperature (T_{mc}), which was derived from NMR relaxation measurements. Furthermore they were able to show that molecular mobility of protein molecules did link to the molecular mobility of the excipients [102], [104], [237]–[240]. It has to be considered that using NMR issues due to radiolabelling of proteins and deuterium exchange in the protein molecule can occur [131].

Electron spectroscopy for surface analysis (ESCA)

Electron spectroscopy for surface analysis (ESCA) permits analysis of the outermost of a powder sample in an elliptical area in the Å (Angstrom) region. With this method the estimation of the composition in terms of molecular species is possible. The solid sample is irradiated in vaccum with monoenergetic soft x – rays during the measurement. Thus, the emitted electrons are sorted by energy [241]. The spectra is imaged as a plot of the number of emitted electrons

per energy interval versus their kinetic (binding energy), As the mean free path of electrons is very small, electrons emitted from deeper layers lose energy by inelastic collisions on the way to the surface and are no longer able to leave the solid phase. Therefore the spectra reflect the composition and chemistry of the outermost 100 Å of the surface [83], [242]. It is used for chemical analysis of freeze-dried protein powders. ESCA can additionally be used for component separation [105], [121].

Solid-state is hydrogen/deuterium (H/D) exchange mass spectrometry

A further method for analyzing the protein structure in the solid-state is hydrogen/deuterium (H/D) exchange mass spectrometry. Site specific information on interactions between protein and excipients in the solid-state can be gathered. Hydrogen atoms in the amides of the backbone of protein are exchangeable with external hydrogens in water. The tertiary structure of proteins is relatively compact and the molecule is folded into a well-defined and unique three dimensional structure. Amide hydrogens located at the surface of the folded structure are exchanged with deuterium very rapidly because external D_2O molecules can access the protein surface easily, whereas hydrogens buried in the folded structure are much more slowly exchanged or not exchanged at all on the typical experimental timescale. This is a result of the lack of protein internal mobility steric hindrance, and the hydrophobicity in the protein interior [243]. Therefore the solid-state protein has to be exposed to D_2O vapor. The latter first adsorb onto the freeze-dried solid, a process that may involve a D_2O phase transition from the vapor to the liquid phase. The adsorbed D_2O then diffuses through the solid particles and comes into intimate contact with individual protein molecules in the solid. The H/D exchange reaction occurs at exchangeable protons on the protein backbone and side chains. Because side chain sites readily undergo back exchange when the solid is dissolved, it is likely that only the backbone amide protons remain deuterated for electrospray ionization mass spectrometry [244], [245]. Thus, hydrogen/deuterium exchange of proteins has been extensively studied by NMR spectroscopy [246]–[248], MS spectrometry [244], [249]–[251] and FTIR spectroscopy [252]–[255] to investigate various phenomena such as protein conformation, folding, dynamics, and ligand binding.French et al. [256] used HD exchange with FTIR analysis to characterize recombinant humane granulocyte colony stimulating factor (rhG – CSF) and recombinant consensus interferon α (ConIFN) in spray – dried powders containing trehalose, using isotopic shifts in the amide II/II' bands. Recently it was shown that H/D exchange can be used to determine the endothermic pre – Tg events, which were found in DSC thermograms, and therefore linked to protein internal dynamics [243]. Desai et al. [257] employed HD exchange with ^1H NMR analysis to study the unfolding of bovine pancreatic trypsin inhibitor (BPTI) upon lyophilization. Moorthy et al [135] showed that ssH/D exchange measurements can be used to predict the protein aggregation during storage of freeze-dried proteins.

Neutron scattering

With neutron scattering it is feasible to obtain molecular dynamics directly. The scattering of neutrons by hydrogen atoms is very intense compared to that of most other elements. The available range of observable time scales makes this technique selectively sensitive to the change in internal motions of hydrogenated materials, such as proteins [258]. A review about characterizing protein dynamics by neutron scattering can be found elsewhere [259].

Positron Annihilation lifetime spectroscopy (PALS)

A further spectroscopy method which can be used in characterizing freeze-dried protein powder is Positron Annihilation lifetime spectroscopy (PALS). The latter can be used to determine voids and defects in solid protein powders. Chieng et al. [260] analyzed density variations in terms of glass dynamics by PALS. The principle of PALS is described in detail elsewhere [261].

Steady-state fluorescence emission spectroscopy

Using steady state tryptophan (Trp) fluorescence emission spectroscopy, it is possible to obtain information about tertiary structure of freeze-dried pharmaceutical proteins [131]. Furthermore phosphorescence spectroscopy or total internal reflection fluorescence (TIRF) spectroscopy as well as NMR can be used to determine protein tertiary structure. These techniques were used on adsorbed protein films as well as solid-state proteins [131], [262]–[265].

X-ray diffraction technique

The solid-state of freeze-dried protein powders can be investigated by X-ray powder diffraction (XRPD). It is the method of choice to identify the solid-state of freeze-dried solids as it provides a valuable insight into possible chemical stability issues. X-ray diffraction of solid-state materials gives important insight, on the degree of long-range order present, into the extent and nature of the crystallinity, microstructure, and nanocrystallinity. This technique has already been used to distinguish between lot-to-lot differences [14]. Detailed information about the principles of X-ray diffraction can be found in standard references [206], [266].

Water content and activity

As residual water is a key factor for stability issues in freeze-dried protein formulations accurate techniques for determination have to be available [267]. Guidelines for the determination of residual water in dried biological products have been issued to describe residual moisture test methods and procedures used to set product residual moisture limits. These guidelines have been published under 21 CFR 10.90 stating the principles and practices of general applicability [268]. Karl Fischer Titration is used to determine the residual water content of lyophilized

protein powder products as it can be automated and is very reproducible [269]. Detailed information about the principles can be found in the literature [270]. Another common and non-invasive method for investigation of the water content is near infrared spectroscopy. A number of chromatographic, thermal and wet chemical methods are applicable as well [271], [272]. Loss on drying (LOD), thermogravimetr ic analysis (TGA) and gas chromatography are widely used [267].

Water activity is a measure of the energy status of the water in a system. The equilibrium relative humidity is determined by water molecule exchanges between sample and air due to an existing partial water vapor pressure in a closed system.

$$a_w = \frac{p}{p0} \qquad \text{Eq. 3}$$

where p is the vapor pressure of water in the substance and p0 is the vapor pressure of pure water at the same temperature. It is possible to investigate T_g as well as to generate sorption isotherms Detailed information about water activity measurements can be found elsewhere [149].

2.6 Conclusion

Freeze-drying of pharmaceutical proteins is a complex process and still a challenge for formulators. Small alterations in processing conditions may result in significantly different lot-to-lot differences which may dramatically affects the outcome of subsequent particulate handling. This review provides an overview of protein and powder properties which can alter after the freeze-drying process and therefore influence the further processing or application. Moreover, techniques to characterize these particle and protein attributes are described to give a complex summary.

2.7 References

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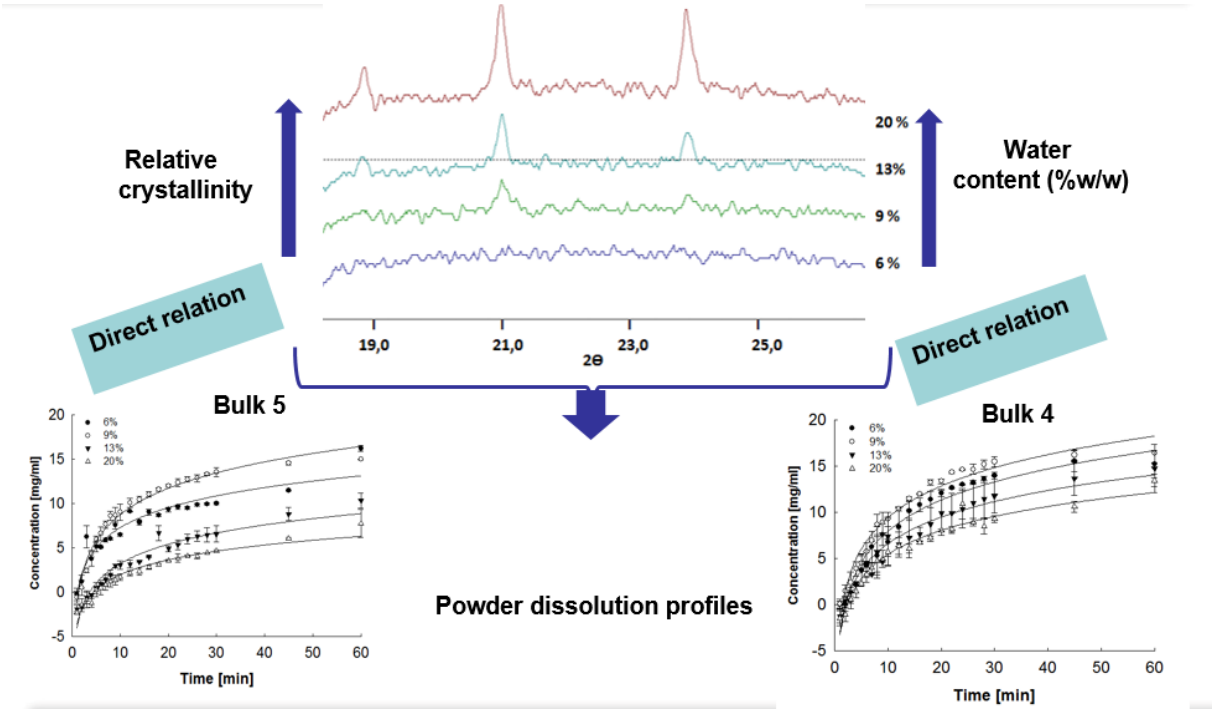
3. Chapter 2: The Influence of Residual Water on the Solid-State Properties of Freeze-Dried Fibrinogen[1]



[1] This Chapter is taken from a Journal article Wahl et al. in the European Journal of Pharmaceutics and Biopharmaceutics (2015)

3.1 Abstract

The purpose of this work was to investigate the influence of residual water in freeze-dried protein powders on the dissolution behavior of the solid-state proteins. To that end, six freeze-dried fibrinogen powder lots were stored at four levels of relative humidity and analyzed with regard to the particle size and shape, the specific surface area, the solid state of protein and the inner surface. Furthermore, the dissolution behavior of the powders was investigated. We clearly identified differences in the specific surface area, specific inner surface area, crystallinity, particle size and shape, which we were able to correlate to the dissolution behavior. These differences were triggered due to the different levels of residual moisture during two weeks of storage. Thus, we were able to show that the storage conditions have significant impact on the processing of pharmaceutical protein materials.

3.2 Introduction

Due to recent advances in biotechnology and our improved bio-molecular understanding, numerous protein-based drugs have been approved for human use or are currently investigated for clinical safety and efficacy. While the development of new small-molecule drugs is still a field of significant interest, biotechnology products are believed to show massive growth over the next decades [1,2]. Despite many advantages of new therapeutic approaches, the complexity of protein molecules creates serious challenges for formulators. Because protein formulations are problematic to handle due to their fragile nature (e.g., maintenance of native structure, stability during shipping and long-term storage), they are often stored in the solid state [3–5]. The most commonly-used method for preparing solid protein pharmaceuticals that enhances product shelf life is freeze drying [6]. The fact, that lyophilization of oral peptides and proteins is a new emerging field [7,8] makes the solid state stability of freeze-dried protein formulations particularly important to the pharmaceutical industry.

Commonly, freeze drying is performed in vials, adding certain amounts of excipient to the protein solution for protection of the protein. The water content of lyophilized protein formulations often determines the chemical and physical stability of the solid state of protein powders [3,9]. The general trend is that the product integrity improves with decreasing water content, and various studies are available reporting on the effect of increased residual water amounts on physical and chemical instabilities [9–11], including recent publications on moisture-induced aggregation of freeze-dried proteins [12–16]. Breen et al. reported growing Asp isomerization with increasing water content [10]. In addition, intermolecular S – S shuffling in response to increasing residual water content was investigated [12–14]. For example, hydrogen - deuterium exchange studies showed that structural dynamics of α – Chymotrypsin increased with a raise in relative humidity [16]. Higher levels of hydration also led to the increased protein conformational mobility [12].

The exact relationship between solid state instability and water content, which depends on the type of degradation reaction, is often difficult to delineate [17]. In the freeze-dried state, water can have various effects on protein destabilization: it can act as a direct reactant with the drug and hence influence the rate and extent of the reaction through its concentration, e.g., a hydrolysis reaction [18]. If water is produced during the course of a reaction, it can possibly contribute directly to the reaction or indirectly through medium effects [18]. Increased water content also favors a higher conformational mobility of the protein molecule [11], making it more susceptible to chemical modifications. It was reported that increased water levels could lead to higher intermolecular disulfide exchange reactions forming covalent aggregates [10,11,13,16]. From a physical perspective, the residual water can act as a medium and plasticizer, resulting in decreased glass transition temperatures and increased crystallization rates [18–20]. While residual water often increases the chemical degradation rates, it can for

single investigated cases also suppress chemical reactions. For example, Pikal et al. showed that the stability of the human growth hormone (hGH) was superior in vials with a higher water contents [21]. Water can influence amorphous protein powder preparations and affect degradation pathways in the solid state in various ways [18,22]. Even when the residual moisture is less than 1%, which is generally considered optimal, chemical and physical degradation reactions may occur [17,23]. However, in some cases a certain amount of water must be present to ensure chemical stability: for example, lipid peroxidation rates decrease when small amounts of water are added [18].

In addition to the protein quality, reconstitution time is another important criteria for protein formulations since a protein powder often has to be rapidly re-dissolved, reconstituted and administered, e.g., in an emergency room. To reduce potential immunogenic side effects and to avoid the injection of protein particulates, the protein drug has to be completely dissolved [24]. A long reconstitution process, which typically involves shaking or turning the vial over, may induce stress due to air-liquid interfaces generated and shear [25]. During bulk production, reconstitution can be critical for the subsequent processing of lyophilized protein powders [26]. The dissolution behavior of powders has been extensively investigated [27,28] but mostly for small- molecule drugs. Little is known about the powder attributes of freeze-dried proteins that influence the reconstitution behavior [26].

The reconstitution process of lyophilized powders depends on a number of factors, such as the cake's morphology, surface area, collapse and meltback, the presence of hydrophobic coatings, the homogeneity of the dry matrix, the formation of channels between the pores and the physical solid state [29].

Since dissolution occurs on the surface of a powder, another crucial parameter is the particle size distribution, which also impacts the specific surface area. The dissolution rate decreases with increasing particle size due to a lower specific surface area according to the Noyes Whitney equation [32]. However, since cohesivity increases, the agglomeration tendency intensifies for smaller particles, forming porous agglomerates. The particle shape and size are critical for powder packing in these agglomerates, which in turn affects the interstitial spaces that penetrating liquids require. Penetration of the liquid is essential since it is responsible for the powder's wetting required for dissolution. It was suggested that since larger particles were more irregularly shaped than smaller ones, they provided more interstitial space and facilitated the dissolution reconstitution [33,34].

Additionally, a high specific surface area, which is typical for small particles, enhances the dissolution time and increases the rehydration rate [35], if agglomeration is not too strong. Furthermore, the crystallinity has a significant effect on the solubility of freeze-dried protein products. In initially amorphous formulations an increase in water content may lead to a tendency to form a crystalline matrix. The dissolution of crystalline powders is generally slower

than that of amorphous structures [36,37]. While amorphous materials have a high water vapor sorption capacity, crystalline solids interact with water via adsorption of moisture on the particle's crystalline surfaces, followed by crystal hydrate formation, deliquescence and capillary condensation [38]. Usually, the above mentioned parameters (e.g. porosity, surface area, crystallinity) can be tuned by varying the freezing rate during the lyophilization process. Furthermore, the formulation itself plays an important role, such as different amount of added exipients [30,31].

To date, the research on lyophilized protein formulations has mainly focused on the influence of the relative humidity on the stability of a protein product [10,15,21]. However, to the best of the authors' knowledge, a correlation between powder characteristics and dissolution behavior as a function of the water content has not been established and only small-molecule pharmaceuticals have been investigated [28,39,40].

In this work we focus on the reconstitution behavior of freeze-dried protein powders using human fibrinogen as a model system. We critically evaluate the influence of powder properties on the reconstitution behavior, which is required for establishing the optimal water content for a rapid dissolution and subsequent processing of the pharmaceutical product.

3.3 Materials and Methods

Freeze-dried fibrinogen powders were provided by Baxter AG, Vienna, Austria. Different lots of the solid lyophilized protein were analyzed in triplicate and the mean and standard deviations were measured. Note that the-freeze dried fibrinogen samples were not pure samples, produced via bulk lyophilization. Small amounts of excipients were present, with polysorbate 80 (PS-80) being the major additive (less than 3% by protein weight) and some proprietary components in small amounts (less than 1% by protein weight). This lead to lot-to-lot variations in terms of varying amounts of additives within the fibrinogen formulation.

To investigate the influence of moisture on the solid-state stability of fibrinogen, the freeze-dried powders were stored under accelerated-storage conditions with controlled humidity. The powders were stored at room temperature at constant levels of relative humidity (RH) in desiccators with different salt solutions. The corresponding RH levels were 12% (LiCl), 52% ($Mg(NO_3)_2$*$6H_2O$), 75% (NaCl) and 93% (KNO_3) [41]. Prior to analyses all powders were exposed to the laboratory environment (room temperature 20±1°C/50±10% RH) for a maximum 1 minute. We showed that this exposure to the environment had no impact on the results (see below).

Moisture Content Determination

Within two weeks of incubation, the lyophilized powder samples were equilibrated to a certain water content and the residual moisture content was determined using Karl-Fischer Titration (Titroline 7500 KF, SI Analytics, Mainz, Germany). The powders (approximately 0.2 mg) were

directly added to the titrator cell. To enhance their solubility, a methanol/formamide mixture (1:1 ratio) was used at lower levels of residual moisture (6-9% water content) to determine the moisture content. All powders with water contents above 10% were studied with methanol only. Residual moisture content measurements were repeated after storing the samples 10 minutes at laboratory environmental conditions.

Image Analysis

The particle size distribution and particle shape were analyzed via the Qicpic System (OASIS wet and dry system, Sympatec GmbH, Clausthal-Zellerfeld, Germany). Millions of particles were included in the analysis, using the principle of dynamic imaging to obtain information about their sphericity and size (EQPC). The QicPic uses rear illumination with a visible pulsed light source that has an exposure time of 1 ns to minimize motion blur. The flash rate of the light source is adjustable from 1 to 500 Hz, and is synchronized with the high-speed camera that operates up to 500 frames per second. Dry powders are fed into the high-speed dry-sample disperser where they are accelerated to a speed of up to 100 m/s via a Venturi tube located in the dispersing line. During this process, dry powders are dispersed and aerosolized by particle–particle, particle–wall collisions and centrifugal forces caused by velocity gradients. Upon exit, particles enter the measurement zone decelerated and are finally collected by a Nilfisk™ vacuum system (Nilfisk-Advance A/S, Sognevej, Denmark). In a typical experiment, at least 50,000 images of the investigated particles are captured by the camera and processed using appropriate image analysis algorithms included in the Windox 5.6.0.0 software.
The particles were dispersed applying a pressure of 2.5 bar. x50 is the median of the particle size distribution (i.e., a particle diameter that corresponds to 50 % of the cumulative undersize Q_3-distribution. The Volumetric Mean Diameter (VMD) is the average diameter based on the unit volume (mass) of a particle. Sphericity (S) and convexity refer to the particle shape. S is defined as the ratio between the perimeter of the equivalent circle (PEQPC) and the real perimeter (P_{real}). The equivalent circle gives the smallest possible perimeter at a given projection area, with the value of S between 0 and 1. The smaller the value is, the more irregular the shape is. Convexity (cv) is the ratio between the projection area of a particle and the area of a convex hull, which is calculated from the particle projection. It describes the compactness of a particle: the smaller the value is, the more concave regions the particle has. This analysis was performed for all powders. However, we were unable to determine the particle size and shape of particles stored with the KNO_3 solution, since the powder was too sticky to be analyzed.

BET Surface Area

The specific surface of the bulk powder was investigated using the Micromeritics Tristar II 3020 (Norcross,USA). The samples were degassed for two days at 30°C in the Micromeritics VacPrep 061 degas unit (Norcross,USA). Since the BET surface of the powder is around $1m^2$, the measurements were performed with Krypton gas. Brunauer, Emmett, and Teller (BET) adsorption theory was used to calculate the specific surface areas, with a pressure of 0.05–0.30 normalized to the saturation pressure of the adsorbate [42] .

Wide and Small Angle X–ray Scattering

Small- and wide angle X–ray scattering was applied to determine the specific inner surface at the nanoscale (1-100nm) and the crystallinity of the powder, respectively. No degassing or other pretreatment was required, i.e., the samples were investigated in their original incubation state. SWAXS measurements were performed at 20 ± 1°C with a S3 – MICRO camera (formerly Hecus X – ray systems, Graz, now Bruker AXS, Karlsruhe) (λ=1.54 Å). The specific inner surface analysis was undertaken via SAXS, following the Porod theory [43] that involves measuring the integral scattering power Q and the decay coefficient k. Since Q and k are proportional to the sample volume and the surface, respectively, the k/Q ratio is proportional to the specific surface ($Å^2/Å^3$). Any interface between domains with different density contributes to the scattering, including inaccessible pores, or areas of different molecular packing, e.g., crystalline vs. amorphous. However, in our case the contrast between solid matter and air was the main effect. Crystallinity was measured via WAXS in the angular range of 17-27°, which corresponds to the real space dimensions of 4.9 and 3.3 Å where the molecular crystals had strong diffraction peaks. To avoid preferential orientation effects of crystallites, the samples held in capillaries of 2 mm i.d. were rotated during the measurement. A typical exposure duration was 1200 sec. WAXS measurements of the samples were repeated after storage of 2 and 18 months at controlled relative humidity. A representative example of SAXS data is given in Figure 1, together with a schematic illustration of the relevant parameters.

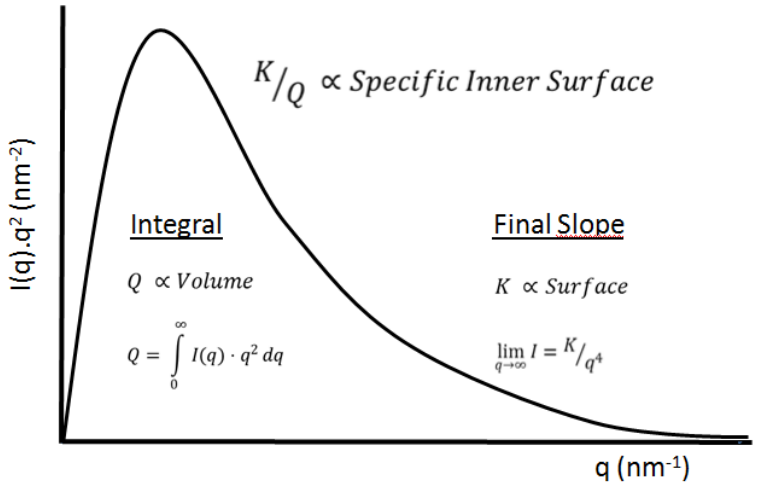


Figure 1: SAXS intensity distribution. Q denotes the integral intensity (strictly the "Invariant" Q, the integral of its second moment. K is the decay coefficient, thus the ration K/Q is proportional to the specific inner surface (Porod's law) (Figure adopted from [44])

Dissolution and UV Spectrophotometry

In order to test the dissolution behavior of the-freeze dried powders, a flow-through cell PT – DZ 1 (Pharmatest, Hainburg, Germany) was used. The amount of material used per cell was 1.2 +/- 0.4 g, with 60ml of a defined dissolution medium consisting of 96.9% Milli–Q water, 3.2% Albumin and 0.25 % Niacinamid. A pH of 7.3 was adjusted using 0.1M sodium hydroxide solution. All experiments were carried out in a closed system mode. The flow-through cell was packed with glass beads. The flow rate of the dissolution medium through the cells was 3000 ml/h at a temperature of 37°C. Samples of 15µL were withdrawn at periodic intervals and their UV absorbance at 280nm was measured using the dissolution medium as a reference with a NanoPhotometer®, P 300 (Implen GmbH, Munich, Germany).

A standard curve of absorbance-versus-concentration was measured using solutions of the freeze-dried protein powder in the dissolution medium with concentration of 0.198-19.83 mg/mL. The initial changes in concentration of the recorded curves were analyzed to determine the dissolution rates.

3.4 Results and Discussion

Usually, bulk quantities of freeze-dried pharmaceutical proteins are exposed to various levels of humidity during storage and processing. The physical powder characteristics (e.g. surface, size, porosity) can critically affect the degree of water sorption. To investigate the consequences of moisture sorption we stored 6 batches of fibrinogen bulk powder under controlled conditions for two weeks over saturated salt solutions in humidity chambers. The corresponding RH levels were 12% (LiCl), 52% ($Mg(NO_3)_2$*$6H_2O$), 75% (NaCl) and 93% (KNO_3) [41]. These RH levels refer to different storage conditions during the production

process of freeze dried proteins, including extreme values such as 93% RH. With these different salt solutions, we obtained protein bulk powders with moisture contents between approximately 5 and 21%. These values seem rather high compared to other freeze-dried protein powder formulations which usually have water contents of 0.5-1% water content. For this particular material water contents of about 7% after freeze-drying are usual.
Table 1 illustrates the moisture content determined at the end of the storage period for all 6 bulks and 4 salts studied including the original water contents.

