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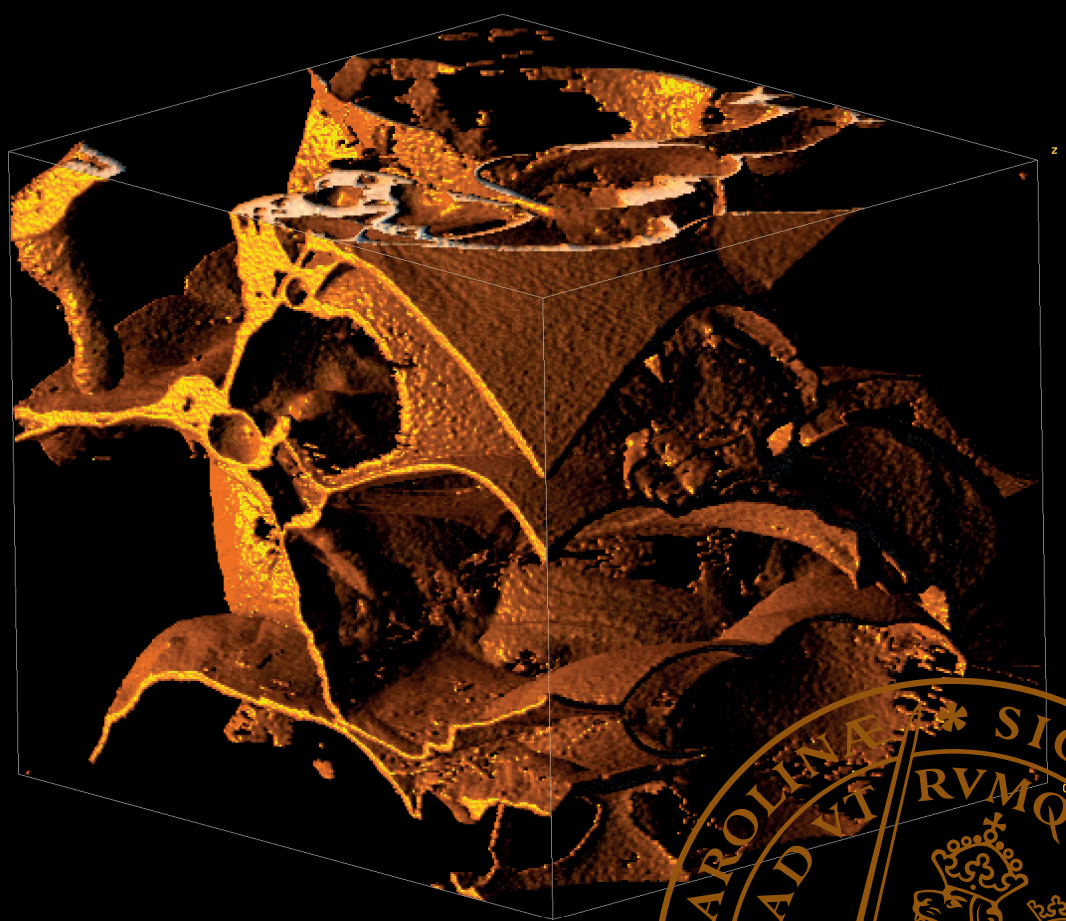

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Daniel Tristan Osanloo

LUNDS UNIVERSITET

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on Wednesday 12th of June at 09.00 in Hall KC:A, Kemicentrum, Lund

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Upon drying, vacuum foam-drying generates a solid foam with thick walls, at least four times thicker than the wall thickness of a freeze-dried material. This is interesting since the thicker walls can fully encapsulate larger biologics such as cells and bacteria. Even though thicker walls, the reconstitution characteristics of the solid foam were good and similar to the corresponding freeze-dried material. Moreover, a glassy matrix was necessary to preserve the stability of the protein. Freeze-drying, spray drying, and vacuum foam-drying processes can all be designed to preserve the stability of proteins in an amorphous matrix. Furthermore, the surface composition of the vacuum foam-dried lamellae is heterogeneous, and forms domains enriched in protein, and other domains enriched in the carbohydrate matrix. The internal composition highlights the phase separation of macromolecular components, protein and polysaccharide polymer, which may result in reduced protein stability.