Table 2: Water content of six different lots under varying humidity conditions

Moisture content [%]	**Bulk 1**	**Bulk 2**	**Bulk 3**	**Bulk 4**	**Bulk 5**	**Bulk 6**
LiCl	6.3 ± 0.4	6.2 ± 0.1	6.2 ± 0.1	6.0 ± 0.4	5.8 ± 0.4	5.2 ± 0.8
$Mg(NO_3)_2$*6H_2O	8.6 ± 0.4	9.5 ± 0.3	9.6 ± 0.2	9.0 ± 0.2	8.8 ± 0.5	9.0 ± 0.1
NaCl	12.6 ± 0.2	13.2 ± 0.5	14.4 ± 1.2	12.4 ± 0.2	12.9 ± 0.6	13.3 ± 0.9
KNO_3	19.8 ± 1	20.5 ± 2	21.1 ± 2	19.02 ± 1.6	19.0 ± 1.9	20.6 ± 0.6
original	7.5	7.7	7.5	7.4	7.4	7.7

As can be seen from Table 1, the maximum s.d. of the moisture content for each bulk (three runs each) was always below 15%, yet typically less than 10%. The resulting moisture values were about 6% (LiCl), 9% ($Mg(NO_3)_2.6H_2O$), 13% (NaCl) and 20% (KNO_3). Wherever possible, these target values will also be used for labeling of figures throughout this report. The measurements were repeated after storing the powders 10 minutes at laboratory environment. No changes in the water content were observed. Since bulk-to-bulk differences in the residual water content were small, as can be seen from Table 1, it can be assumed that the water sorption was similar. In Figure 2, adsorption isotherms for the 6 bulk materials are presented.

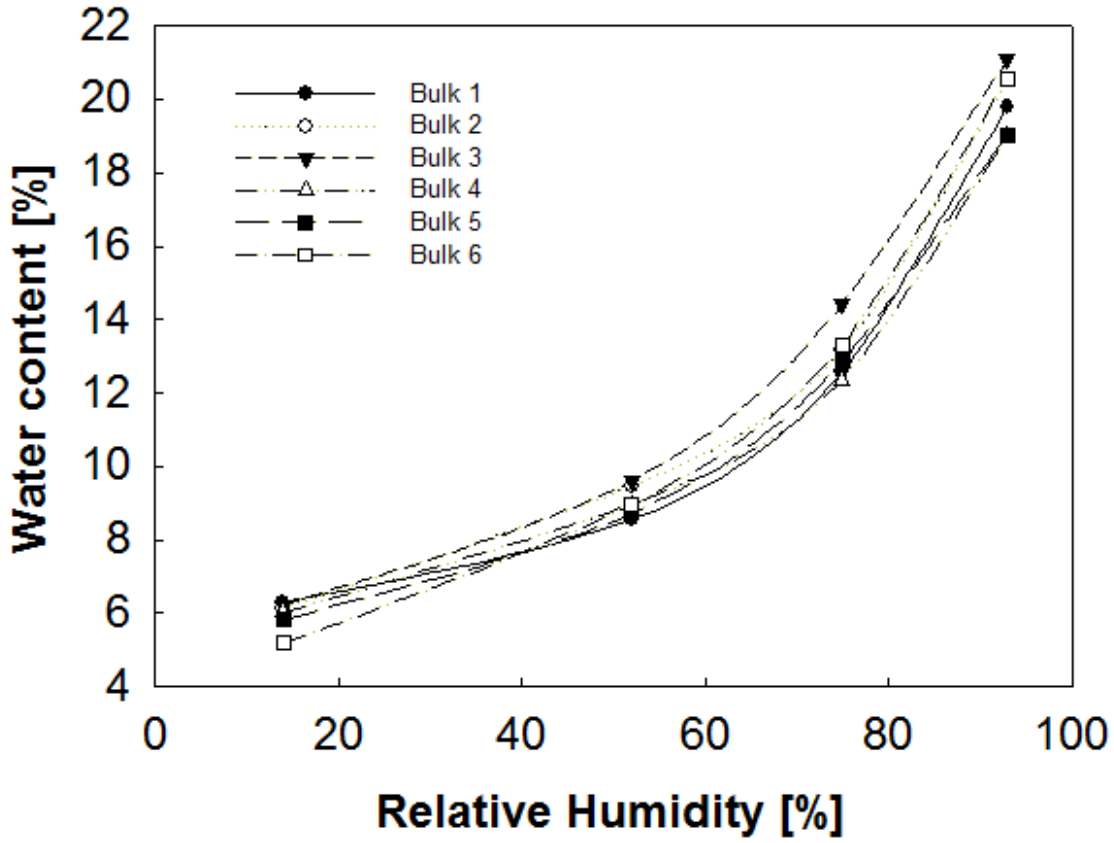


Figure 2: Adsorption isotherm of six bulk protein powders.

For small molecules, water content was proven to be a major factor influencing reconstitution [40,45,46]. From a stability perspective, the generally accepted view about water content in freeze dried powders is “the drier the better” (exception: tissue type plasminogen activator (tPA)) [47]. Although it was found by Pikal et al. [21] that oxygen can have significant impact on freeze-dried material, the influence of oxygen was excluded for this study as the fibrinogen powder is exposed to oxygen during its whole production process. To evaluate the influence of water content on the reconstitution behavior of fibrinogen bulk powder, dissolution tests were performed in a flow-through cell (USP 4). The protein powder was dissolved in a well-defined dissolution medium and the UV absorption (280nm) that represents the dissolved protein fraction was monitored over time. Results for two representative bulks are presented in Figure 3. In general, a linear increase in the concentration was observed during the first 8 to 10 minutes before the curve flattened out. This initial phase of a linear concentration increase was used to calculate the dissolution rate, as shown in Figure 4.

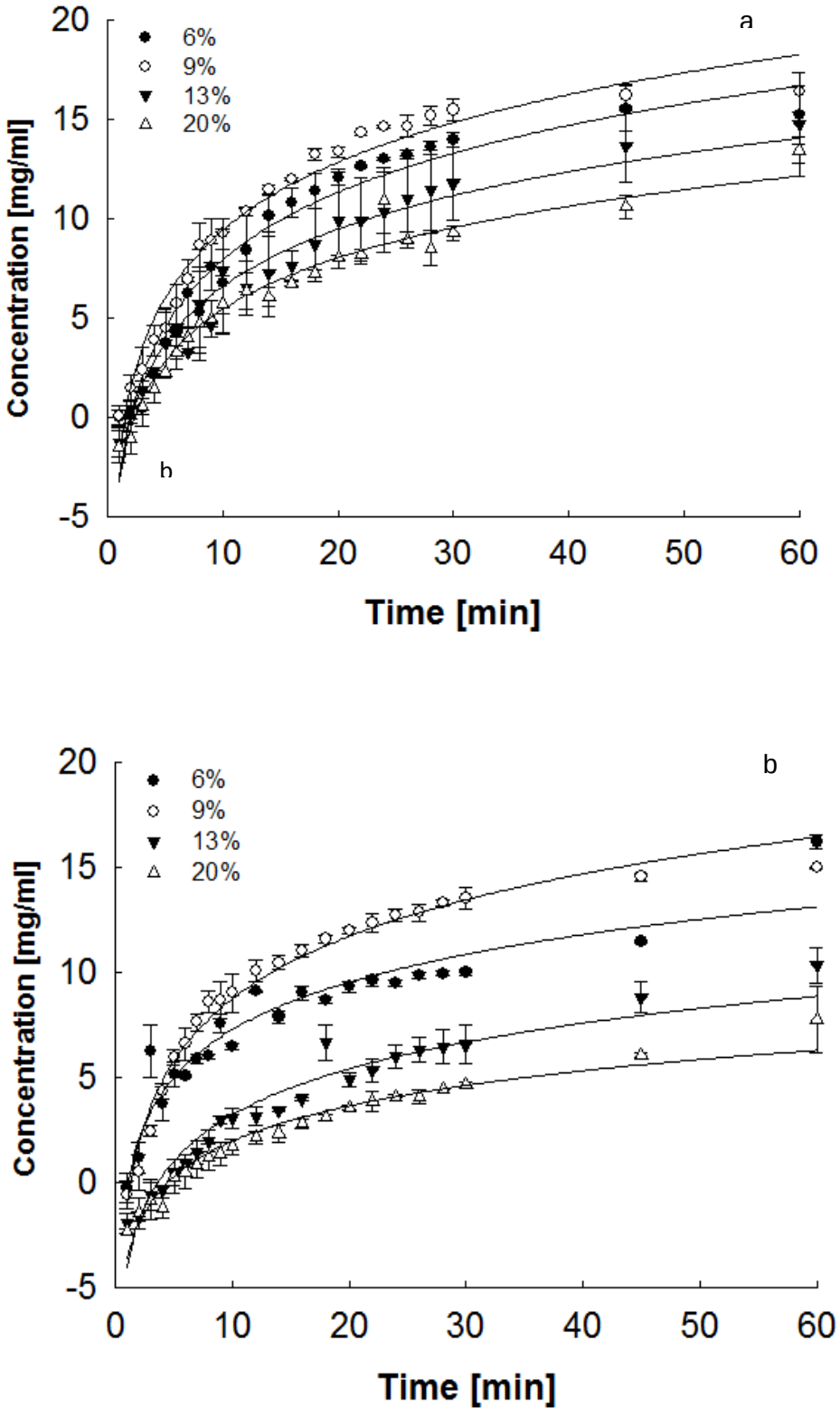


Figure 3: Dissolution profile of two typical bulks, i.e., (a) 4 and (b) 5. The curves represent different water contents of the lyophilized powders

Interestingly, we observed distinct differences between bulks equilibrated at different humidity levels. As can be seen in Figure 4 there are large variations in the dissolution rate in terms of

both the initial dissolution rate and the amount of dissolved protein after 60 minutes. Figure 4 presents the summarized results for all 6 bulks with 4 water contents. Remarkably, the fastest dissolution was achieved at 9% water content, and the dissolution rate decreased with the decreasing and increasing water content. One bulk (bulk 2), however, behaved differently, with the sample with the water content of 6% exhibiting the fastest reconstitution. Additionally, bulk 3 had the highest dissolution rate at a water content of 13%. Nevertheless, although the standard deviations were quite high for certain replicates, the overall trend was evident. Thus, the highest dissolution rates were obtained for material stored at intermediate moisture levels.

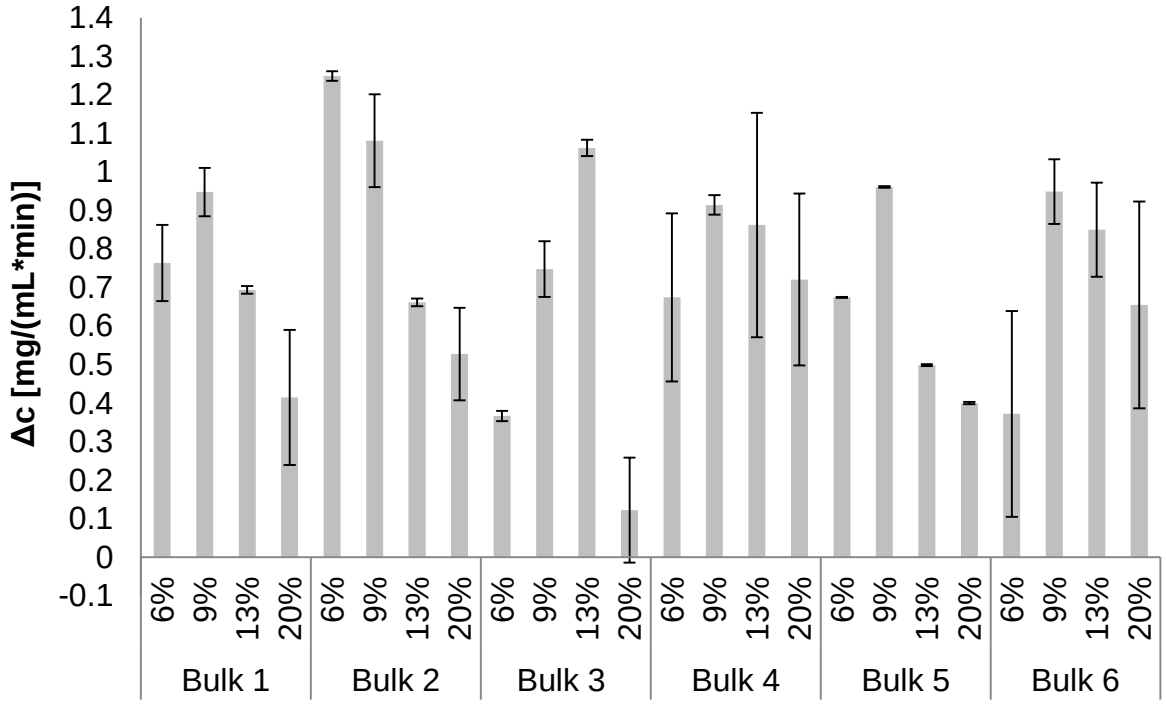


Figure 4: Initial dissolution rates expressed via the change in protein concentration over the initial linear absorption increase. Bulks with a water content of 9% typically had the fastest dissolution rate. However, bulk 2 and 3 were an exception, with the highest dissolution rate at a water content of about 6% and 13%, respectively.

The dissolution rate depends on many factors, including the particle size distribution (PSD). To further investigate the correlation between the water content and dissolution behavior, various physicochemical powder attributes that are known to influence dissolution characteristics were studied [32,48,49]. Since smaller particles have a higher specific surface area, they typically dissolve faster than larger particles [35]. However, the smaller the particles are, the higher the agglomeration tendency is due to the increasing cohesivity [50]. Moreover, the particle shape and size affects the powder packing and the interstitial spaces that are crucial for liquid penetration [51], which is responsible for powder wetting necessary for dissolution. Lippold et al. stated that there is a correlation between the wetting behavior of a

substance and the drug dissolution rate [52]. In general, larger particles are more irregularly-shaped than smaller ones and provide more interstitial space that enhances dissolution [33]. However, in earlier studies particles of the same size were investigated with different levels of irregularity in the particle shape. It was demonstrated that the dissolution rate decreased with the increasing flakiness and irregularity due to the increasing thickness of the average hydrodynamic boundary layer [48]. Those examples demonstrate that the influence of particle size and shape is a complex issue since the same powder characteristic can be a trigger for different phenomena. Furthermore, it was shown that the solubility was higher when the particle surface was convex and lower when the surface was concave [53].

In our study the particle size and shape were determined via dynamic image analysis with the focus on size and the shape factors (i.e., sphericity and convexity). As mentioned above, bulks with a water content of about 20% could not be investigated since the powders were too sticky and formed agglomerates that could not be dispersed. Figure 5 shows the mean particle size for different bulks as a function of the moisture level. As can be seen there is a significant difference of particle size between the bulks. In addition, there is a (smaller) dependence on the moisture content. As can be seen the larger the moisture level is, the larger the particles are. However, the dependence on moisture is not strong. The reason for this increase in size could be either swelling or agglomeration.

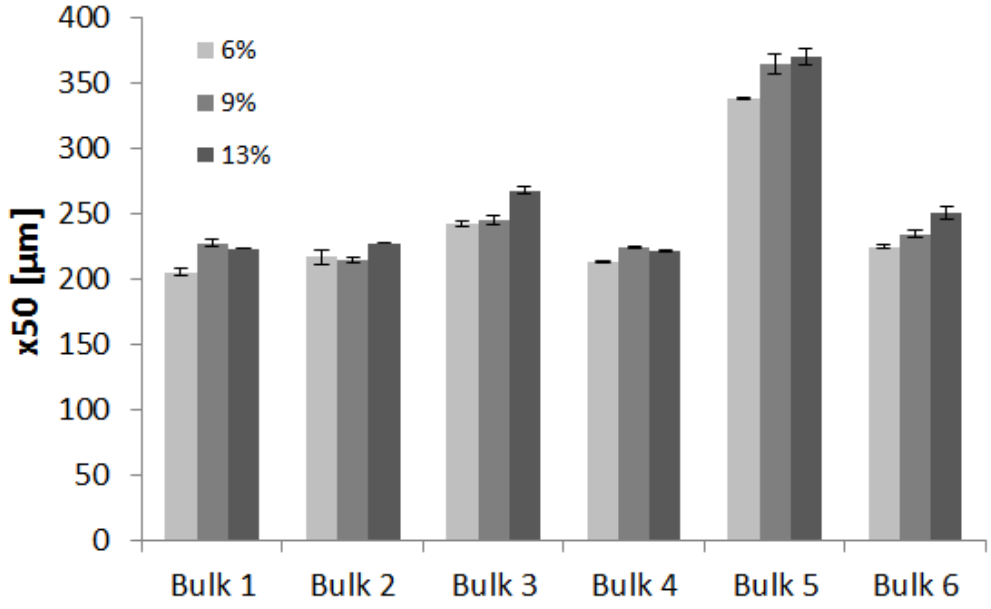


Figure 5 Comparison of the particle size (x_{50} [µm]) of different residual moistures. In general, the particle size slightly increased with the increasing water content.

Table 2 presents the x_{50}, the VMD and the shape factors s_{50} and cv_{50}. In each bulk, the variations were below 2% for x_{50} and below 3% for VMD. For s_{50} and cv_{50}, the variations were below 1%. The span of the particle size distribution slightly decreased (data not shown) with the increasing water content, indicating that the particles become more similar in size as the

water content increased. Although bulk 5 had a significantly higher particle size than other bulks, indicating a bulk-to-bulk variability in the particle size, its sorption and dissolution behavior was the same as in the other bulks.

The sphericity of the fibrinogen bulk powders increased with the increasing water content (Table 2), while the span of sphericity decreased (data not shown). In addition, convexity (Figure 6) and the extent of convexity (data not shown) decreased with increasing water content. Thus, it can be assumed that the particles become more similar in shape as the residual water level increased. In summary, the particle size and shape data suggest that the particles swelled with increasing water content.

Table 3: Particle size and shape

		*VMD [µm]**	*x50[µm]***	*s50****	*cv50*****
	6%	*284.6*	*205.8*	*0.77*	*0.83*
Bulk 1	***9%***	*308.9*	*228.0*	*0.77*	*0.83*
	13%	*309.2*	*223.9*	*0.78*	*0.84*
	6%	*298.2*	*217.4*	*0.73*	*0.81*
Bulk 2	***9%***	*283.1*	*214.9*	*0.75*	*0.82*
	13%	*298.2*	*228.1*	*0.78*	*0.84*
	6%	*304.8*	*243.1*	*0.72*	*0.81*
Bulk 3	***9%***	*313.2*	*245.5*	*0.74*	0.82
	13%	*336.3*	*268.2*	*0.76*	*0.83*
	6%	*296.8*	*213.3*	*0.77*	*0.83*
Bulk 4	***9%***	*313.5*	*224.4*	*0.78*	*0.84*
	13%	*307.4*	*221.9*	*0.79*	*0.84*
	6%	*380.3*	*338.6*	*0.71*	*0.81*
Bulk 5	***9%***	*407.9*	*365.0*	*0.73*	*0.83*
	13%	*412.5*	*370.6*	*0.76*	*0.84*
	6%	*284.5*	*225.1*	*0.74*	*0.81*
Bulk 6	***9%***	*293.2*	*234.8*	*0.75*	*0.82*
	13%	*346.5*	*251.3*	*0.77*	*0.83*

Standard deviations were below 3%, if not stated otherwise, ** Standard deviations were below 2%, *; **** Standard deviations were below 1%.*

Dissolution of pharmaceutical powders is often described by the Noyes-Whitney equation,

$$\frac{dC}{dt} = k_d\,(c_s - c) = \frac{k_i A}{V}(c_s - c)$$

where *dC/dt* is the dissolution rate, k_d is the dissolution rate constant, c_s is the solubility and *c* is the drug concentration in the bulk solution. k_i is the intrinsic dissolution rate, A the surface area and V the volume of the dissolution medium.

The surface area A in this equation is strictly speaking the external surface area, which is proportional to the particle size. The specific surface area of particles scales with the inverse

of the equivalent diameter. Clearly, also the internal surface area contributes to the dissolution, however, in a more complex way, as the penetration of the dissolution medium into the pore space, the internal dissolution, transport of solutes in the pore space, increase of pore sizes and swelling of the solid matrix impact the dissolution rate. Thus, the Noyes-Whitney equation does not apply to porous materials, as in our case. BET analysis reports both the external and internal surface area, the last one typically dominating for particles in the μm-range. Figure 6 shows the BET surface area of all protein powders analyzed. As can be seen the BET areas are in the order of 1 m^2/g and thus, rather small. Thus, most of the surface area is internal and the dissolution rate should correlate more with the internal surface area and not with the external one. On average, the largest specific surface area was measured for fibrinogen powders, which were stored at a water content level of 9%, the overall highest value occurring for 9%. At higher moisture levels, the specific surface area decreased with the increasing relative humidity. All bulks had the smallest specific surface area at the highest water level (around 20% water content). Except two bulks (bulk 2 and 3), all fibrinogen batches dissolved fastest with a water content of about 9% and slowest with a water contents of about 20%.
Thus, dissolution rates correspond quite well with the BET surface area of the powders. Higher moisture levels lead to lower BET values and thus, to lower dissolution rates. Obviously, a water content of around 9% gave the highest BET surface values, corresponding to the highest dissolution rate. Likely explanations for this behavior may be that either with water present swelling occurs due to the integration of water in the tertiary structure thus reducing internal pore space. However, another possibility is, that the protein powder denaturates leading to a collapse as visually observable by partial fusion of the powder cake at samples with a water content of 20% or that due to increased crystallization and the associated increase in specific volume pore space disappeared [54].

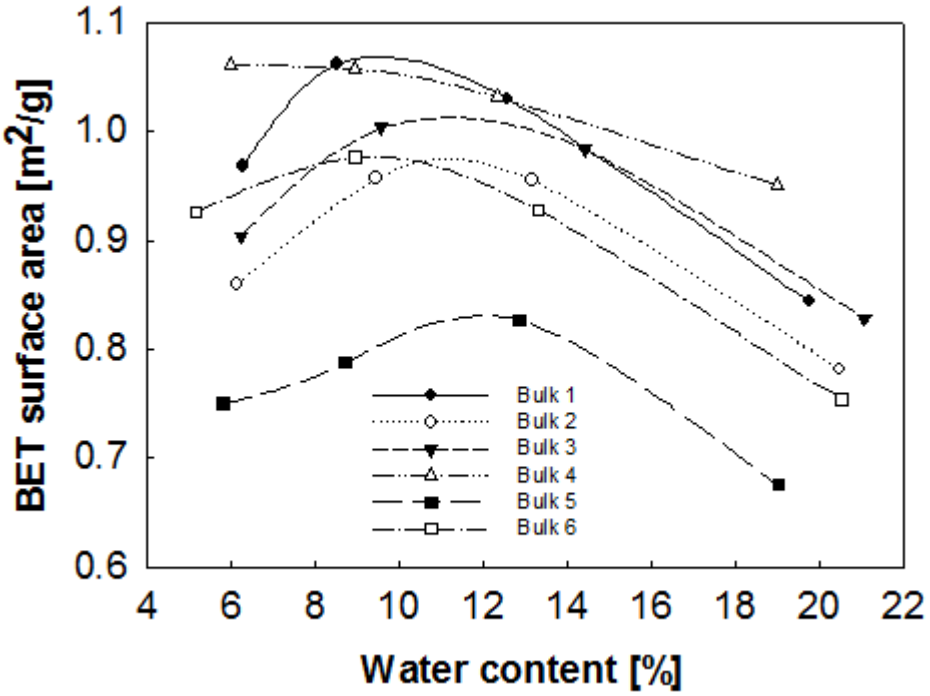


Figure 6: Correlation between the BET surface of dried protein powder and water content during storage. At low residual moistures, the BET surface size increased with the increasing water content. Subsequently, a decrease in the specific surface area occurred.

Lot–to-lot variations can be explained by the varying amount of PS – 80 in the powder bulks (see supplementary part). In addition to the BET analysis, the inner surface of the lyophilized fibrinogen powders was examined. The small angle X-ray scattering (SAXS) results showed a similar tendency as the BET surface area data as can be seen in Figure 7. Note that for the BET analysis, the powders were completely dry. For SAXS measurements, powders remained in their equilibrated state as established during storage and the water content thus remained constant. When expressing the inner surface of the fibrinogen powders via k2/Q, the same trend was observed: in general, bulks stored in $Mg(NO_3)_2$*$6H_2O$ with a final moisture level around 9% had the highest inner surface. For lower and higher moisture levels the k2/Q values decreased (Figure).

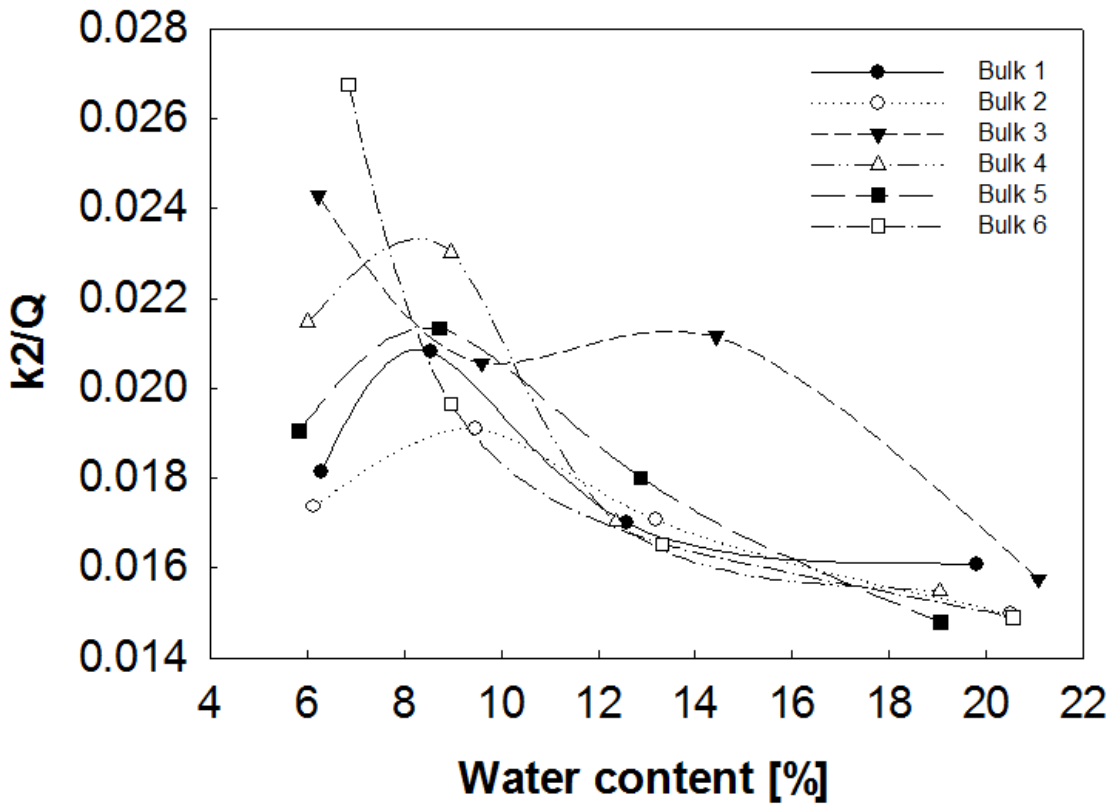


Figure 7: Inner surface of the stored fibrinogen powders. For most powders the inner surface is at a maximum at 9% water content.

Bulks 3 and 6 were the exceptions: their highest inner surface value was not obtained for a water content of 9%. However, this may be statistical outliers as the dissolution data followed the same trends as observed for the remaining powders.

According to the literature, crystallinity also plays a major role in dissolution since crystalline particles dissolve slower than, and not as well, as their amorphous counterparts. Several studies indicating enhanced crystallization of amorphous freeze-dried proteins with increasing water content have been reported [18,55]. If a more stable crystalline state exists, an amorphous material can crystallize when sufficient molecular mobility exists. On the one hand, water acts as a plasticizer and enhances structural mobility. On the other hand, it forms building units of hydrated crystals, to stabilize labile substances and thus, reducing mobility [18]. In order to investigate these phenomena, we investigated the solid state structure of the freeze-dried protein. To determine the crystallinity of the samples, wide angle X-ray scattering (WAXS) measurements were performed, which indicated that crystallinity increased with higher levels of water content. This is shown in Figures 8 and 9. However, since it was not possible to calibrate the method with a 100% crystalline sample, no quantification of the crystalline content was performed. The WAXS crystallinity was calculated based on the ratio of the integrated intensities of the diffraction peaks by subtraction of the baseline (Figure 8).

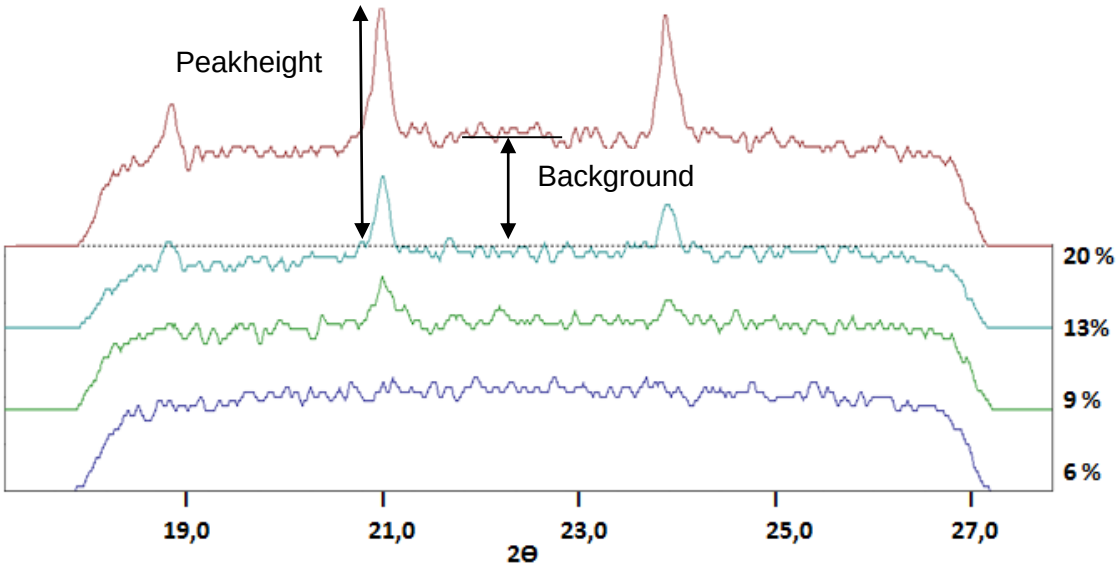


Figure 8: WAXS spectrogram. The WAXS crystallinity was calculated based on the ratio of the integrated intensities of the diffraction peaks by subtraction of the baseline

As can be seen in Figure 8 and 9, particles that were stored in lithium chloride (lowest moisture) seemed to be close to amorphous and protein powders equilibrated in potassium nitrate (highest moisture) had a high crystalline content. In the beginning, a linear correlation was observed. When the water content reached about 13%, the curves began to flatten (with the exception of bulks 1 and 3), at which point the maximum crystallization degree was achieved. Bulks 1 and 3 seemed to have a slower crystallization rate than the rest of the fibrinogen lots. Non-treated (not stored at a certain humidity level) fibrinogen powders were measured as well. The solid state proteins were amorphous after freeze drying and before storing them on the saturated salt solutions. Control measurements of the samples stored at controlled humidity showed that no changes in crystallinity occurred within 18 months.

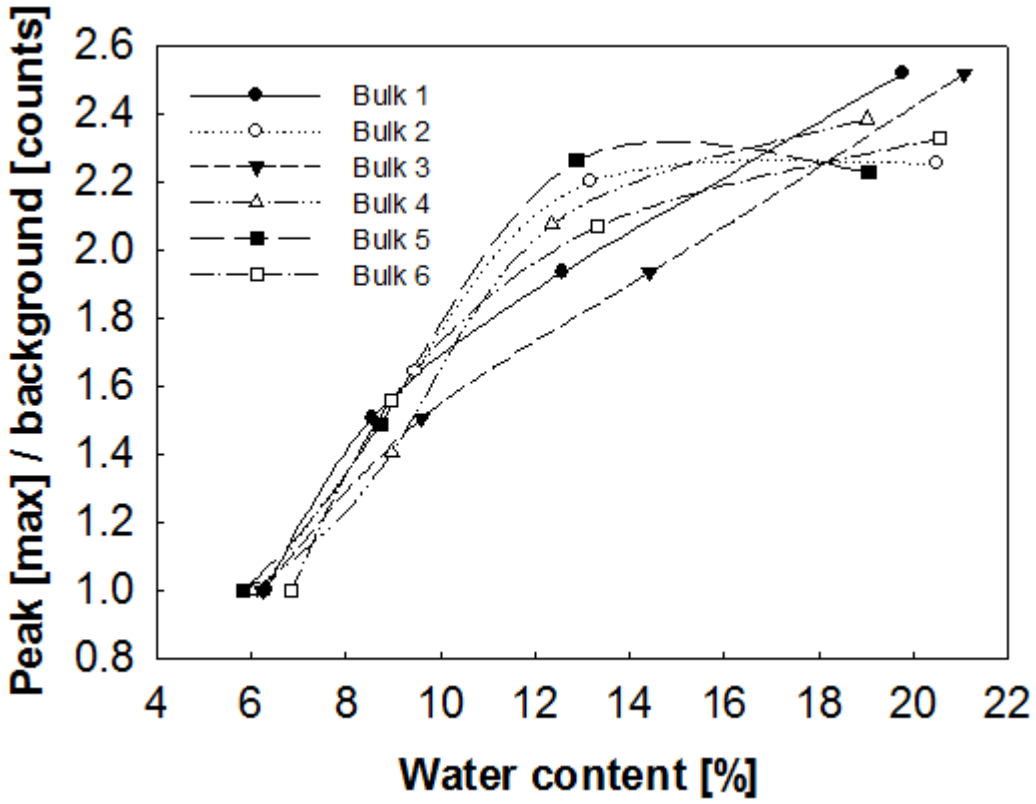


Figure 9: Crystallinity vs. water content. A linear correlation exists until the water content reaches about 13% for most of the bulks (except for Bulks 1 and 3). Subsequently, the curve flattened. The y-axis was calculated by subtracting the background (counts) from the maximum of the crystallization peaks at certain angles.

These WAXS reflections do not correspond to any known signals from additives leaving the protein as only possible origin. Since amorphous drug forms have no internal crystal structure, they are in a higher energy state and have higher solubility, and thus, dissolution rates than crystalline forms [40,55]. The obtained WAXS data indicate that the dissolution rate decreases with the increasing water content and thus with crystallinity as expected. When correlating the crystallinity data with the BET surface area, it may be concluded that the specific surface area decreases with the increasing crystallinity.

In summary, according to our data, the surface area increased at a water content between 6% and 9% and subsequently dropped. At the same time the crystallinity increased with water content. A similar trend in the BET surface vs. crystallinity was reported in the literature [56]. The crystalline state is structured and has a well-defined long-range order of molecular packaging. In contrast, the amorphous solid has no long-range order of molecular packaging or well-defined molecular conformation if the constituent molecules are conformationally flexible [55]. Amorphous solids have the tendency to absorb relatively high amounts of water vapor, which can cause phase transitions and lyophile collapse [57]. Often, there is a critical humidity at which the glass transition occurs for a given temperature [54]. As such, the BET

surface decreases when the crystallinity increases according to the changed molecular packaging of the samples.

Comparing the SAXS and WAXS data showed that an increase in crystallinity was accompanied by a decrease in the nanostructure e.g. fluctuations at the scale of 1-100nm, of the samples. Since less capillaries and inner pore space was provided for the solvent, the dissolution rate of the fibrinogen powder dropped. The correlation between dissolution and inner surface was quite pronounced, i.e., a p-value of about 10^{-16} was obtained using ANOVA. p-values of less than 0.05 are considered to be statistically significant, and thus, a statistically significant correlation between the inner surface and the dissolution rate was established.

Together, the results indicate that different levels of residual moisture in a solid-state protein during storage lead to variability in pore structure, inner surface and crystallinity, affecting strongly the dissolution behavior due to physico-chemical changes. The BET and inner surface correlate with the investigated dissolution rates showing an anomaly maximum at intermediate water content. Such biphasic behavior cannot be observed with particle size and crystallinity.

3.5 Conclusion

In this work we demonstrated that differences in the water content during the storage of solid state proteins lead to differences in the dissolution behavior and the dissolution rate. These differences are due to a change in the BET and inner surface of the freeze-dried protein powder and the accompanying changes in the crystallinity during storage. Specifically, we found that:

- the particle size slightly increased with increasing water content, most likely due to swelling and the higher specific volume of crystalline materials in comparison with the amorphous starting material
- the particles became more similar in shape (convexity, sphericity) with increasing water content for similar reasons
- the inner and the BET surface had a maximum at a water content of 9%
- the crystallinity was increasing with increasing water content
- the dissolution rate had a pronounced maximum at intermediate moisture levels

These results demonstrate – up to our knowledge for the first time - the influence of physico–chemical parameters of freeze dried proteins on their dissolution behavior. Moreover, these results are important for storing and processing protein powders. In general, dry powder and water contents higher than 10% did not improve the dissolution performance. Therefore, the general accepted concept of “the drier the better” is not appropriate for freeze dried fibrinogen in terms of processing. Similar results were also demonstrated for tissue type plasminogen activator (tPA) [47], were higher water contents increased the stability of the product. Thus, the experimental evidence suggests that identifying the ideal water content for subsequent

process steps is useful for improving the processing performance of solid-state protein formulations.

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3.7 Supporting Information

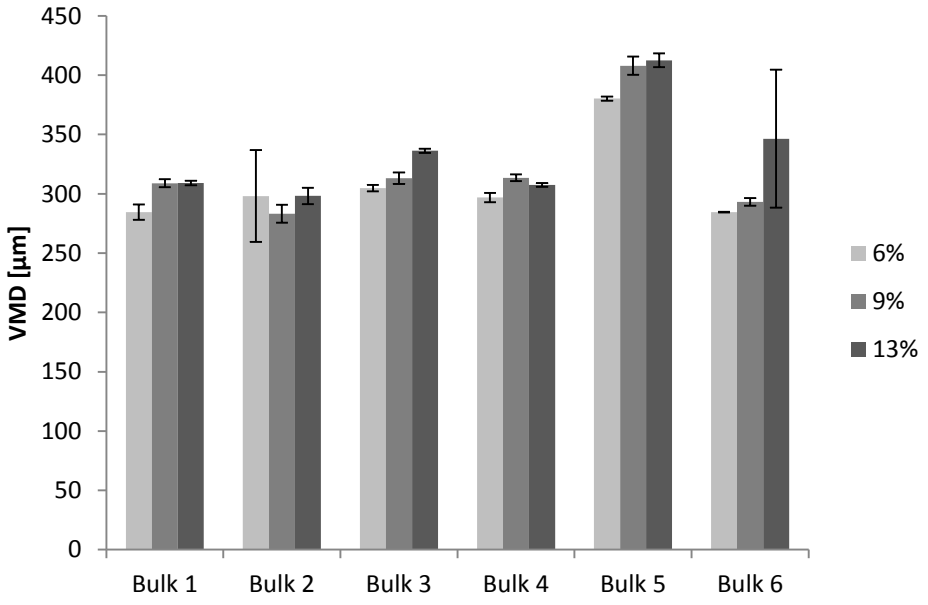


Figure 1: Comparison of the VMD (Volume mean diameter) [µm] of different water contents.

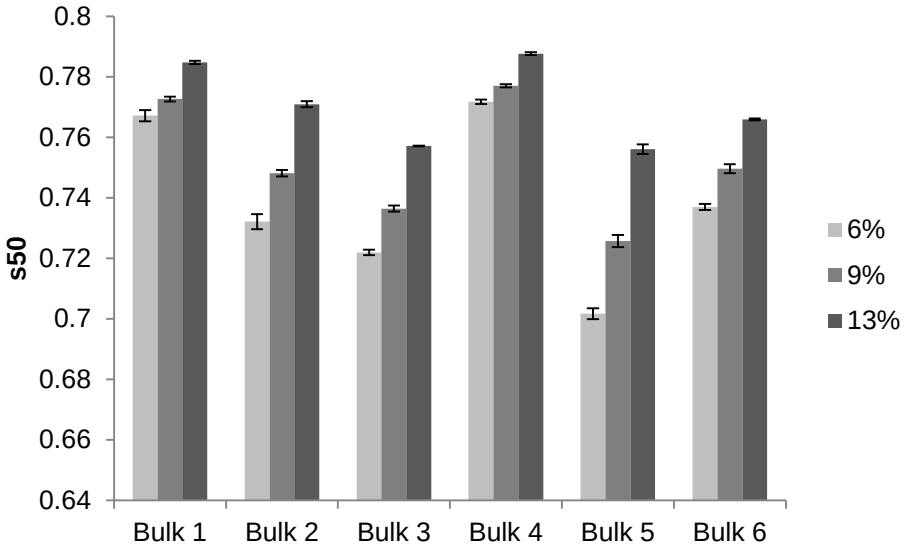


Figure 2 Comparison of the sphericity (s50) of different residual moistures. Sphericity increases with the increasing water content and particles become more regularly-shaped when sorbing water.

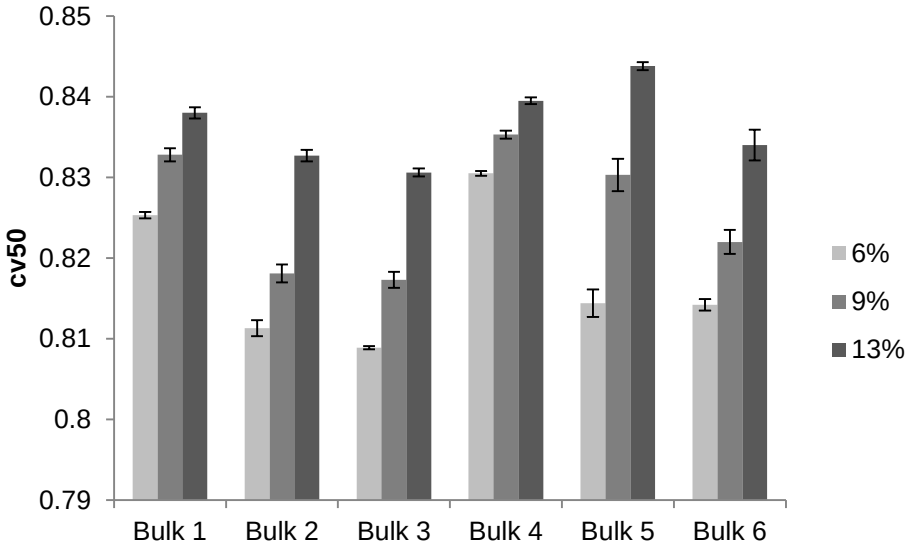


Figure 3: Convexity of the particles (cv 50). Convexity increases with the increasing water content.

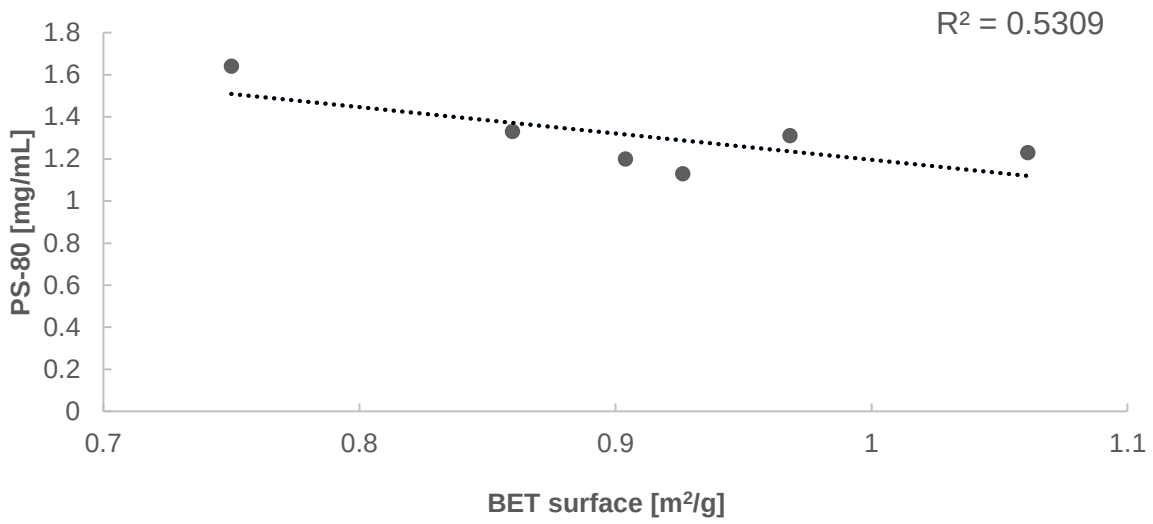
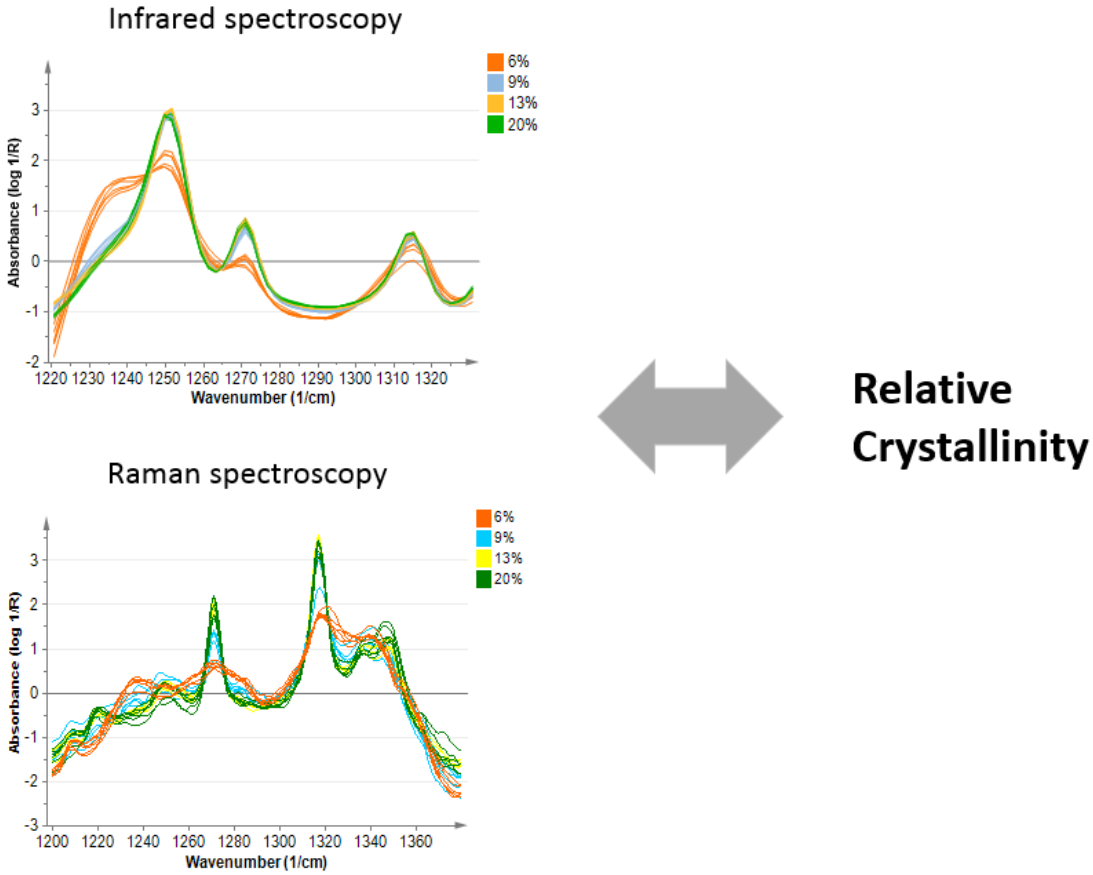


Figure 4: Correlation BET surface – amount Polysorbate 80.

4. Chapter 3: The influence of residual water on the secondary structure and crystallinity of freeze-dried fibrinogen[2]



[2] This Chapter is taken from a Journal article by Wahl V. et al from the International Journal of Pharmaceutics (2015)

4.1 Abstract

The purpose of this work was to investigate the influence of water content on the secondary structure of a freeze-dried protein (fibrinogen) after a storage period of two weeks. To that end, Attenuated Reflectance Fourier Transformed Infrared (ATR-FTIR) and Raman spectra were generated and evaluated and the crystalline state of the fibrinogen bulks was determined via X–ray diffraction. First, a PCA (principal component analysis) of the spectral data was performed. While the α–helix and β–turn contents were increasing with the increasing water content, the β-sheet content was decreasing. A partial least squares (PLS) model was developed to correlate the mid-infrared and Raman spectral changes with the degree of crystallinity. The obtained R^2 value of 0.953 confirmed a correlation between changes in the secondary structure and crystallinity of the samples. The results demonstrated that the combined ATR-FTIR and Raman approach could be used to predict the crystalline state in freeze-dried fibrinogen products.

4.2 Introduction

Many biopharmaceuticals are known to be more stable when stored in the dried state, therefore they are routinely freeze dried to increase shelf life. However, freeze drying can damage proteins as they are exposed to various kinds of stresses (Griebenow and Klibanov, 1995; Wang, 2000). While most macromolecular therapeutics are stored in the amorphous state, small molecule drugs are typically produced in the crystalline form (Hancock and Zografi, 1997) that offers higher storage stability, purity and reproducibility with respect to a drug's physical, chemical and therapeutic properties. In contrast, the amorphous state is not well defined and various types of long-range orders may occur, i.e., different amorphous forms exist, depending on the process (Young et al., 2007; Yu, 2001). The amorphous state is a thermodynamically unstable state with higher energy, i.e., the materials have the tendency to transform into a more stable crystalline form during storage. The remaining water content of solid-state proteins can influence the protein powder as medium and plasticizer, resulting in decreased glass transition temperatures, increased crystallization rates and increased molecular mobility (Hancock and Zografi, 1997; Pikal et al., 2007; Shalaev and Zografi, 1996; Yoshioka and Aso, 2007). As such, during storage the solid state of proteins and peptides can be affected by moisture (Flores-Fernández et al., 2010).

Bulk quantities of lyophilized pharmaceutical proteins are exposed to various levels of humidity during storage and processing, which influence their long-term chemical and physical stability (Costantino et al., 1994; Hageman, 1988; Liu et al., 1991). Water plays a critical role with regard to the protein stability because it is involved in the degradation processes and, in particular, in aggregation and structural changes. During the transformation from a dry solid protein to the fully hydrated one, substantial changes in protein structure may occur (Costantino et al., 1994; Hageman, 1988; Liu et al., 1991). Upon reconstitution, these (partially) unfolded proteins can form aggregates, which may cause severe immune reactions and reduce the therapeutic efficacy (Jain and Roy, 2008). Therefore, it is important to examine the stability of proteins under pharmaceutically-relevant conditions (Costantino et al., 1994).

Recent studies reported changes in the secondary structure associated with various levels of water content (Flores-Fernández et al., 2010, 2009). Infrared and Raman spectroscopy are widely used for investigating the secondary structure of proteins(Beer et al., 2009; Luypaert et al., 2007; Pieters et al., 2013; Wen, 2007). Both are non-invasive, fast and sensitive methods for investigating the vibrational state of chemical bonds present in the analyzed samples, which require virtually no sample pretreatment. Raman spectroscopy measures the changes in the scattered light frequencies based on the interaction between the monochromatic laser light and different vibrational states of the analyte (John M. Chalmers and Griffiths, 2002). In infrared absorption spectroscopy the molecules absorb infrared radiation and transit from the ground state to the excited vibrational state to generate an IR absorption spectrum (Barth, 2007).

Infrared spectral interpretation of the secondary protein structure generally relies on the amide I (1600-1700 cm^{-1}), amide II (1510-1580cm^{-1}) and amide III (1235-1300 cm^{-1}) bands. The amide III band is attributed to N-H in plane bonding, C-N vibrations in stretching modes and weak contributions by C-C stretching and C=O bending (Haris and Chapman, 1992; S and J., 1986). A variety of chemometric and statistical techniques have been employed to extract information from raw spectroscopic data, including multiple linear regression (MLR) analysis, principal component analysis (PCA) and partial least squares regression analysis (PLS) (Mitchell H. Katz, 1999; Pieters et al., 2014; Roggo et al., 2007).

Determining crystallinity is important with regard to qualifying raw materials, intermediates and end products. In the last years, also process control via in-line PAT tools started to be an area of intense interest. There are several methods for investigating crystallinity, such as X-ray diffraction, density determination, solid–state NMR and water vapor absorption (Buckton and Darcy, 1999). Changes in crystallinity can be detected using DSC and isothermal microcalorimetry. These analyses can only be performed off-line. Recently, near infrared spectroscopy was used to quantify the amorphous and crystalline parts of solid-state materials, e.g., glycine, raffinose, lactose (Bai et al., 2004; Beer et al., 2009; Buckton and Darcy, 1999; Gombás et al., 2003; Hogan and Buckton, 2001).

The goal of our study was to examine changes in the protein structure due to moisture sorption via infrared and Raman spectroscopy and to correlate these changes with the crystallization state of human fibrinogen. To that end, we used the amide III band of ATR-FTIR and Raman spectra to characterize the secondary structure of lyophilized fibrinogen since the influence of water in the ATR-FTIR spectra is negligible in this region. This method can be used, in theory, to monitor online the change of crystallinity in stored bulks and during production.

4.3 Materials and Methods

Materials

The analyzed freeze-dried protein powders were provided by Baxter AG, Vienna, Austria. Six lots of the solid lyophilized protein were examined in triplicate. The freeze-dried fibrinogen samples were not pure samples. Small amounts of excipients were present, with polysorbate 80 (PS-80) being the largest additive (less than 3% by protein weight) and some other components in small amounts (less than 1% by protein weight).

To investigate the influence of moisture on the solid-state stability of fibrinogen, the freeze-dried powders were stored under accelerated-storage conditions with controlled humidity. The powders were stored at room temperature at constant levels of relative humidity (RH) in desiccators with different salt solutions. The corresponding RH levels of the air were 12% (LiCl), 52% ($Mg(NO_3)_2*6H_2O$), 75% (NaCl) and 93% (KNO_3) (Rockland, 1960).

Residual moisture analysis

After two weeks of incubation, the lyophilized powder samples were saturated and the residual moisture content was determined with Karl Fischer Titration (Titroline 7500 KF, SI Analytics, Mainz, Germany). The powders (approximately 0.2 mg) were directly added to the titrator cell. To enhance the solubility of powders and to assess the samples with low levels of residual moisture content (6-10% water content), a methanol/formamide mixture (1:1 ratio) was utilized. The other powders (13-20% water content) were investigated using pure methanol.

Wide- and small-angle X–ray scattering

Wide-angle x–ray (WAXS) scattering was applied to determine the powder's crystallinity. WAXS measurements were carried out at 20 ± 1°C in a S3 – MICRO camera (formerly Hecus X-ray systems, Graz, now Bruker AXS, Karlsruhe). Crystallinity was measured in the angular range between 17 and 27°, corresponding to real space dimensions of 4.9 and 3.3 Å, where molecular crystals have strong diffraction peaks. To avoid preferential orientation effects of crystallites, the samples held in capillaries of 2 mm in diameter were rotated during the measurement. The typical exposure time was 1200 sec.

Attenuated Total Reflected – Fourier Transformed – Infrared Spectroscopy

The secondary structure of the freeze-dried protein was acquired using a Bruker Optics Vertex 70 (Rheinstetten, Germany) apparatus. A spatula tip of the bulk powder was recorded with a DLaTGS detector and an ATR unit (MVP Pro star, Diamond crystal). A total of 500 scans were performed at wavenumbers ranging from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}. Before examining each sample, a reference measurement without a sample was performed to reduce the background noise. Three samples were collected from each batch and subjected to IR measurements.

To eliminate the influence of water vapor and sorbed water, the protein's secondary structure was analyzed in the amide III region (1220 – 1330cm^{-1}), in which the absorbance of water is negligible(Griebenow and Klibanov, 1995) and which has well-characterized spectral features that provide higher resolved bands for quantitative analysis than other amide regions.(Cai and Singh, 1999)

Raman spectroscopy

A RamanRXN2™ Hybrid Analyzer (Kaiser Optical systems, Ann Arbor, MI, USA) equipped with a 785 nm laser and a PhAT probe was used to investigate the secondary structure of the freeze-dried fibrinogen powder. The PhAT probe generates a 6 mm diameter spot that covers most of the solid-state protein powder, making it possible to analyze a representative surface of the sample. Spectra were recorded using iC Raman™ software. Raman scattering in the range between 100 and 1890 cm^{-1} was recorded for twenty seconds three times and averaged.

The amide III band (1200-1380 cm^{-1}) Raman spectra were analyzed in terms of the secondary structure using SIMCA 13.0, Umetrics (Umeå, Sweden).

Chemometric analysis

To interpret the spectroscopic measurements, multivariate chemometric methods were applied. The spectra were analyzed using SIMCA 13.0, Umetrics (Umeå, Sweden).

A SNV-correction (standard normal variate) of the amide III region was performed to compensate for the scatter–induced baseline offsets (scale: 5 ctr). Afterwards, the second derivative with Savitzky – Golay smoothing (second order polynomial, kernel: 5 points) of the spectra was calculated. PCA was used to identify high-variability areas in the spectral region. IR and Raman spectra were analyzed separately. Subsequently, the obtained principal components representing the IR and Raman spectra via a reduced number of variables were used to develop a hierarchical model. These values were correlated with the crystallization data.

In this study, PLS was applied to predict the crystallization state of the lyophilized fibrinogen powder based on spectral data: the preprocessed Raman and infrared results were used as input variables (X) and crystallisation data as output variables (Y). As input for the PLS regression (X), the PCA score values (rather than the complete measured spectral data) were used to equalize Raman and IR spectra regardless of the number of involved variables. The hierarchical values of all measured spectral data (X) were applied in a further PLS regression to minimize the amount of data and to establish a uniform weighting for all measured properties, i.e., both the spectral and the univariate material properties.

4.4 Results and discussion

To investigate the influence of moisture on the solid-state instability of fibrinogen, six batches of freeze-dried powders were exposed to accelerated storage conditions of controlled humidity. The powders were stored at room temperature at constant levels of relative humidity (RH) of 12% (LiCl), 52% ($Mg(NO_3)_2$*$6H_2O$), 75% (NaCl) and 93% (KNO_3),(Rockland, 1960) resulting in protein bulk powders with moisture contents between 6 and 20% measured via Karl Fischer titration. The target values for moisture content were 6% (LiCl), 9% ($Mg(NO_3)_2$*$6H_2O$), 13% (NaCl) and 20% (KNO_3). The exact values of the final water content and their standard deviation were reported elsewhere. (Wahl et al., n.d.) When possible, these target values were used to label the Figures throughout this study. Since no significant differences in the water content between batches were observed, the water sorption behavior seemed to be similar for each lot. (Wahl et al., n.d.)

Fibrinogen is a large, multi-domain glycoprotein composed of three non-identical polypeptide chains. The secondary structure composition was quantified as 40% α-helical structures and

minor segments of β-sheets (18-20%), β-turns (14%) and unassigned structures (16%). These values were previously determined via Circular Dichroism (CD) and ATR-FTIR spectroscopy. (Azpiazu and Chapman, 1992; Gorinstein et al., 2003) According to already published data the water content was proven to be a major factor influencing secondary structure of proteins.(Flores-Fernández et al., 2010, 2009; Georget and Belton, 2006) Here, ATR-FTIR and Raman measurements were performed to evaluate the secondary structure of various equilibrated fibrinogen bulk powders.

With regard to infrared measurements, the amide III (1220 -1330 cm^{-1}) spectral region, where water and water vapor have a negligible impact and which was often used to obtain information about the secondary structure of proteins (Cai and Singh, 2004; Carrasquillo et al., 2000; Griebenow and Klibanov, 1995), was of special interest as water and water vapor have a negligible impact in this spectral area. Therefore, this region is often used complementary to information derived from the amide I band, to gain insights into the secondary structure of proteins (Cai and Singh, 2004; Carrasquillo et al., 2000; Griebenow and Klibanov, 1995). The different secondary structures of proteins generally have higher resolved differences in the amide III spectra and thus were selected for this study (Cai and Singh, 2004; Griebenow and Klibanov, 1995). The spectra were analyzed using SIMCA 13.0 and pretreated as described before.

A common method of multivariate data analysis, principal component analysis (PCA), was performed to identify similarities and differences between the batches and varying water contents. PCA transforms multidimensional axes (i.e., wavenumbers) presented in the original data into a set of new orthogonal axes known as principal components (PCs), detecting trends and structures in the data sets by retaining the variability of the data. Based on loading vectors, qualitative information about the samples can be obtained by describing what type of information characterizes them. PCA separates the original data matrix into score and loading matrices that are used to interpret the PCA via means of score and loading plots. The score plot represents coordinates of the data in the new PCs axes. Provided that the plot captures a sufficient amount of spectral variability, data clustering in the score plot reflects their similarity. The loading matrix can be viewed as a set of weights applied to the original variables in order to obtain PCs (Beebe et al., 1998; Gemperline, 2006).

In the score scatter plot in Figure 1, the obtained infrared data are grouped by similarity. Bulks conditioned with lithium chloride (water content 6%) were different from those conditioned with potassium nitrate (water content 20%), sodium chloride (water content 13%) and magnesium nitrate (water content 9%) in the first principal component (PC 1). Powders with a water content of 20% discriminated in the second principal component (PC 2). Since PC 1 explained 91.2% of the data and PC 2 explained 7.29%, both principal components reflected well the significant changes in the infrared spectra of the fibrinogen powder. These changes were assigned to

changes in the secondary structure of the freeze-dried fibrinogen as, according to literature, changes in the secondary structure due to various water contents were recognized in other proteins (Flores-Fernández et al., 2010, 2009).

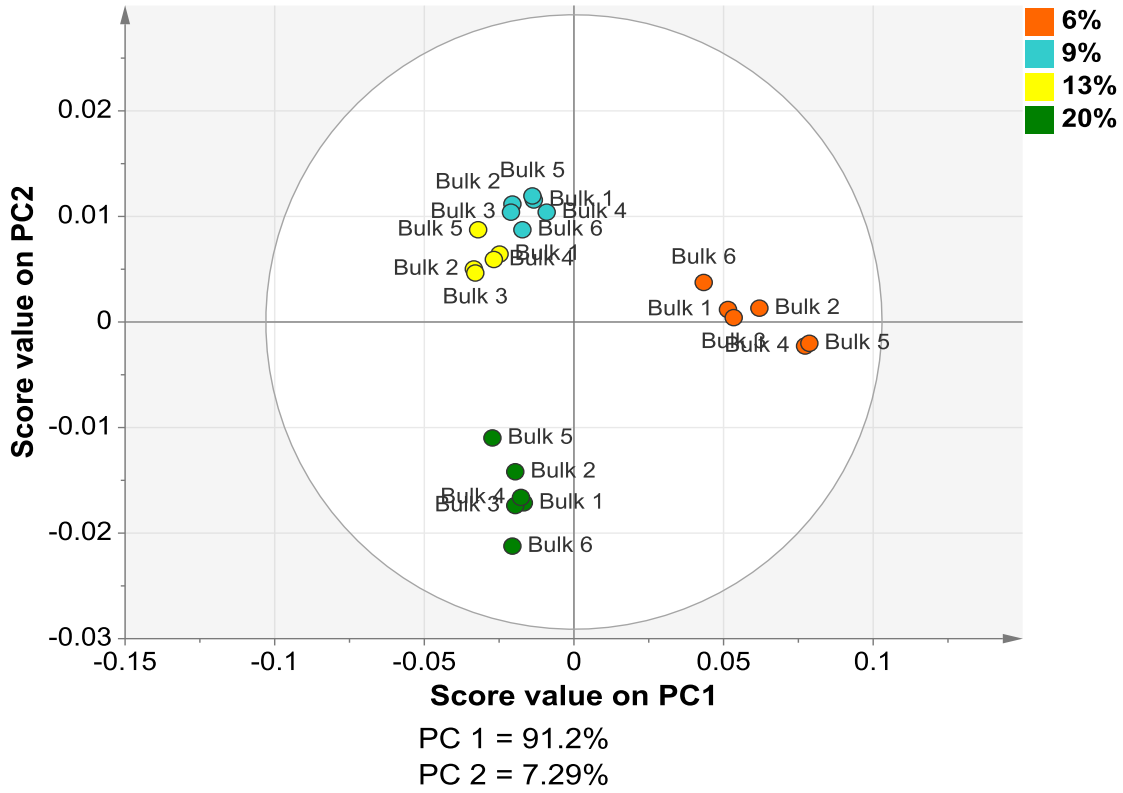


Figure 1: Scores scatter plot based on pre-treated ATR-FTR measurements recorded for all samples. Bulks are grouped by the levels of residual moisture. They have differences in the secondary structure according to the amount of remaining water. Bulks stored in LiCl [water content 6%] discriminate in the first principal component from the rest of the batches, while bulks stored in KNO_3 [water content 20%] differentiate in the second principal component from bulks stored in NaCl [water content 13%], LiCl [water content 6%] and $Mg(NO_3)_2*6H_2O$ [water content 9%].

To identify these differences, first, the pretreated spectra were visually analyzed (Figure 2). SNV-corrected spectra can be found in the appendix.

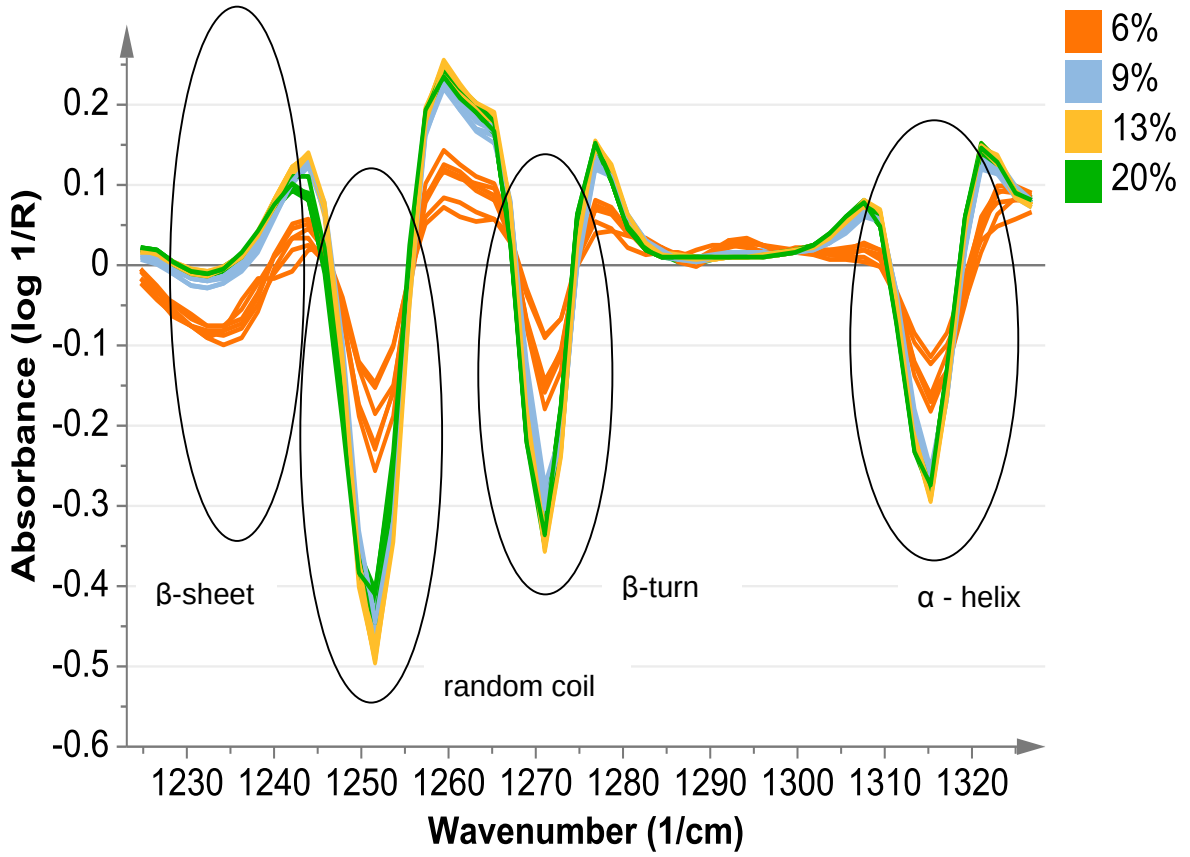


Figure 4: The second derivative of the ATR-FTIR spectra (amide III band: 1220 – 1330 cm-1) after incubation at various relative humidity levels. The intensity of the peaks varies depending on the levels of water content. The recorded IR spectra were attributed to various forms of the secondary structure,(Cai and Singh, 2004, 1999) and changes in these structures due to water contents were observed.

The shape of the spectra obtained from powders with a water content of 6% was different from that of powders stored at higher RHs. An increase in the IR absorption with the increasing residual moisture was observed in the area corresponding to the α-helix region (1330-1295 cm^{-1}), while the absorption in the β-sheet-specific regions of the spectra (1250-1220 cm^{-1}) decreased. The absorption in the β-turn region (1295-1270 cm^{-1}) increased with the rising residual moisture, similar to the increase in the random coil area (1270-1250 cm^{-1}). Clearly, structural changes in the fibrinogen powder occurred at elevated levels of residual water. Thus, incubation of the samples at higher relative humidity levels results in substantial spectral changes (and a changed secondary structure) characterized by an increase in the β-turn, α-helix regions and random-coil and by a decrease in the β-sheet.

As can be seen in Figure 1, the second principle component of the group of bulks stored on KNO_3 (20 %) is different for that of the other powders. Shifts to lower wavenumbers in the entire spectra of bulks with a water content of 20% were observed. Although not visible in Figure 2, this can be clearly seen in the raw spectra shown in Figure 11 (see Appendix), leading to a

difference in the second PC. Based on the shifts to lower wavenumbers, it was concluded that the binding energy decreased in the fibrinogen powder bulks with the highest water content. Thus, less energy input is required during the measurement to excite the protein molecules containing a higher level of water. To visualize this assumption, loadings of the first and second principal component (PC) of the analyzed infrared data are compared in Figure 3. Since bulks with a lower moisture (6-13%) do not exhibit differences in the PC2, PC2 explains the effects of higher moisture. A shift to lower wavenumbers can be clearly seen in Figure 3. The loading plot for each PC can be used to identify variables that have a large influence on the investigated system.(Beebe et al., 1998; Gemperline, 2006)

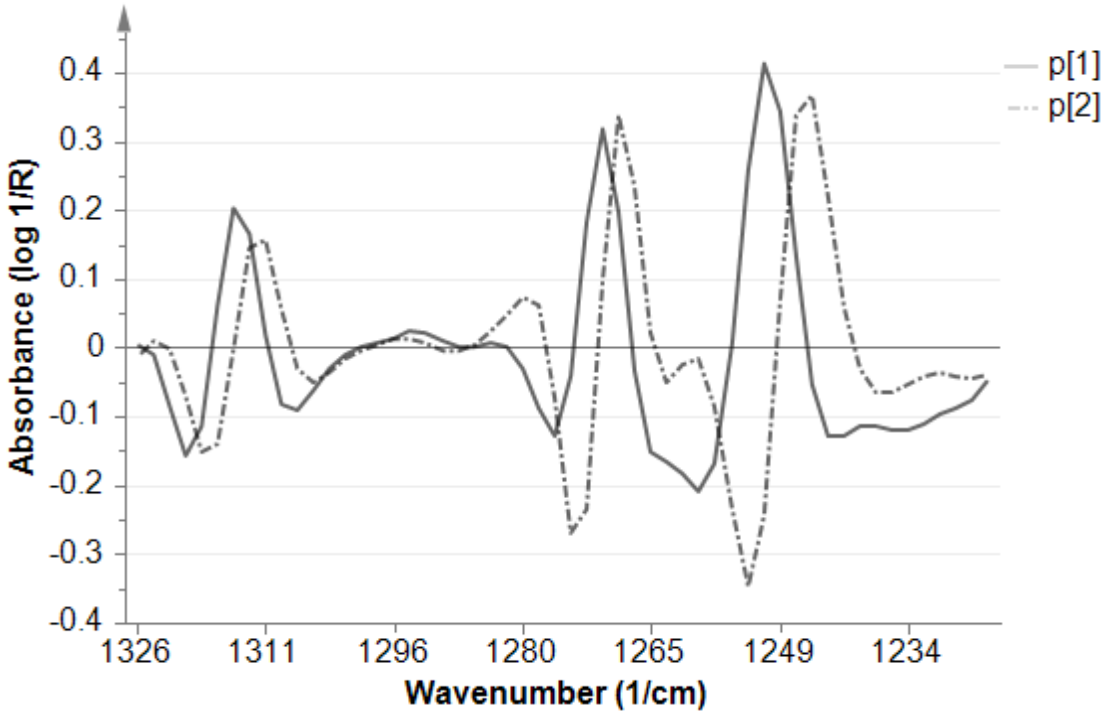


Figure 3: Comparison of the first and second principle components of the ATR-FTIR pre-treated spectra of the 24 samples. Since the second principal component shifted to lower wavenumbers, decreasing binding energies with the increasing water content can be expected since less energy input is required during the measurement to excite the protein molecules with a higher level of water

The hypothesis about the wavenumber shifts is also supported by findings in the literature: frequency shifts to lower wavenumbers in the amide A/II band due to heat denaturation of pepsin (Liu et al., 1994) and lowering of the frequency upon hydrogen bonding between ribonuclease and sucrose were detected via Raman spectroscopy.

While differences of the powder batches (clustered according to their water content – see Figure 1) in the first principal component is caused by changes in the secondary structure due to differences in the water content, changes in the second principal component were induced

by changes in the binding energies of the molecules. For bulks stored in potassium nitrate (20%), changes in the binding energy were observed that could be due to protein molecule unfolding. When molecule unfolding occurs because of the increasing water contents, a general weakening of the intra-molecular hydrate bonding strength occurs because the protein losing its cooperatively folded structure (Cooper, 2000). Since the native protein conformation is thermodynamically stabilized, changes in the microenvironment of the protein (e.g., higher water contents) may disturb the equilibrium. On the surface, the residual water is typically bound to charged and polar groups and to the peptide backbone groups (Chang et al., 1993; Separovic et al., 1998). Thus, with increasing water content, the system becomes globally unstable, causing a transition to (partially) unfolded states that will be energetically preferred.(Ragoonanan and Aksan, 2007) Water plays a critical role with regard to the stability of proteins since it is involved in deleterious processes and, in particular, aggregation and structural changes (Jain and Roy, 2008). Other spectral ranges (amide I [1700 – 1600 cm^{-1}], amide II [1500 – 1600 cm^{-1}] band) that were analyzed suggest a distortion of the secondary protein structure. However, since water can have a significant influence in these regions, the data are ambiguous and were thus ignored.

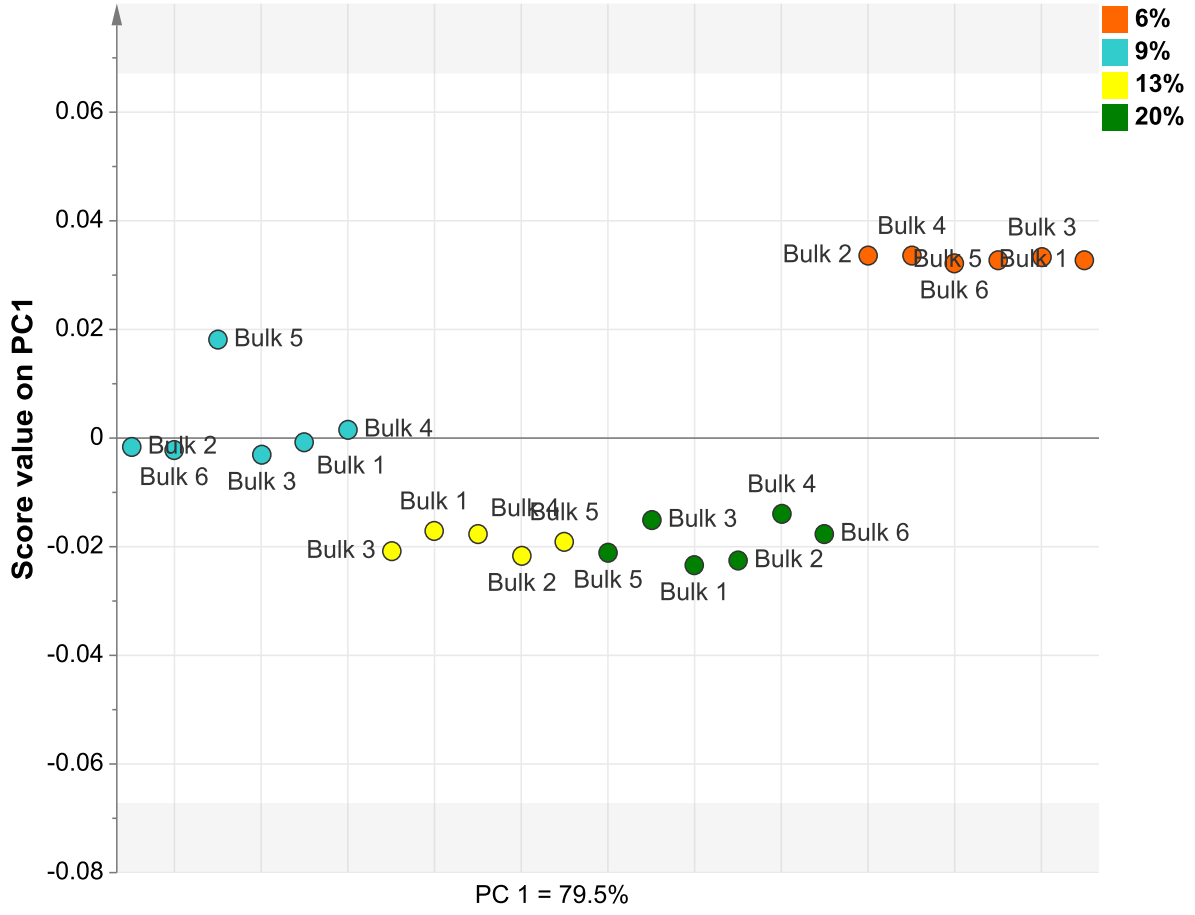


Figure 4: Scatter plot of Raman spectra. The bulks differ only in terms of the first principal component.

Using a complementary approach, Raman spectra were taken in the amide III band (1200 - 1380 cm^{-1}) that is highly sensitive with regard to the secondary structure (Cai and Singh, 1999). We used this amide band for comparison reasons since the same vibrational modes (N-H in plane deformation; C-C-N stretching) are involved in Raman and IR. The spectra were processed identically to the infrared spectroscopy data. The scatter plot in Figure 4 shows that 79.5% of the data are explained via the first principal component. Thus, only one principal component shows significant impact in the Raman spectra of the freeze-dried fibrinogen powder. These changes can be indicated as changes in the secondary structure due to changes in the water content.

As before, the second derivatives of the spectra were interpreted visually. Changes in the secondary structure were found in the Raman spectra. Shifts in the peak maxima and peak intensities were observed (Figure 5).

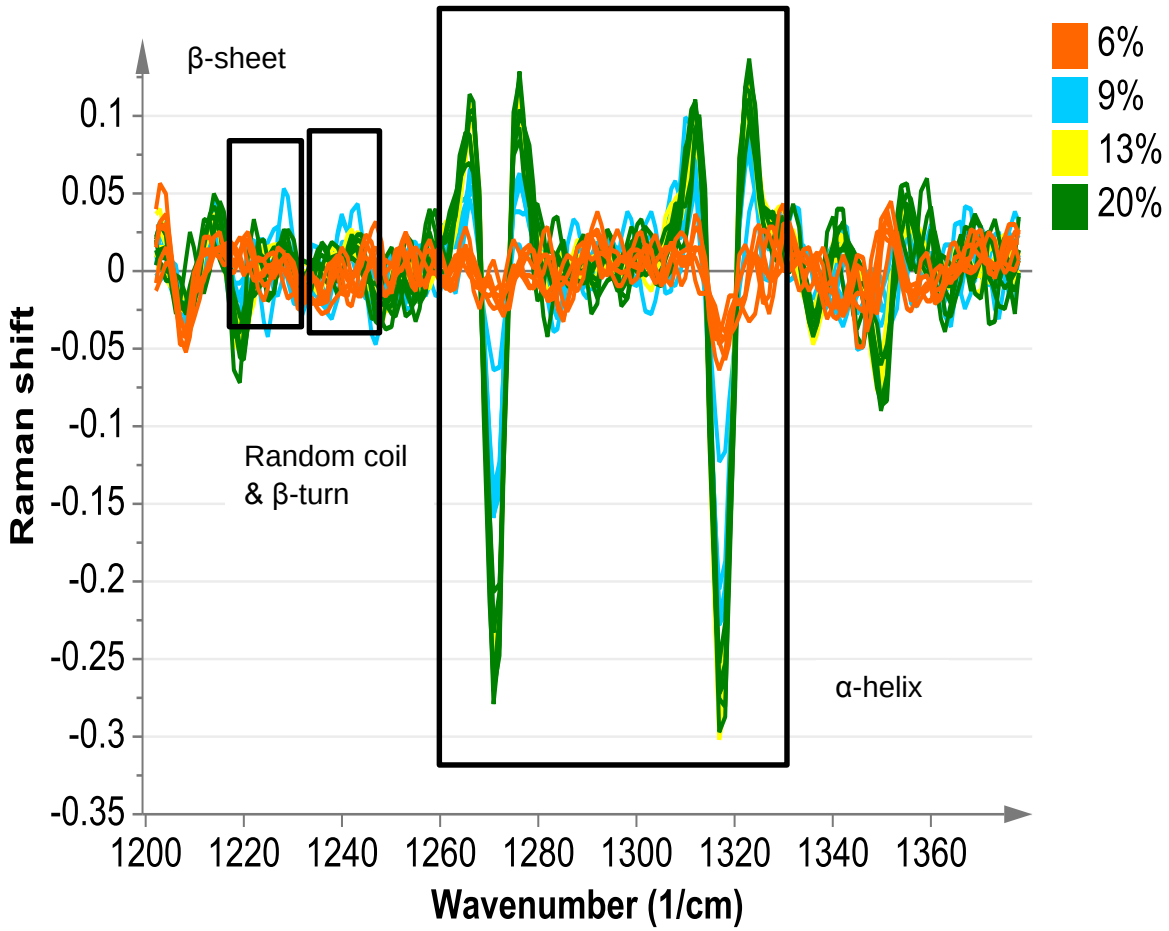


Figure 5: Second derivative of amide III (1200-1380 cm-1) region of the Raman spectra. Clearly, the α-helix and β-turn regions are increasing with the water content in all bulks.

As the residual moisture increased, there was a small decrease in the beta-sheet (1230–1245 cm^{-1}) and peak shifts, but no changes in the random coil (1245-1255 cm^{-1}) occurred. Furthermore, an increasing water content led to an increase in the beta-turn (1254-1280 cm^{-1}) and the alpha-helical structures (1280-1320 cm^{-1}).(Herrero, 2008; Tantipolphan et al., 2008)

These results confirm the relevance of the amide III band to the infrared spectroscopy data. A visual inspection of SNV corrected and original spectra (Appendix) underline the presented results.

Since water has no significant influence on the amide spectral regions in the Raman spectra, amide I (1600 – 1700 cm^{-1}), C–C stretching (890 – 1060 cm^{-1}) and amide IV (730 – 745 cm^{-1}) were analyzed separately. Similar trends with regard to changes in the secondary structure conformations were detected (data not shown).

In summary, changes in the secondary structure due to water contents were identified visually and confirmed via MVDA (multivariate data analysis) of the ATR-FTIR and Raman spectra. To complete the picture, the effect of water on the protein powder was further elucidated. On the one hand, water acts as a plasticizer and enhances structural mobility. If a more stable crystalline state exists, an amorphous material can crystallize when sufficient molecular mobility is present.(Shalaev and Zografi, 1996) On the other hand, it forms building units of hydrated crystals. Recently it was shown that crystallinity can be determined via NIR and Raman.(Bai et al., 2004; Beer et al., 2009; Gombás et al., 2003; Hagemam and Snyder, 1989) Therefore, the crystallization state of all freeze-dried fibrinogen bulks was determined via Wide Angle X–Ray Scattering (WAXS). The WAXS spectra confirmed that crystallinity was increasing with the increasing water content. Particles that were stored in lithium chloride (6%) appeared to be amorphous and protein powders equilibrated with potassium nitrate (20%) had a higher crystalline content. However, since it was impossible to calibrate the method with a 100% crystalline sample, no quantification of crystalline content was performed. WAXS crystallinity was calculated as the ratio of the maximum of the diffraction peaks divided by the baseline height. Details, including figures representing the WAXS pattern can be found elsewhere (Wahl et al., n.d.).

Since amorphous powders have no internal crystal structures, they represent a higher energy state (Yu, 2001). Furthermore, molecular rearrangement of the material that lowers the binding energy can occur due to moisture in the sample (Waterman et al., 2012), which explained the peak shifts in lower wavenumbers in the second principal component observed in ATR-FTIR. Based on these observations, the crystallization data were correlated with the infrared spectra (Figure) via projection to latent structures (PLS), which is a commonly used chemometric data analysis tool. The most common form in science and technology is the two-block predictive PLS version that relates two data matrices, X and Y, via a linear multivariate model and goes beyond traditional regression in that it also models the structure of X and Y (Eriksson, L., Kettaneh-Wold, N., Trygg, J., Wols, 2004).

PLS regression generalizes and combines PCA features and multiple regressions to predict or analyze a set of dependent variables based on a set of independent variables or predictors (Abdi, 2007) via modelling of variable correlations arising from a small set of latent variables.

All measured variables are modelled as linear combinations of these latent variables (Eriksson et al., 2006). The first step was to correlate all wavenumbers with the crystallization results and select the most significant wavenumbers for the analysis.

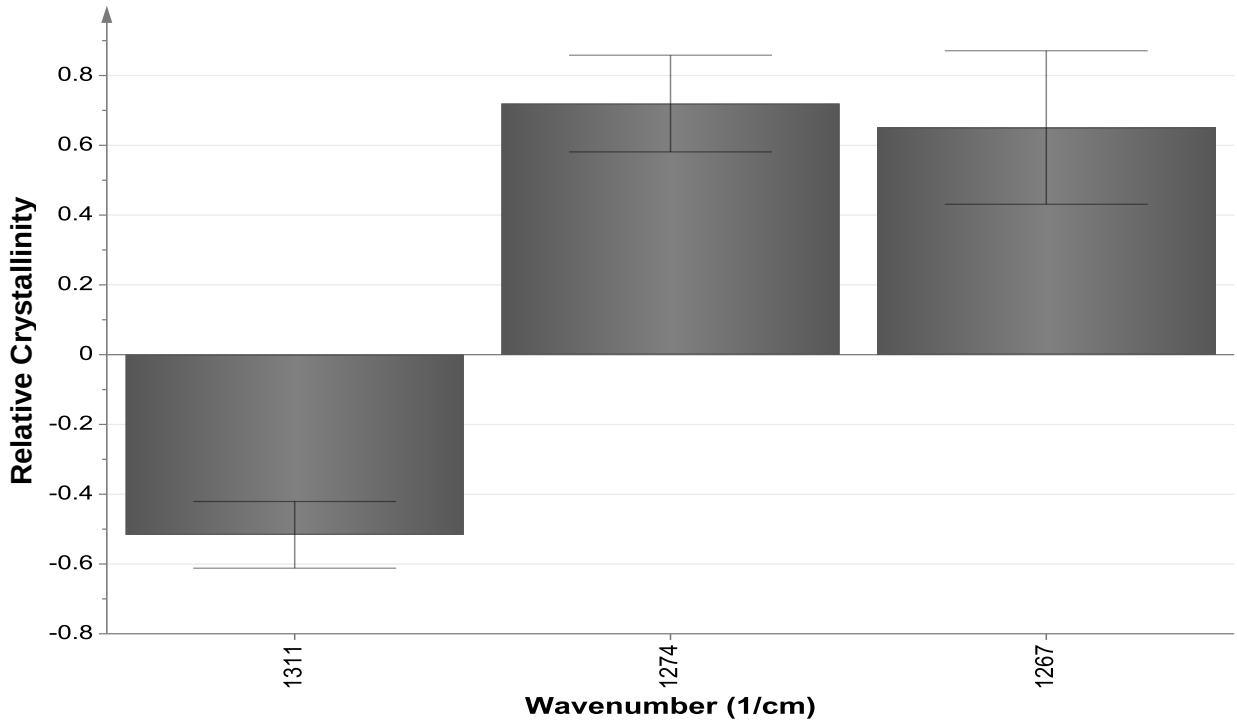


Figure 6: Coefficient plot correlation of the second derivative ATR-FTIR spectra with the crystallization data. First, all wavenumbers of the amide III band were correlated with the crystallization values. Next, the most significant ones were chosen. The correlation is good with R^2 of 0.948.

A correlation of the three selected wavenumbers (1311 cm^{-1} = α – helix; 1274; 1267 cm^{-1} = random coil) with the crystallization outcome was excellent (R = 0.948). On that basis, we created a model to predict the crystallization state of the freeze-dried fibrinogen according to changes in the secondary structure identified via mid-infrared spectroscopy.
Moreover, the amide III band of the Raman spectra was correlated with solid-state data. Again, the most significant wavenumbers were chosen for the model (Figure 7).

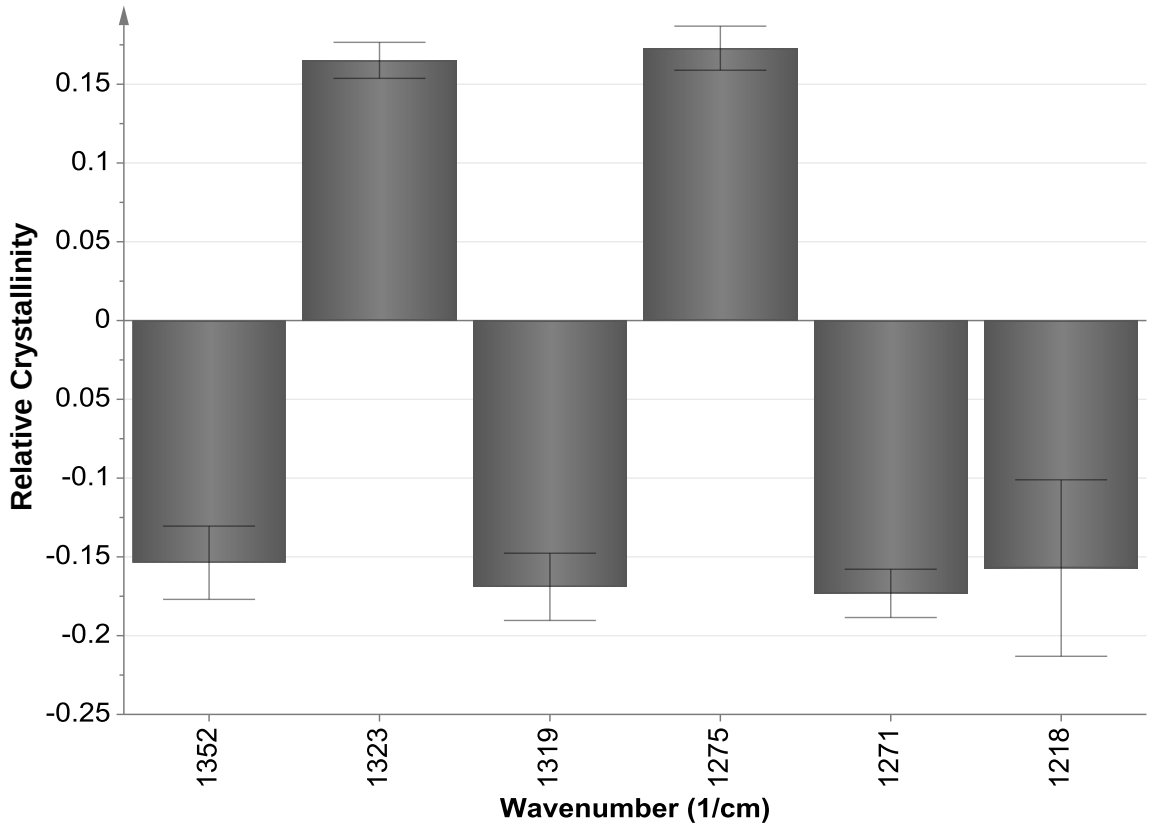


Figure 7: Coefficient plot of the correlation between the chosen second derivative Raman spectra wavenumbers and the crystallization data. First, all wavenumbers of the amide III band were correlated with the crystallization values. Next, the most significant ones were chosen. Using these six wavelengths, R^2 of 0.902 was generated.

Based on the six chosen wavenumbers, a statistical significant correlation (R^2 = 0.902) was generated. Subsequently, hierarchical models with infrared and Raman data were compiled. A hierarchical PCA is a technique that simplifies the interpretation of multiple variables, with each block of variables first summarized by its scores. These scores are collected and combined into a new matrix, consisting of scores and further univariate data (in our case crystallization). The new matrix is then used as input for a new PCA or PLS (Eriksson et al., 2006).

This reduced data set was correlated with the crystallization results via PLS. The generated coefficient plot is shown in Figure 8.

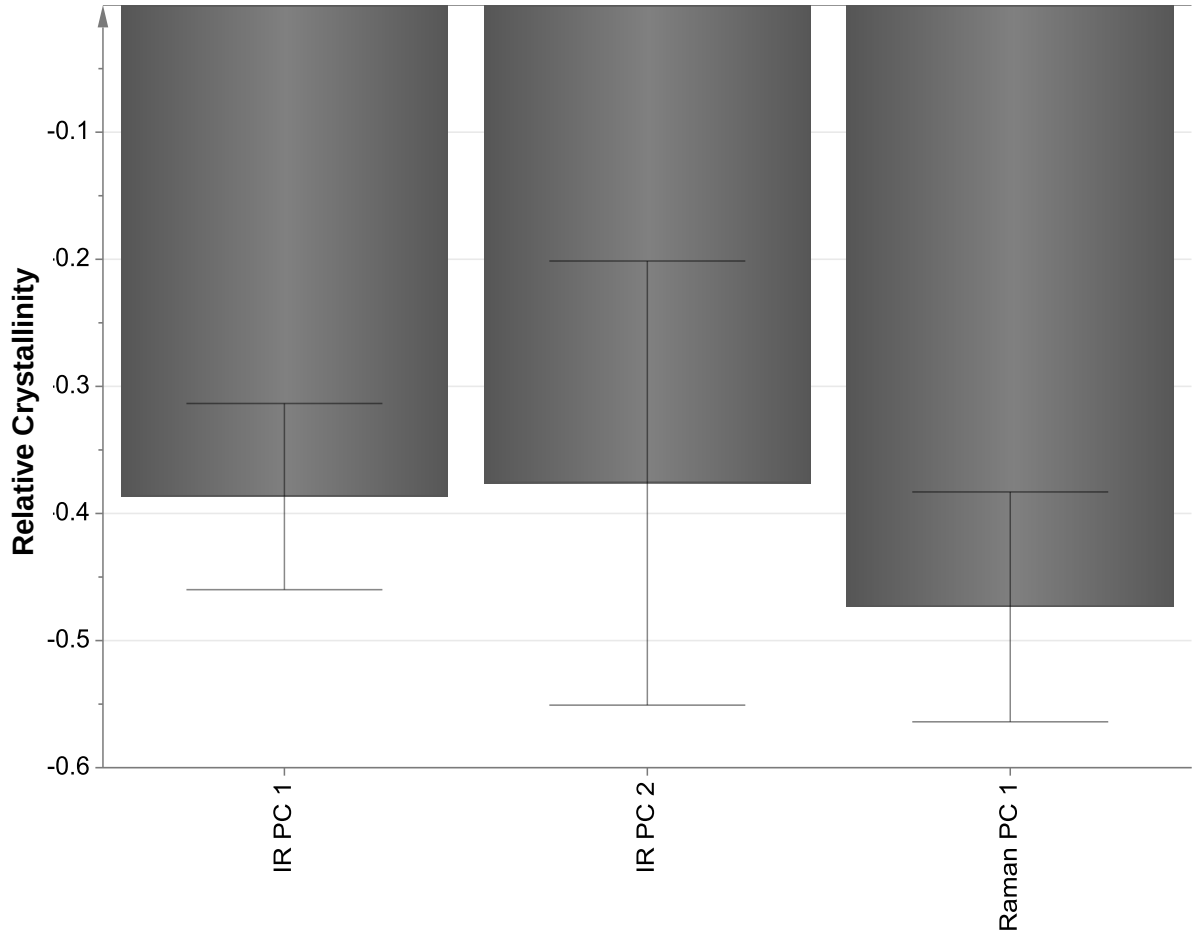


Figure 8: Coefficient plot of the hierarchical model including Raman, infrared and crystallization values. R^2 is statistically significant (R^2=0.953). IR PC 1 indicates the first principal component of the hierarchical data set of the IR spectra while IR PC 2 explains the second principal component. Raman PC 1 index the first principal component of the Raman data.

Significant influence of the first and second principal components of the IR data and of the first principal component of the Raman data on the degree of crystallinity were observed. The R^2 of 0.953 indicates that the crystallization state of fibrinogen can be determined via mid-infrared and Raman measurements. To assess the created model, a cross validation was performed by generating four groups. Each group contains samples with the same water content. According to this cross validation a Q^2 = 0.877 was calculated indicating a predictive model. A cross validation is a model validation technique for assessing how the results of a statistical analysis will generalize to an independent data set.

Based on the hierarchical mid-infrared and Raman data and the crystallization results, an additional PLS analysis was performed correlating the spectral outcome with the water content of freeze-dried powders and a valid model was created (R^2=0.984). The second principal component of the Raman spectra had a significant influence on the model. Since the structural

changes in the protein were due to changes in the water content, we expected to obtain this correlation.

Significant differences in the secondary structure of solid-state fibrinogen generated via ATR-FTIR and Raman spectroscopy correlates well with to the crystallization state of the investigated fibrinogen powder. Since different water content levels lead to different crystallization states, they can be investigated based on changes in the secondary structure of the protein in the amide III band. Control measurements were performed after an incubation time of 4 weeks. No changes in the correlation were obtained.

Such changes in the crystallinity, measured by changes in the secondary structure, can provide knowledge about subsequent process and stability performance. Moreover, stored bulk materials can be easily tested using hand-held devices that can then be used to predict the level of crystallinity and time-dependent changes in the bulk.

4.5 Conclusion

This work demonstrated that exposure of lyophilized human fibrinogen to moisture caused structural changes in the solid state. Depending on the relative humidity value, these structural shifts had the following effects: with an increase in RH, an increase in α–helix, β-turn and random coil and a decrease in β-sheet contributions were observed. Furthermore, WAXS measurements were applied to characterize the crystallinity state of the freeze-dried samples. A good correlation (R^2 = 0.953) was established by combining the mid-infrared and Raman data with the WAXS outcome. Up to our knowledge, such a correlation using freeze dried protein powders – was done for the first time.

Since freeze-dried products are often exposed to varying levels of relative humidity during storage and shipping, changes in their secondary structure can occur. These changes can influence the subsequent processing of freeze-dried proteins, e.g., dissolution behavior and filtration performance. Therefore, in order to control the protein stability, changes in the solid state of freeze-dried proteins and their secondary structure should be analyzed. Using the model proposed in this paper, the related predictions can be made in a non–invasive and fast manner, i.e., via hand-held devices, allowing the monitoring of the quality of stored and shipped material.

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4.7 Appendix

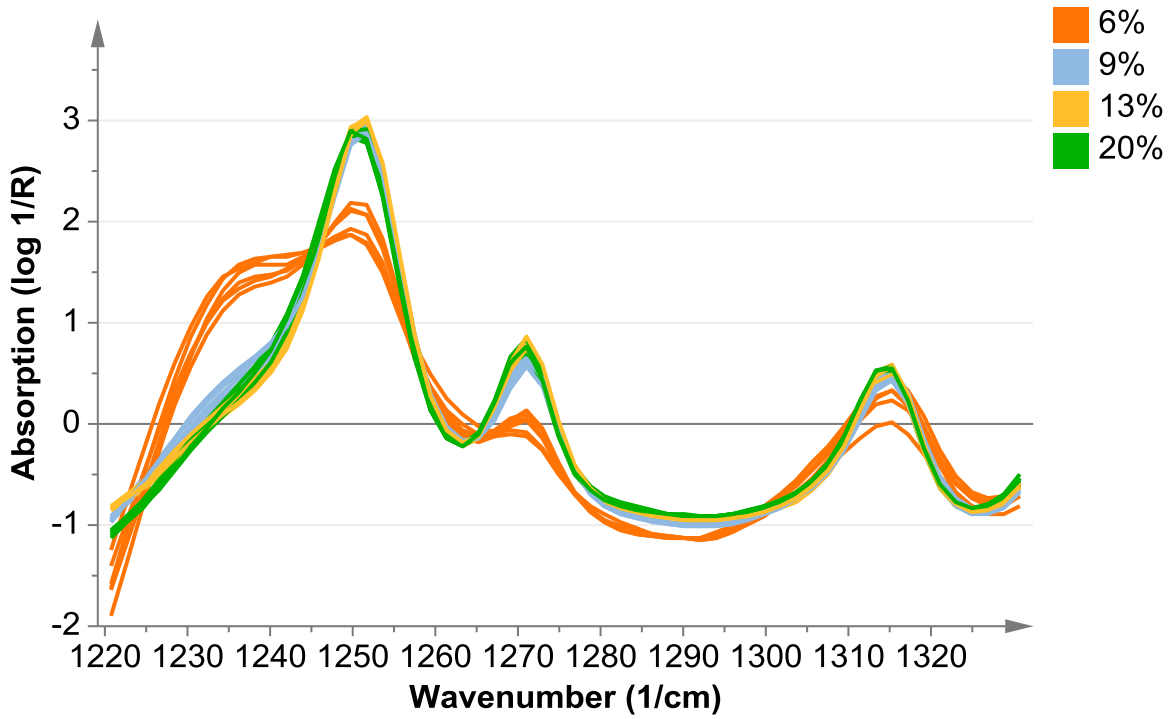


Figure 1: FT-IR-spectra- SNV corrected amide III band

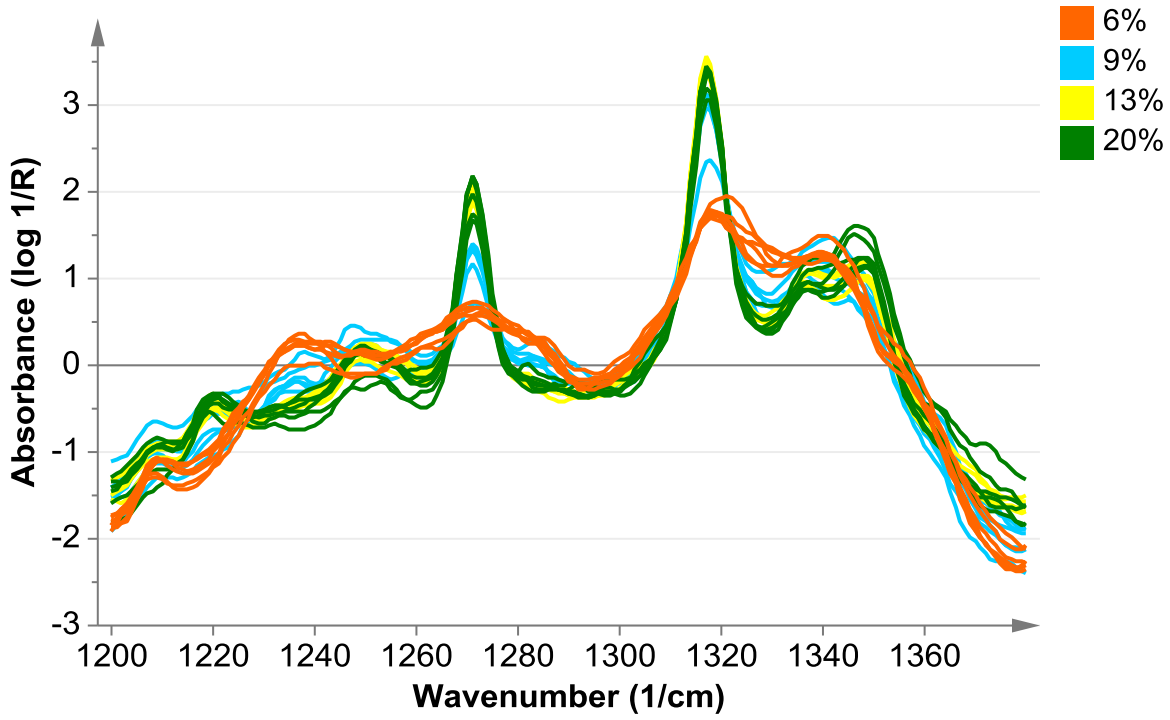


Figure 2: Raman spectra – SNV corrected amide III band

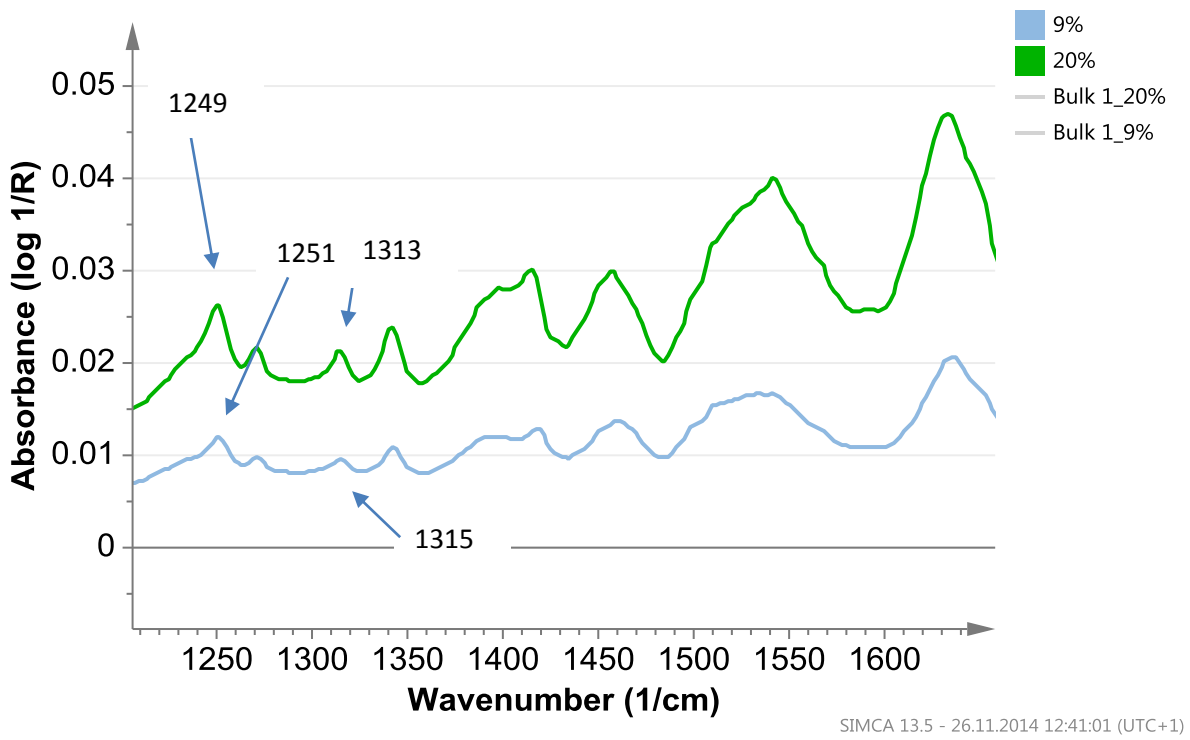
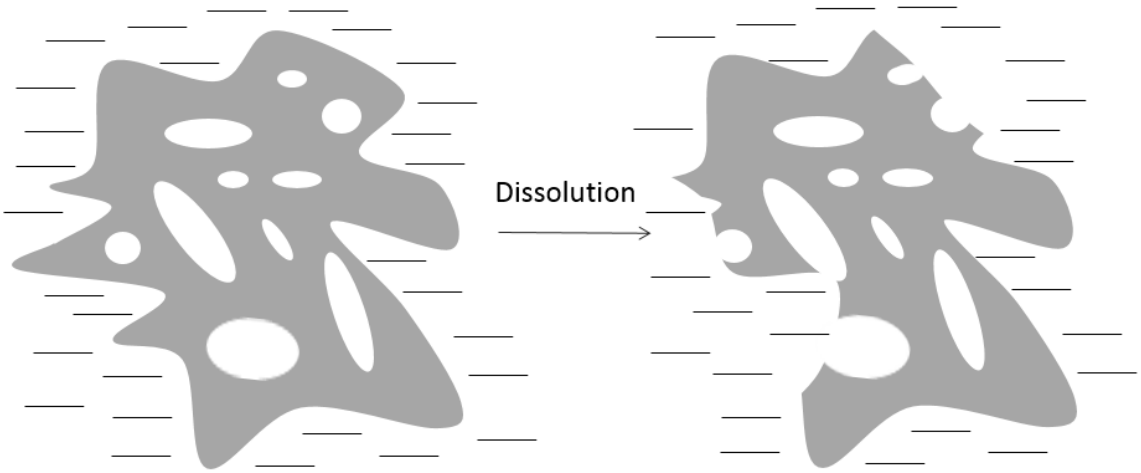


Figure 3: Raw spectra IR – Peak shift

5. Chapter 4: Specific Surface, Crystallinity, and Dissolution of Lyophilized Fibrinogen.- A Study by Combined Small- and Wide-Angle X-Ray Scattering (SWAXS)[3]



[3] This Chapter is taken from a Journal article by Wahl et al. in the European Journal of Pharmaceutics and Biopharmaceutics (2015)

5.1 Abstract

The nano-structural properties of six different batches of lyophilized fibrinogen at various contents of residual humidity (6 – 20%) were studied by small-and wide-angle X-ray scattering (SWAXS) and related to the dissolution properties. As structural parameters, the specific surface and relative degree of crystallinity, from SAXS and WAXS, resp., were used, and correlated to the dissolution rates. BET surface area and electron microscopy were used as ancillary methods. The results show a complex, biphasic behavior: above 9 % water content the crystallinity increased, and the specific surface decreased with increasing water contents; at the lowest water contents (6%), however, where the WAXS patterns showed amorphous structure of the fibrinogens, the specific surface and dissolution rates diverged over a wide range of values. Systematic correlations could be established between specific surface and dissolution rates for the water contents below 13%: the dissolution rates were found to decrease with increasing specific surface, most notably in the amorphous form, in contrast to expectations from classical thermodynamics. Protein conformational changes and hydrophobic surface formation upon depletion of water could be possible causes. This is supported by the protective effect of the high-HLB surfactant PS-80, which was found to enlarge the specific surface.

5.2 Introduction

Recent advances in pharmaceutical biotechnology have led to increasing numbers in protein-based drugs approved for human use or under investigation for clinical safety and efficacy on the market [1,2]. The most commonly used method for preparing solid protein pharmaceuticals is lyophilization, or freeze-drying [3,4], which is a combination of various dynamic and non-equilibrium processes. Besides the advantage of better stability, freeze-dried formulations also provide easy handling during shipping and storage [10]. It can, however, be very challenging to achieve a stable and reproducible final product [3,5,6]. In industrial practice, bulk samples of freeze-dried pharmaceutical proteins are commonly exposed to various levels of humidity during storage and processing [7–9]. Residual moisture content has a significant impact on the solid-state stability of biopharmaceutical products. With increasing moisture, the initially dry and amorphous lyophilisates tend to revert to the thermodynamically more stable crystalline state during storage [10]. The dissolution of crystalline powders is usually slower than that of amorphous ones [10,11]. In addition to the protein quality, one of the most important quality concerns for protein formulators is the reconstitution time, i.e. the rate of dissolution. To reduce immunogenic side-reactions and to avoid potential injection of protein particulates, the protein drug has to be dissolved completely before use [12] and particulate formation needs to be minimized. It has to be ensured that the protein solution can be reconstituted rapidly - minutes - as they often have to be administered quickly, e.g. in an emergency room.

Lot-to-lot variations often raise quality issues during pharmaceutical development [6,13–15]. However, differences between freeze-dried proteins are difficult to characterize as they are often small and indistinct, and not visible crystallographically. Other characterization methods, such as FTIR or Raman spectroscopy would be necessary to investigate such variabilities. Such variations obviously can also affect the dissolution rates of freeze dried protein formulations [13,16], and hence, sensitive analytical methods are necessary to control the quality.

Conventional dissolution testing methods are laborious and time consuming, and it would be of great benefit to developers and producers to find a convenient and reliable technique to predict the rehydration behavior from solid state properties. One obvious key property seems to be the surface area of the solid, as expected from classical thermodynamic laws [17]. The BET technique [18,19] based on gas sorption has become a widely used tool in pharmaceutical solid-state analytics. The need for ‘baking’ the sample, however, limits its practical usefulness, especially with sensitive materials such as protein powders.

In this study, freeze-dried fibrinogen was used a model substance. Fibrinogen is a large, multidomain glycoprotein composed of three nonidentical polypeptide chains (340kDa). It is the principal protein involved in vertebrate blood clotting and therefore, therapeutically used as an adjunct to hemostasis [20].

SAXS (small-angle X-ray scattering) which measures the specific surface rapidly and noninvasively, i.e. irrespective of the presence or absence of adsorbed water, is a promising candidate in this respect. SAXS 'sees' all interfaces between zones of different density, irrespective of whether they are externally accessible or closed pores. With state–of–the–art SAXS instruments the analysis is very fast: significant data sets can be obtained within few minutes. With simultaneous wide-angle diffraction signal detection, as provided by the SWAXS technique [21], the characteristic powder diffraction peaks from crystalline contents can be can be measured. Thus, in combination with SAXS information on both the crystalline and the amorphous parts are provided [10,11,22]. Recently, a novel application of SWAXS in the assessment of aspirin and lactose content in a binary pharmaceutical powder formulation was published [23]. Further, the effect of process variables on SWAXS patterns of powders, granules and pharmaceutical tablets was investigated [24].

The present study has been focused on the exploration of this technique as an analytical tool in the screening of dissolution of lyophilized fibrinogen formulations with different degrees of residual water.

5.3 Materials and Methods

Samples

Six lots of freeze-dried fibrinogen powders of production grade (courtesy of Baxter AG, Vienna, Austria) were analyzed as reported elsewhere [25]. To investigate the influence of moisture on solid-state stability of fibrinogen, the freeze dried powders were exposed to accelerated storage conditions of different controlled relative humidity (RH), by vapor equilibration with saturated solutions of the following salts: LiCl, $Mg(NO_3)_2*6H_2O$, NaCl, and KNO_3 , respectively, corresponding to 14% RH (LiCl), 52% ($Mg(NO_3)_2*6H_2O$), 75% RH (NaCl) and 93% RH (KNO_3), resp. [26]. After two weeks of incubation, the lyophilized powder samples the residual moisture of the samples was determined by Karl Fischer titration (Titroline 7500 KF, SI Analytics, Mainz, Germany) as previously described [25]. The residual moisture values are summarized in Table 1. In addition to residual moisture, the samples contained small amounts (2-3%) of Polysorbate 80 (Tween 80) (HLB-value = 15.0) and tnBP (tri-teritiary butylphosphate).

Table 1: Residual moisture in the fibrinogen samples 'Bulk 1-6' used for this study (modified from [25]*)*

	% H20					
Equilibration medium	**Bulk 1**	**Bulk 2**	**Bulk 3**	**Bulk 4**	**Bulk 5**	**Bulk 6**
LiCl	6.3 ± 0.4	6.2 ± 0.1	6.2 ± 0.1	6.0 ± 0.4	5.8 ± 0.4	5.2 ± 0.8
$Mg(NO_3)_2*6H_2O$	8.6 ± 0.4	9.5 ± 0.3	9.6 ± 0.2	9.0 ± 0.2	8.8 ± 0.5	9.0 ± 0.1
NaCl	12.6 ± 0.2	13.2 ± 0.5	14.4± 0.2	12.4 ± 0.2	12.9 ± 0.6	13.3 ± 0.9
KNO_3	19.8 ± 1	20.5 ± 2	21.1 ± 2	19.02 ± 1.6	19.0 ± 1.9	20.6 ± 0.6

Dissolution and UV spectrophotometry

For testing the dissolution behavior of the freeze dried protein powders a flow-through cell PT – DZ 1 (Pharmatest, Hainburg, Germany) was used, as previously described [25]. Aliquots of approximately 1.2 g of the powders were dissolved in a well-defined dissolution medium (water, human serum albumin and niacin amid) and the UV absorption at 280 nm was used as a representative value for the amount of dissolved protein and followed over time. The total measuring time per sample was 60 min.

SWAXS

Simultaneous small-and wide-angle X-ray scattering (SWAXS) measurements were performed in a point-focussing camera system (S3-MICRO, formerly Hecus X-Ray Systems Graz, now Bruker AXS, Karlsruhe) equipped with two linear position sensitive detectors to cover the real space resolution ranges of 10-1500 Å (SAXS) and 3.3–4.9 Å (WAXS). Samples were rapidly transferred from the equilibration atmosphere to sealed X-ray capillaries, and the SWAXS patterns were measured under constant capillary rotation (Hecus SpinCap 0.5 Hz) to avoid preferred orientation or texture artifacts. SWAXS measurement times were generally 600s. All X-ray data have been normalized to the same transmission, i.e. scattering mass. For the evaluation of SAXS data, see Appendix.

BET Surface Area

The BET specific surface of the bulk powder was measured by using the Micromeritics Tristar II 3020 (Norcross,USA). The samples were degassed for two days at 30°C at the Micromeritics VacPrep 061 degas unit (Norcross,USA). The measurements were performed using Krypton gas as the BET surface of the powder is around 1 m^2/g. Brunauer-Emmett- Teller (BET) adsorption theory was used to calculate the specific surface areas, with a pressure range of 0.05–0.30 normalized to the saturation pressure of the adsorbate [18].

Environmental Scanning Electron Microscopy (ESEM)

Scanning electron microscopy (SEM) images were recorded using an ESEM FEI Quanta 600 FEG (Hillsboro, Oregon) environmental scanning electron microscope in high – vacuum mode (HV 5.0kV) equipped with a Large Field Detector (LFD). Using ESEM, wet samples can be investigated in their “natural” state.

5.4 Results

Nanostructure at Different Water Contents

Representative SAXS curves are shown in Figure 1. The main scattering power is observed at q<0.1 $Å^{-1}$ corresponding to real-space distances of >60 Å (>6 nm), which is also the characteristic size range of the density fluctuations, pores, water pockets etc.

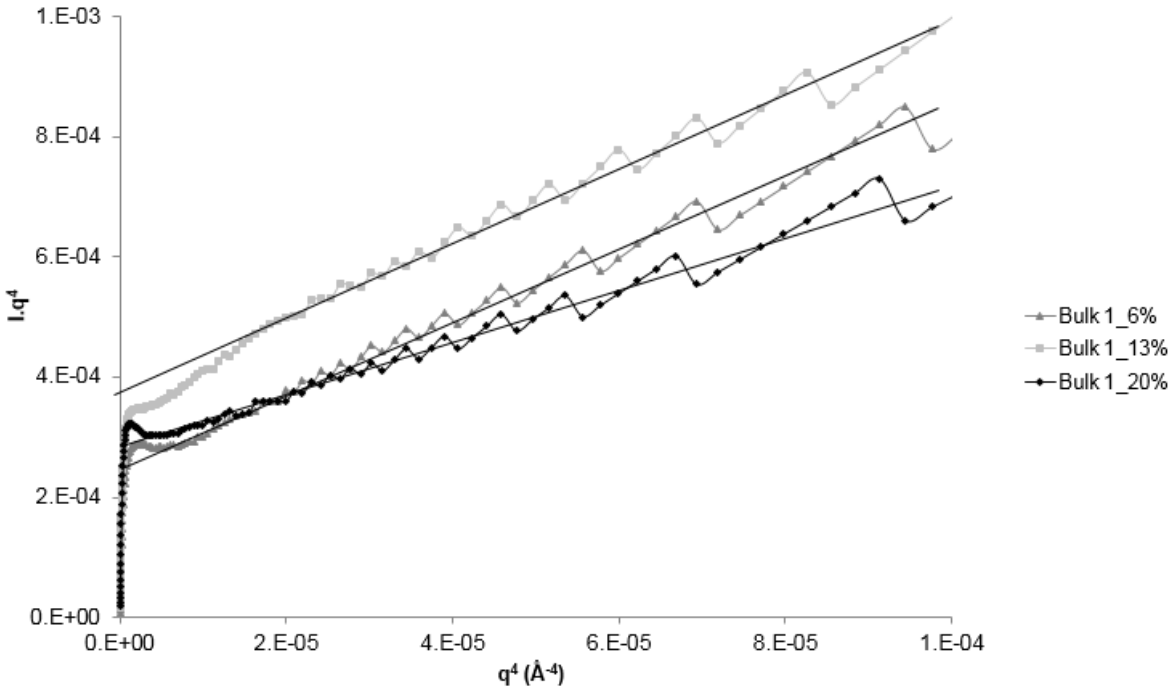


Figure 1: Typical SAXS Porod plots (Iq^4 vs q^4) for three selected samples (Bulk 1 6%, 13%, 20% water); data normalized for scattering mass. The intersections at q=0 indicate the different surfaces. Exposure time was 300 s.

The WAXS patterns show strong variations in crystallinity (Figure 2). At the lowest water contents, no discrete Bragg peaks could be observed and hence, the systems are crystallographically amorphous. With increasing water content, there appear three sharp peaks at 2Θ of 19°, 21.3° and 24°, resp. As there is no reference record of fully-crystallized fibrinogen powder diffraction available, it is not possible to calibrate for the true percentage of crystallinity. As an operative parameter, therefore, a crystalline/amorphous ratio (crystallinity) was determined from the signal-to-background ratio, signal/(signal-background), of the strongest peak at 21.3° relative to the continuous background between this and the 24°-peak, indicated Fig.2. These WAXS reflections do not correspond to any known signals from additives (Tween 80, tri-teritiary butylphosphate) leaving the protein as only possible origin, although no reference data on crystalline fibrinogen powder exist to positively confirm the protein origin of the signals.

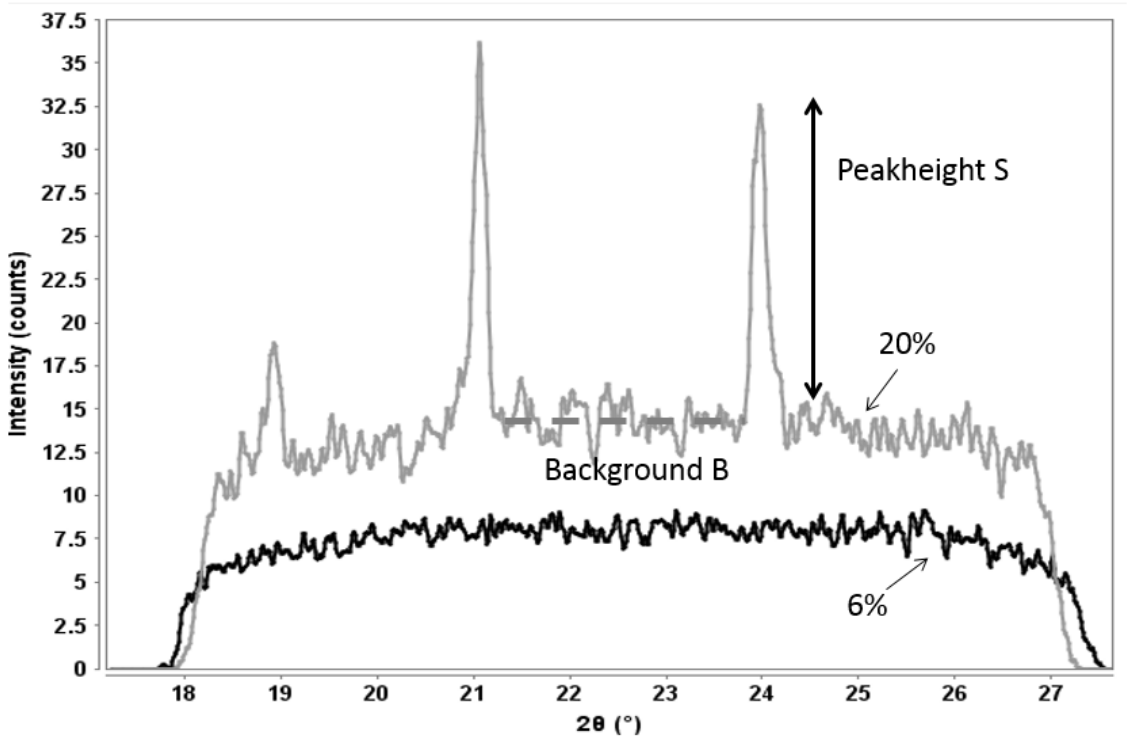


Figure 2: Typical WAXS patterns of the fibrinogen lyophilisate. The arrows indicate the measurement of the crystalline/amorphous ratio S/(S-B). For better visibility the two curves are differently magnified.

The parametric results of SAXS and WAXS as functions of water content are shown in Fig 3. The S_i values from SAXS were found to fall in the range of 140-280 m^2/g. This is dramatically higher than the BET values (see Fig.6) which were found to be around 1 m^2/g, in good agreement with the findings by Hageman [7],. With several lyophilized proteins Hageman observed that the N_2 sorption areas, typically between 1 and 6 m^2/g, were substantially below the H_2O vapor sorption areas, which were in the range of ~ 200 m^2/g. Such large differences between BET and SAXS are not a general phenomenon, since earlier studies on inorganic/ceramic materials have shown much better agreement [27]. It seems that lyophilized materials, especially proteins, are sensitive to “baking”.

The SAXS specific surface was found to decrease with increasing water content to about 60% of the maximum values. Correspondingly, the average chord length (Fig. 3b) increases from around 60 to 80 Å, indicating that there are on average larger domains of homogeneous medium, both protein and voids.

A strong deviation from this trend occurs at the lowest water contents, where the samples are crystallographically amorphous. There, the specific surface values scatter over the range of 170-270 m^2/g, and the average chord length from 45 to 75 Å, far beyond any cumulative error range of the different preparations and analytical methods. Hence the results show a qualitative structural change at the limit of dehydration and full amorphicity.

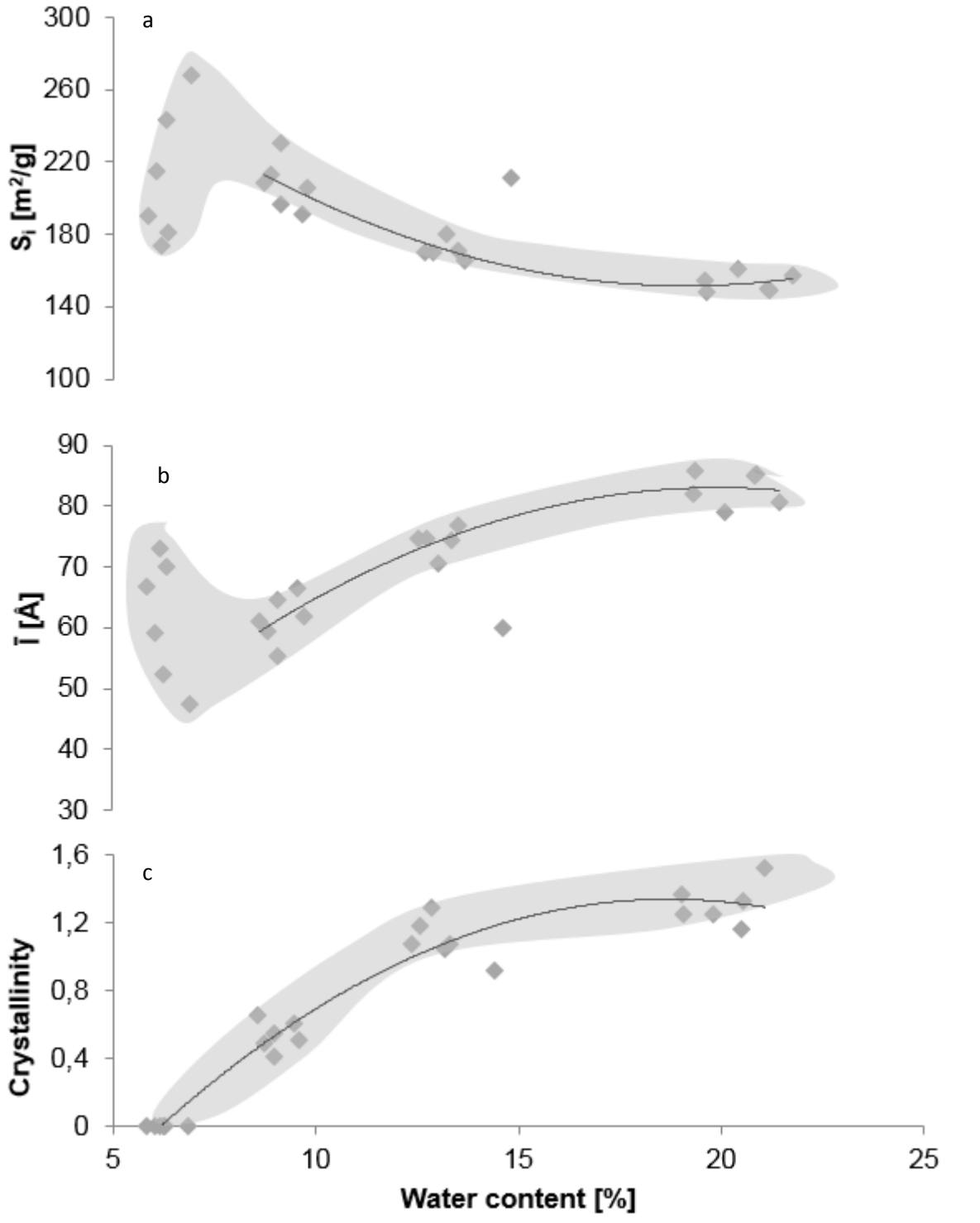


Figure 3: (a) Specific surface S_i, (b) average chord length and (c) crystallinity, as functions of water content

In parallel to the decrease in specific surface at higher water contents, a systematic increase in crystallinity is observed from the WAXS patterns (Fig. 3c). This is also clearly shown also by the correlation-plot of specific surface and crystallinity (Fig. 4) indicating a reciprocity between S_i and crystallinity. Similar observations, increasing crystallinity with growing water contents were

reported elsewhere [28]. However, at the limit of zero crystallinity, i.e. in the amorphous form, the S_i values diverge strongly for the different samples.

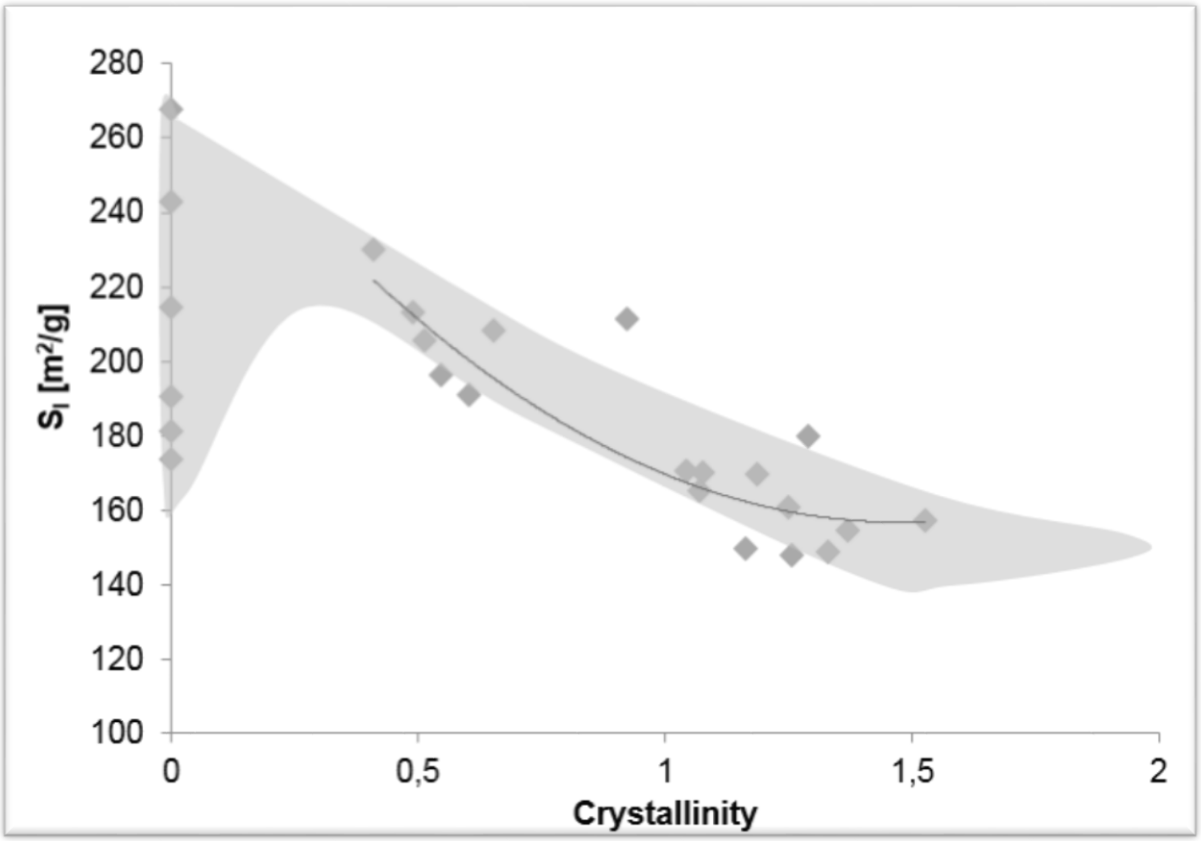


Figure 4: Correlation plot of specific surface and crystallinity.

To visualize the difference in morphology of different lyophilized fibrinogen powders, ESEM (environmental scanning electron microscopy) images were taken. As representative samples, pictures from the extreme values, with water contents of 6% and 20%, respectively, were chosen (Fig. 5).

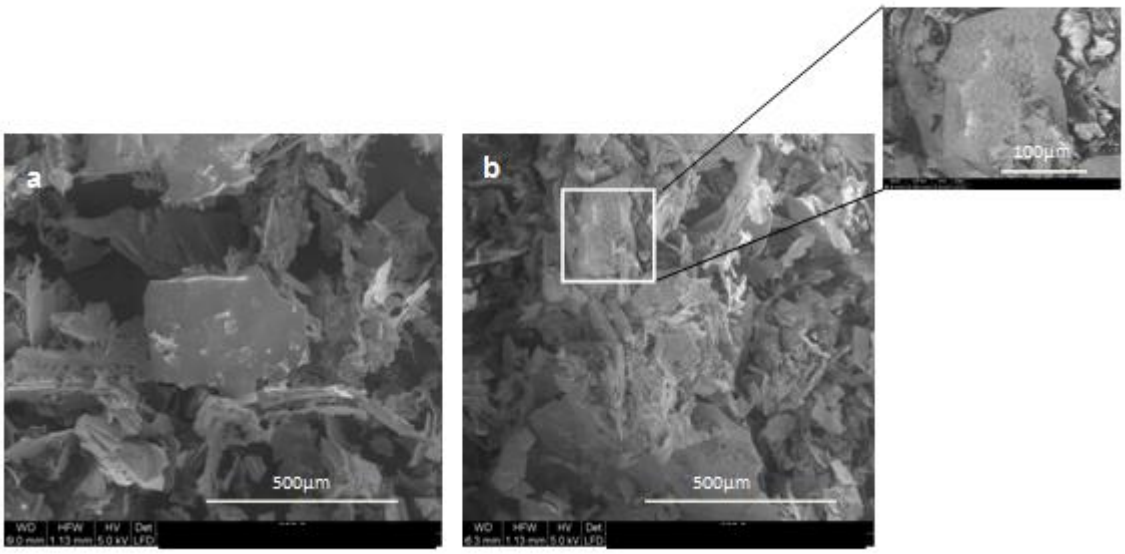


Figure 5: ESEM images of bulk 1: a) 6% water content b) 20% water content. The fibrinogen "flakes" of the 6% sample has a smooth surface area while the 20% water content sample reveals crystals on the powder surface.

The ESEM images show that the particles with lower water content have a relatively smooth surface. At higher residual moistures (20%), crystals can be detected at the surface of the protein "flakes". Note, that the observation window of these ESEM pictures is in the µm-mm range, while that of SAXS and WAXS is in the nm-range, i.e. by 3-6 orders of magnitude smaller. It is interesting to note, that BET measurements performed on the same set of samples showed a generally similar trend, i.e. decreasing BET surface with increasing humidity, but at about hundred-fold lower surface values, around 1 m^2/g (Fig.6). A possible reason for this discrepancy could be that SAXS measures both, the open and closed pores, while BET only captures the open ones. Perhaps even more important seems to be the prolonged (48 hours at 30°C) degassing in vacuum necessary for BET, which is likely to lead to collapse of fluffy, freeze dried materials, as previously documented Schersch et al. [29–31] and literature therein.

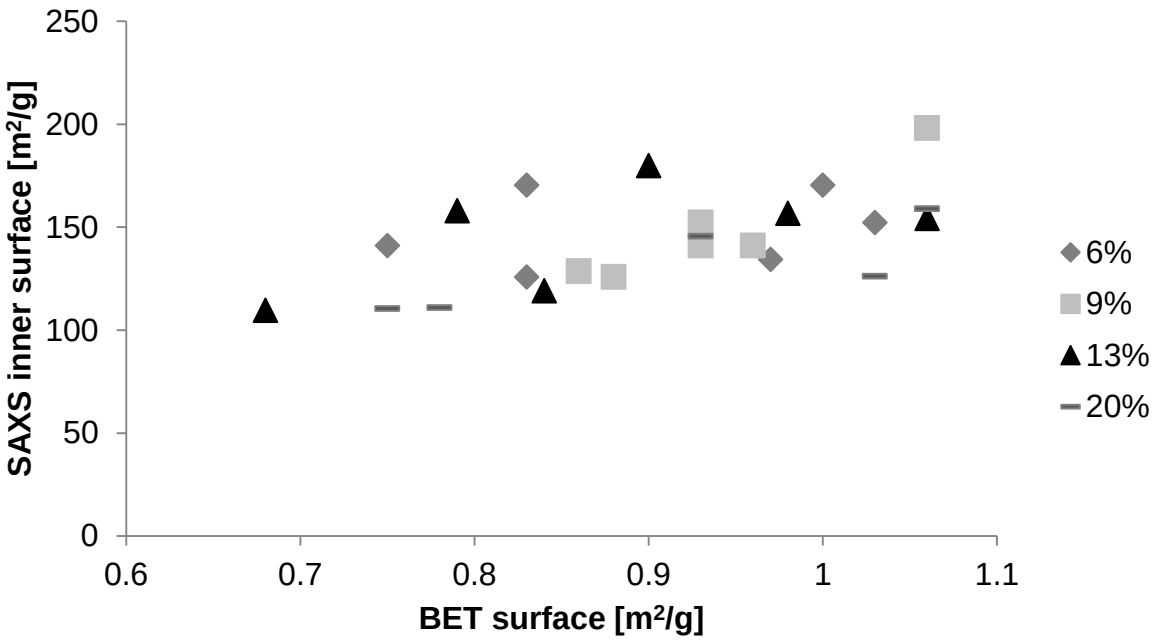


Figure 6: Juxtaposition of SAXS specific surface and BET surface for all samples studied. Original water contents are indicated by the different symbols

Dissolution Rate and Structure

The dependencies of dissolution rate on crystallinity on the one hand, and on specific surface on the other, are shown in Fig 7. A decrease of dissolution rate is observed as crystallinity increases, in agreement with the higher thermodynamic stability of the crystalline state. However, this behavior diverges at the limit of zero-crystallinity, i.e. amorphicity, where also the specific surface results also show anomalous scatter. For the dissolution rates, crystallinities and specific surface values, resp., compiled for all samples and hydrations, the scatter is too wide as to allow any statistically significant correlation (Fig 7b).

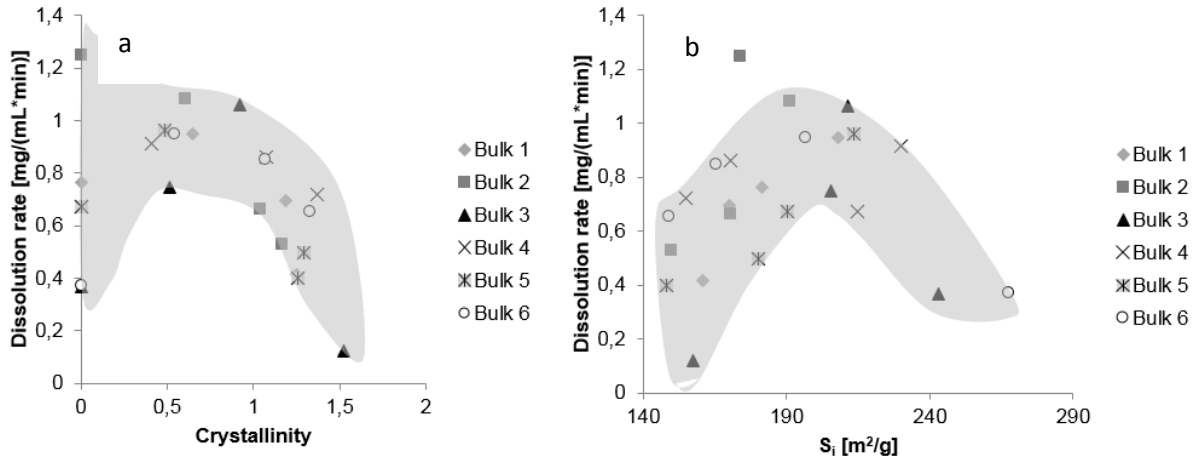


Figure 7: Dependence of dissolution rate on crystallinity (a), and on specific surface S_i (b), compiled for all different sample lots (Bulk 1-6) and all water contents.

Clear correlations, however, become apparent if the data from individual water contents are inspected (Figure 8). This disentanglement of the data reveals the following: for the amorphous form, at the lowest water content (6%), the dissolution rates decrease with growing specific surface over a wide range between 170 and 270 m^2/g. A similar behavior was also found at 9% and 13% water content, resp., however, within narrower ranges of specific surface. The inserts to Figure 8 show, that these variations are correlated to the relative amount of PS-80 present in the samples.

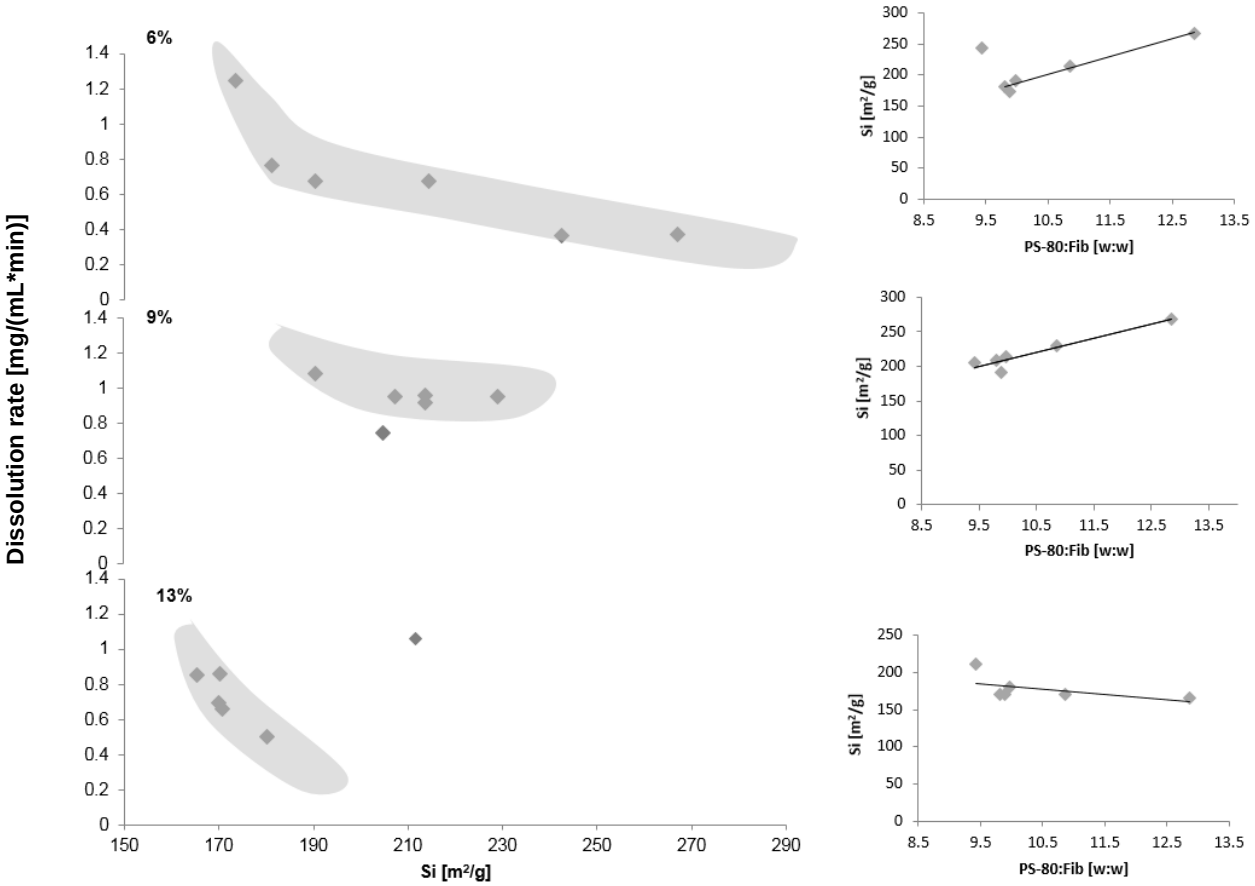


Figure 8: Dependence of dissolution rate on specific surface S_i at 6%, 9% and 13 % water content, respectively. Insert: Specific surface as a function of PS-80 / fibrinogen molar ratio.

5.5 Discussion

The primary objective of this study was to relate the nanostructure of lyophilized fibrinogen preparations, as revealed by SAXS, to their dissolution behavior. Twenty-four samples at six different residual water contents were analyzed by combined SWAXS to screen for specific specific surface and crystallinity, from SAXS from WAXS, respectively. In a previous study [25]

the solid-state properties of freeze-dried fibrinogen have been extensively studied by various techniques, thus, providing a solid foundation for the present work. The starting assumption was that the specific surface as readily determined by SAXS can serve as a critical parameter, since dissolution is thought to be strongly influenced by the surface properties.

The specific surface, with a maximum around 9%, was found to decrease systematically at higher water contents and with crystallinity. This can be understood on the basis of a compaction or sintering of the crystalline domains (analogous to the behavior of dry and wet snow flakes) or by a condensation of the system when stored at higher relative humidities. Since the crystallized form is more stable than the amorphous one, the dissolution rates are also expected to decrease. With water contents above 9%, the observed dissolution rates follow this behavior. Approaching the amorphous form, however, this classical model turns out to fail.

The most striking observation was the unsystematic scatter of the specific surface values in the ‘X-ray amorphous’ state (6% water content), paired with the systematic, *inverse* correlation between specific surface and dissolution rate. This scatter is far beyond any possible accumulation of systematic errors in preparation and analysis, especially, in view of the fact that it does not occur to a comparable degree with the same samples at higher water contents and crystallinities. This behaviour is counterintuitive and cannot be explained on a classical thermodynamic basis (Noyes-Whitney [17]) whereby an increase in surface area would primarily drive dissolution. To rationalize these observations, we first consider the possible reasons for the anomalous scatter of the specific surface of the different samples in the amorphous form. It is tempting to speculate that hitherto undefined structural events occur during the steps of amorphization, which remain kinetically locked in the dehydrated state. This is tantamount to the notion that the amorphous form does not have one defined, invariant structure, but can vary in its degree of compactness, which is correlated to functional properties, in this case to dissolution. One likely strong variable seems to be the detergent-to-protein ratio as demonstrated in figure 8.

As the amorphous form is thermodynamically unstable and therefore, has the tendency to move towards equilibrium, a higher molecular mobility can be expected [32,33]. Molecular mobility involves translational and rotational motions of entire molecules, or segments of the protein [34,35]. Thus, it can be presumed that in the amorphous form conformational transitions of the protein occur and lead to specific surface variations.

A possible reason for the inverse correlation between specific surface and dissolution rate, as observed especially in the amorphous form, might be a hydophobization through protein

conformational changes[4]. Depleting the protein of the minimal water layer that stabilizes the 'native' conformation may lead to partial or complete unfolding, thus, increasing the specific surface and at the same time leading to an entropically hindered approach of water to hydrophobic surfaces, which in turn slows down dissolution. The observation of a 'protective' effect of the detergent PS-80, which was found to enlarge the specific surface, also points in this direction, as this additive with a high HLB-number (hydrophilic-lipophilic balance) of 15 is expected to counterbalance the effects of water depletion.

5.6 Conclusion

The variations in dissolution properties of amorphous protein powders prepared by lyophilisation and stored under different conditions, in particular humidity, can be attributed to the specific surface at the nano-scale such that higher specific surface leads to decreased dissolution rates. The true reasons for this seemingly anomalous reciprocity are still unclear and certainly need further investigation. In the technologically most interesting amorphous form at lowest residual moisture, however, our present results show, that dissolution rates as well as specific surface can vary widely. It appears also to be a general trend that dissolution rates decrease with increasing crystallinity.The most important result of this study is that SAXS presents a practical tool to characterize the compactness and density fluctuations in the amorphous form by the specific surface at the nano-scale. SAXS is, therefore, an essential tool to characterize such systems complementary tool to X-ray powder diffraction. This is of great practical importance as no other method, measurement of water content, nor crystallographic analysis, is sufficient in this situation. From the correlations between S_i and dissolution rates for different batches in the amorphous form, dissolution properties can be positively predicted from SAXS, without the need for lengthy dissolution tests.

The present findings also suggest that amorphous preparations of fibrinogen can attain different stabilities and nano-structural states as a consequence variations in protein folding between the different bulk powders, depending e.g. on variations in the production processes [36].

[4] a paper on: „Changes in secondary structure of freeze dried fibrinogen due to changes in water content" is in preparation

5.7 Appendix

The Concept of Specific Surface Determination by SAXS

SAXS is sensitive to (electron) density contrast within the sample at the nano-scale, commonly between 1 and 100 nm (10-1000 Å). In random two-phase systems, SAXS analysis allows to infer important quantities relating to the inner structure of the particles within the powder. In the following, a brief summary of SAXS shall be given, as this is not general knowledge and important to understand the present results.

The integral intensity, the so-called 'Invariant' Q,

$$Invariant\ Q \equiv \int_0^\infty I(q)q^2 \cdot dq = V.(\Delta\rho)^2 \cdot \varphi_1\varphi_2 \cdot 2\pi^2 \qquad Eq.1$$

where I(q) is the recorded scattering intensity, and q is the angular argument $q = 4\pi\frac{sin\theta}{\lambda}$, with λ…wavelength, 2$\theta$…scattering angle. V is the total scattering volume of the sample, $(\Delta\rho)^2$ the squared electron density contrast, $\varphi_1\varphi_2$ the product of the volume fractions of solid and void, respectively. In the present case, the overwhelming contribution to the contrast comes from electron density differences between the protein powder and air, so that internal variations e.g. between crystalline and amorphous, or hydrated vs. unhydrated domains, become negligible.

The second important value measured from the scattering curve is the intensity decay, measured by the decay coefficient, according to Porod's law [37]

$$\lim_{q\to\infty} I(q) = S \cdot (\Delta\rho)^2 \cdot \frac{2\pi}{q^4} \qquad Eq.2$$

which is proportional to the total system surface. In practice, the scattering data are fitted by $I(q) = K_1 + K_2/q^4$, where K_1 is a constant background. This equation is used to extrapolate to $q\to\infty$ in equation 1 from the 'final slope region', where the q^{-4} - law is found to hold.

Combining equations 1 and 2 leads to the specific surface. The ratio between the decay coefficient K_2 and the integral scattering power Q is proportional to the specific surface S_i, since

$$\frac{S}{V} = Si = \frac{K_2}{Q} \cdot \varphi_1\varphi_2 \cdot \pi \cdot \bar{v} \qquad Eq.3$$

For the present purpose, the term $\varphi_1\varphi_2 \cdot \pi \cdot \bar{v}$ (with the specific volume $\bar{v}$ = 0.74 cm^3/g for fibrinogen) is set to unity, hence the specific surface is calculated as $S_i = 10^4 . K_2/Q$, in m^2/g.

A simple geometric argument shows, that the average chord length $\bar{l}$ can be obtained from the reciprocal specific surface, according to $\bar{l} = Q/K_2 \cdot 4/\pi$. This is the average chord length

through solid and pore space, resp., in any direction throughout the particle (Fig. 9b). No knowledge of the volume fraction of pores is required for the determination of $\bar{l}$. These theoretical principles are summarized in Figure 9.

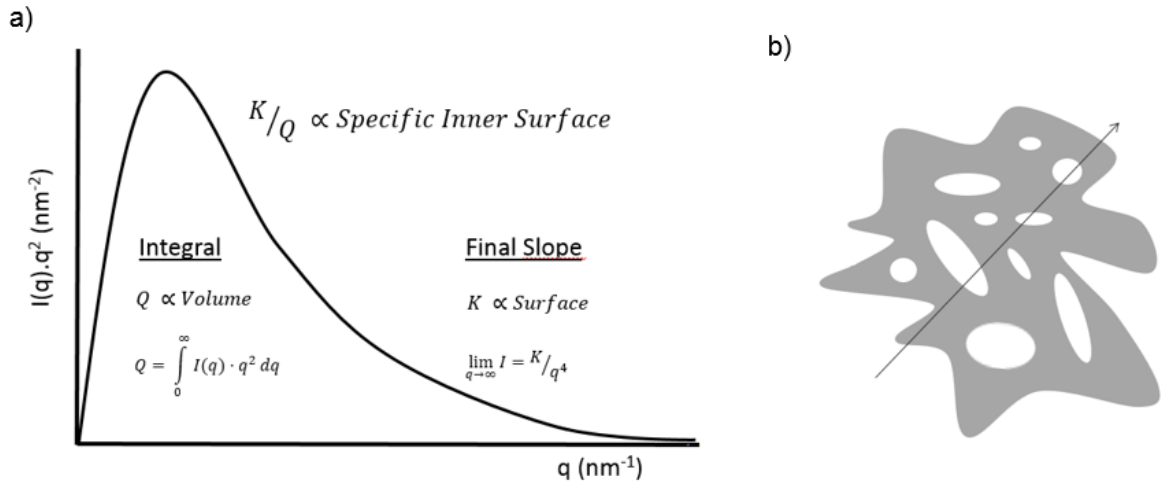


Figure 9: (a) graphical scheme of the determination of specific surface S/V by SAXS according to Porod's law; (b) scheme of the chord distribution. As the system is isotropic and all particle orientations are representated, the averages are taken over all directons indicated by $\langle 1/\bar{l} \rangle = 1/l_1 + 1/l_2$. *Chord lengths in the two phases are* l_1 *and* l_2, *respectively, the total average chord length is* $\bar{l}$.

The two parameters derived from SAXS analysis on powders, specific surface and average chord length, resp., are assumed to be key determinants for the kinetics of dissolution. The surface controls wetting and solvent attack, and the average chord length between adjacent surfaces measures the degree of dispersion, hence the accessibility. In the process of dissolution, initially closed pores are opened and new area is presented to the solvent. A graphical model of this scheme is given in Figure 10. In order to avoid confusion, it has to be emphasized, that the term 'inner' surface, originating from liquid matter characterization, includes both, the inner pores within the powder particles and the outer surface.

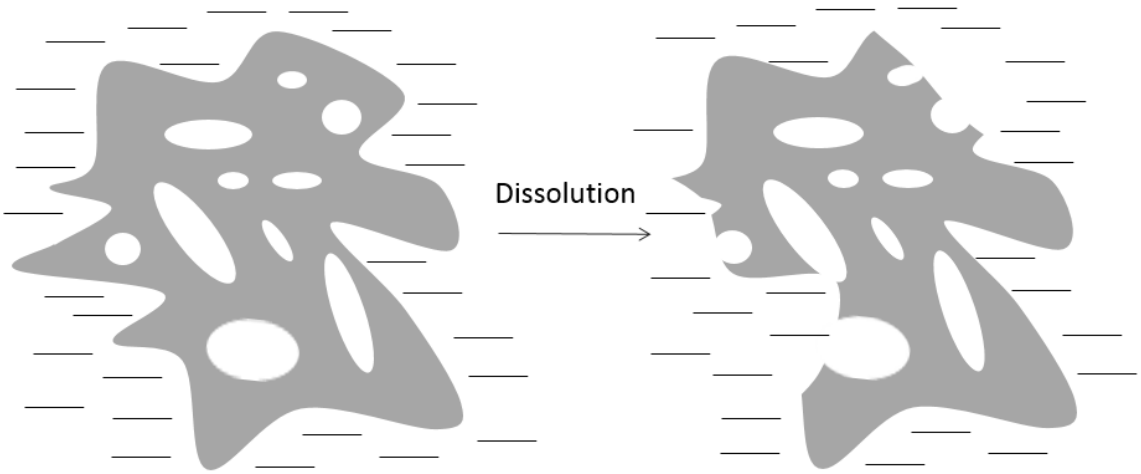
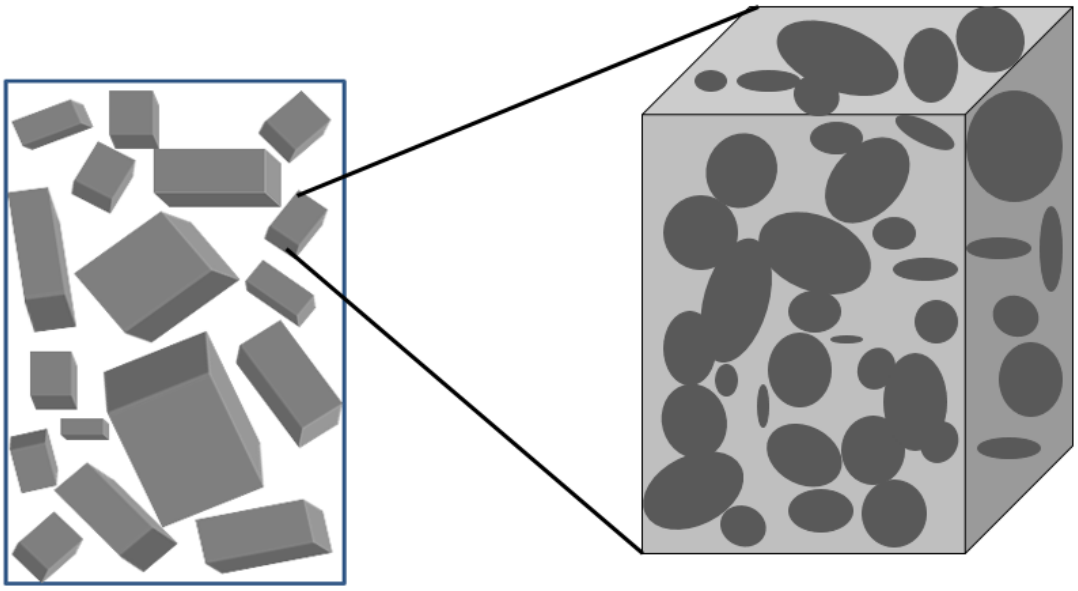


Figure 10: Model of a porous particle before and during the process of dissolution: initially closed pores become accessible, thus offering new area for solvent attack

Comparison of BET and SAXS Surface, resp.

It is necessary to emphasize the difference between outer surface as determined e.g. by BET, and specific surface as measured by SAXS. The outer surface is the envelope surface of the particles delimiting their space occupied mechanically, as measured by optical particle sizing. For spheres or polyhedral geometric bodies there are simple scaling laws relating the specific surface to the particle size, e.g. for spheres it holds that $S/m = 6/(r.\Phi.\rho)$, where r is the radius, Φ is the packing volume fraction, and ρ is the solid matrix density). A model calculation for random, close packing of spheres (where $\Phi \sim 0.65$), as shown in Figure 11, demonstrates that in typical powders of particle sizes in the range of 1-100 µm the specific outer surface is in the range between 0.1 and 10 m^2/g, in agreement to what is generally found by BET.

(a)



Outer Surface
Particle Envellope
(Size Range µm – mm)

Inner Surface
Pores – Cavities
(Size Range nm - µm)

(b)

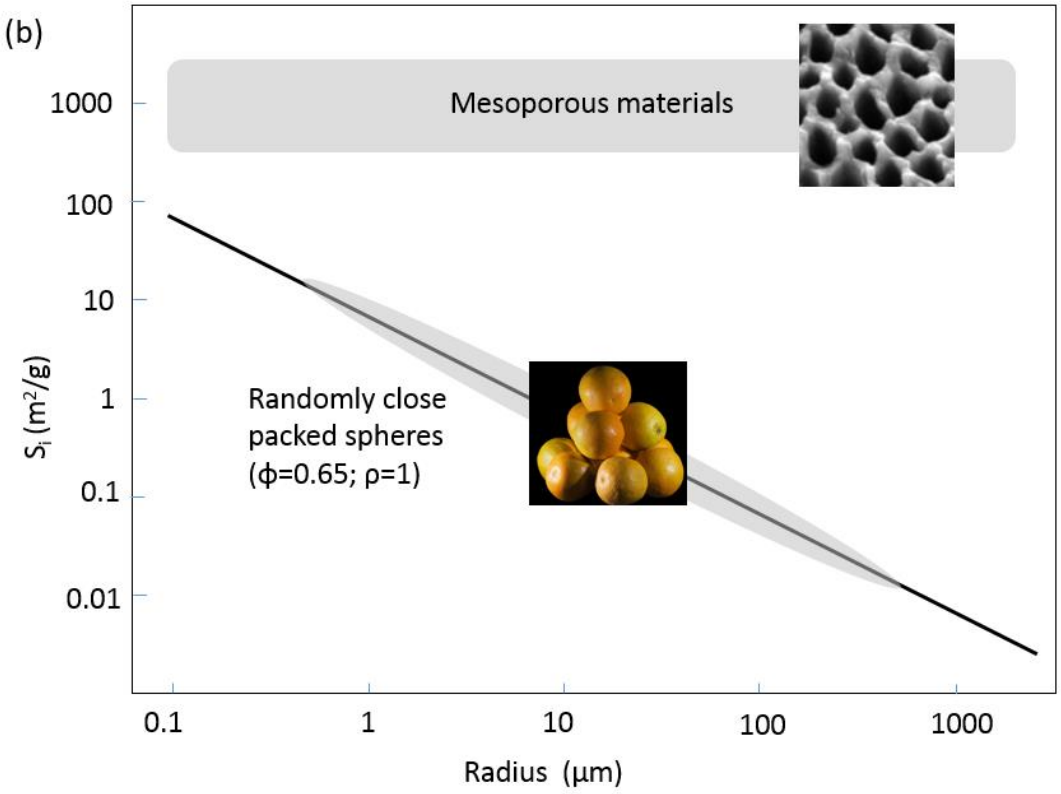


Figure 11: (a) Scheme of outer and specific surface; (b) specific outer surface calculated for randomly close packed spheres with a solid volume fraction Φ of 0.65 and a matrix density ρ of 1; the hatched area indicates the typical range for the specific surface of mesoporous materials.

The specific surface measured by SAXS refers to the outer particle surface *plus* pores, cavities and fractures within the particles. The typical pore dimensions are a fraction of the envelope size, i.e. in the nm to µm range. Hence, for a sponge-like model the specific surface can be by orders of magnitude larger than the outer surface, often reaching several hundreds of m^2/g.
The advantage of SAXS over other methods for surface determination is that no sample pretreatment, like drying or "baking" is required. Hence, hydrated, dispersed or even reacting or decomposing samples can be investigated. This is important when it comes to the measurement of solids composed of temperature- and humidity-sensitive materials such as freeze dried proteins, in the case of hydrates and in all other cases, where drying would inadvertently change the (surface) properties of the investigated material [38]. SAXS can also asses the nanostructured surface of closed pores which are not accessible to the BET method.

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6. Conclusion

This work aims to investigate the influence of residual water of freeze dried fibrinogen on its powder properties. Especially the investigation of changes in the dissolution behavior to determine the influence on subsequent processing was evaluated.

In the introduction a general overview about lyophilization/freeze-drying is provided, including general aspects as well as bulk lyophilization. Furthermore, the role of fibrinogen in the human body is discussed.

Chapter one is dealing with powder and protein properties that may influence the subsequent processing of freeze-dried protein material. A special focus is set on lot-to-lot variations. Therefore, a detailed review about powder and protein properties which can vary is given. Moreover, the techniques to investigate these attributes are listed and described.

In the second chapter the influence of residual water on powder properties of freeze-dried fibrinogen on its dissolution behavior is discussed. It was demonstrated that differences in water content of solid state proteins lead to differences in dissolution behavior. This can be explained by differences in particle size and shape as well as BET surface and the inner surface of the freeze dried protein powder. To identify the ideal water content for further processing steps, lyophilized fibrinogen powder was stored on different relative humidities. In general water contents higher than 10% are not suitable for improving the dissolution performance. Moreover, it was demonstrated that a direct correlation of the BET and the inner surface of the particles to the dissolution behavior is evident. The results provide experimental evidence which suggests that the identification of the ideal water content for subsequent process steps could be very useful in the development of solid – state protein formulations to improve their processing performance.

Chapter three deals with the investigation of the freeze- dried fibrinogen powder via RAMAN and Infrared Spectroscopy. Furthermore, the relative crystallinity of the product was investigated. A partial least squares (PLS) model was developed to correlate the mid-infrared and Raman spectral changes with the degree of crystallinity. The obtained R^2 value of 0.953 confirmed a correlation between changes in the secondary structure and crystallinity of the samples. In order to control the protein stability, changes in the solid state of freeze-dried proteins and their secondary structure should be analyzed. Using the model proposed, the related predictions can be made in a non–invasive and fast manner, i.e., via hand-held devices, allowing the monitoring of the quality of stored material.

In Chapter four the nanostructural changes at various contents of residual humidity (6 – 20%) were studied by small-and wide-angle X-ray scattering (SWAXS) and related to the dissolution properties. Surprisingly, it was found that higher specific surface leads to decreased dissolution rates. The true reasons for this are still unclear and need further investigation. Furthermore, it

was found that with increasing crystallinity the dissolution rate decreases. The most important result of this study is that SAXS presents a practical tool to characterize the compactness and density fluctuations in the amorphous form by the specific surface at the nano-scale. From the correlations between Si (specific surface) and dissolution rates for different batches in the amorphous form, dissolution properties can be positively predicted from SAXS, without the need for lengthy dissolution tests.

7. Outlook

To develop a more detailed understanding for the optimal storage conditions and dissolution behavior of freeze dried fibrinogen, further research would be necessary. Not only storage on different relative humidities, but also on various temperatures would be interesting to study. Thus, to investigate the formation of an insoluble particle fraction during the dissolution/reconstitution of freeze–dried protein formulation and to rationally correlate with the physico-chemical properties of freeze-dried protein-powders, upstream powder (pre-)-treatment and processing parameters. Beyond the formation of insoluble particles, other physicochemical problems can arise during the reconstitution, e.g. precipitation, recrystallization, protein aggregation and foaming, as well as gel formation. Therefore, a comprehensive characterization of the lyophilized protein formulation at molecular, micromeritic and surface level would be necessary (Figure 1).
Furthermore, the impact of various handling and environmental conditions (T, RH, time) on the powder characteristics, and thereby, on the formation of insoluble particulates is of particular interest.

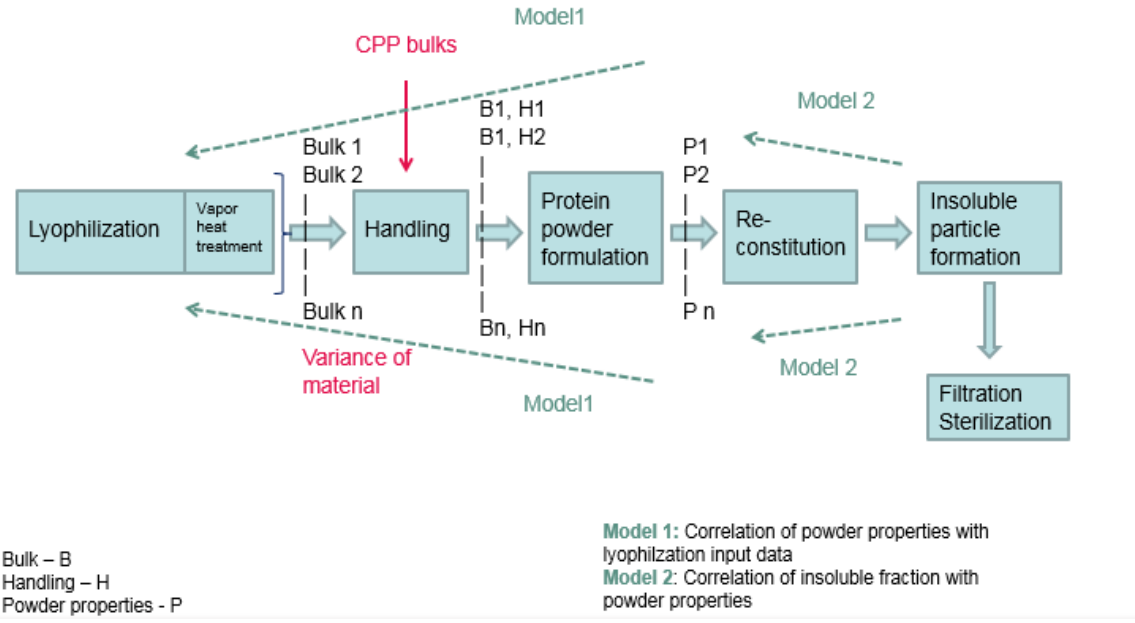

Figure 1: Overview Outlook

A goal of further research should be the establishment of a rational correlation using generated data and to develop a predictive model for the extent of insoluble particulates formation during the reconstitution process. Thus, online monitoring methods in order to analyze the reconstitution/dissolution steps in detail have to be investigated.

Additionally, lyophilization process data should be used to establish a model predicting the impact of the freeze drying process on the protein formulation attributes relevant to reconstitution. The ultimate research goal will be the understanding of the formation of insoluble particulates and – based on this understanding – to optimize the upstream process to minimize problems during downstream operation.

Furthermore, the contradictory behavior identified for the specific surface – dissolution correlation (higher surface area leads to decreased dissolution rates) has to be clarified. Therefore, deep understanding of the mechanism of lyophilization excipients on the formulation has to be developed.

8. Publications

Peer – reviewed Paper

Wahl, V., Scheibelhofer, O., Roessl, U., Leitgeb, S., deBeer, T., Khinast, J., 2015. The influence of residual water on the secondary structure and crystallinity of freeze-dried fibrinogen. Int. J. Pharm. 484, 95-102

Wahl, V., Leitgeb, S., Laggner, P., Liebminger, A., Pichler, H., Khinast, J., 2015. The Influence of Residual Water on the Solid State properties of Freeze dried Proteins. Eur. J. Pharm. Biopharm., 91. 1-8

Wahl, V., Saurugger, E.M., Khinast, J.G., Laggner, P., 2015. Specific Surface, Crystallinity, and Dissolution of Lyophilized Fibrinogen. A Study by Combined Small- and Wide-Angle X-Ray Scattering (SWAXS). Eur. J. Pharm. Biopharm. 89, 374–382.

Talks

Wahl, V.; Scheibelhofer, O.; Rößl, U.; Leitgeb, S.; deBeer, T.; Khinast, J.: The influence of residual water on the secondary structure and crystallinity of freeze dried fibrinogen.- in: 8th PSSRC Annual Meeting. Ljubljana am: 16.09.2014

Poster

Wahl, V.; Leitgeb, S.; Laggner, P.; Khinast, J.: The influence of residual water on the reconstitution behavior of lyophilized human fibrinogen.- in: 6th International Congress on Pharmaceutical Engineering. Graz am: 16.06.2014

Wahl, V.; Leitgeb, S.; Khinast, J.: The influence of water on the solid state properties of freeze dried proteins. - in: 9th Worldmeeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. Lisbon am: 31.03.2014

Wahl, V.; Piller, M.; Leitgeb, S.; Khinast, J.:Einfluss des Restwassergehaltes auf das Aggregationsverhalten lyophilisierter Proteinbatches.- in: 9. Minisymposium der Verfahrenstechnik in Leoben. Leoben am: 17.04.2013

Wahl, V.; Piller, M.; Leitgeb, S.; Khinast, J.: Optimization of processing by characterizing critical protein powder parameters.- in: Freeze Drying of Pharmaceuticals & Biologicals. Breckenridge am: 07.08.2012

Wahl, V.; Leitgeb, S.; Khinast, J.: Characterizing critical protein powder parameters for optimization of further processing.- in: 8. Minisymposium der Verfahrenstechnik. Linz am: 02.05.2012

Application notes

Laggner P., Saurugger E-M., Wahl V., Leitgeb S., Khinast J.: SWAXS in Protein Drug Development: Specific Surface, Crystallinity and Dissolution of Freeze-Dried Fibrinogen -Bruker