In conclusion, vacuum foam-drying shows promise as an alternative drying technology for proteins, and if used correctly may be used as a concept for most dry biologics.

Key words: Vacuum foam-drying, Freeze-drying, Spray drying, Spray-freeze drying, Matrix former, Glass transition temperature, Residual water content

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“Oh, what a day, what a lovely day!”

–Nux

Table of contents

Abstract

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In conclusion, vacuum foam-drying shows promise as an alternative drying technology for proteins, and if used correctly may be used as a concept for most dry biologics.

Populärvetenskaplig sammanfattning

Biologiska läkemedel (t.ex. proteiner, mRNA, DNA, celler eller bakterier) utvecklas till en början som vattenlösningar. Kostnaderna är förhållandevis låga och formuleringarna enkla. Men på grund av besvär med stabilitet, förvaring och transport är vattenlösningar sällan en långsiktig lösning. Typiska källor med skadlig utgång är solljus, värme och omrörning. Alla dessa källor leder förr eller senare till en ofrivillig nedbrytning. Torkning är ett sätt att komma runt dessa skadliga källor. Målsättningen med att torka biologiska läkemedel: är bevarad aktivitet; låg vattenhalt; och god upplösning vid givet tillfälle t.ex. en injektion. Att bara torka ett biologiskt läkemedel själv leder sällan till en framgångsrik produkt. Produkten är visserligen torr men torkning är en påfrestande process för biologiska läkemedel. Därför tillsätts hjälpämnen i formuleringen. Hjälpämnena kan delas in som matrisbildare och ytkonkurrenter. Matrisbildarens huvudsakliga uppgift är att bli amorf (avsaknad av ett ordnat kristallmönster) och ersätta vattnets interaktion med det biologiska läkemedlet. Genom att matrisen blir amorf kan det biologiska läkemedlet bäddas in och skyddas från skadliga källor, samtidigt som fördelaktig matris-biologiskt-läkemedel-interaktion bevarar och stärker matrisens skyddande egenskaper. Många biologiska läkemedel såsom proteiner, enzymer och antikroppar är ytaktiva, vilket innebär en drivkraft att söka sig till ytor, till exempel ytan av en vattenlösning. Matrisen har ej möjlighet att förhindra detta och därför anses det klokt tillsätta en ytkonkurrerare i formuleringen. Ytkonkurrentens huvudsakliga uppgift är att istället för det biologiska läkemedlet söka sig till vattenytan först och därigenom erbjuda en skyddande barriär.

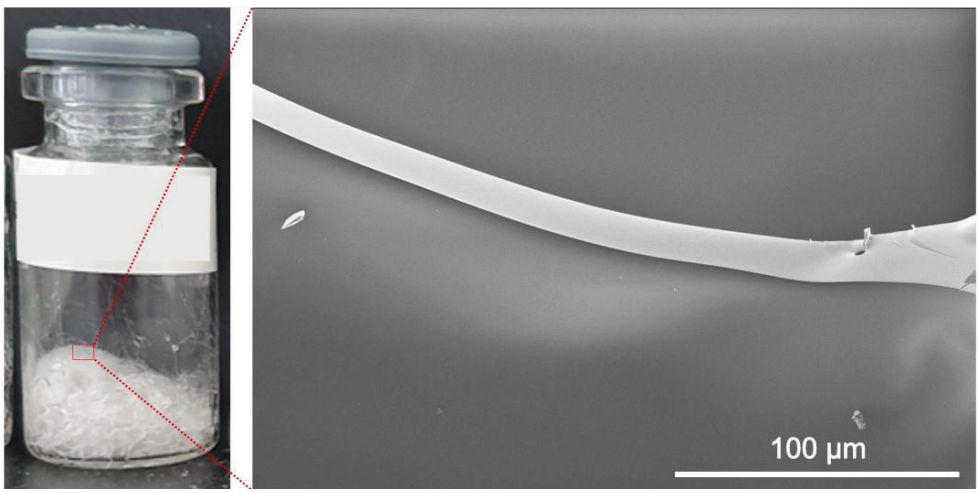
Frystorkning är den vanligaste torktekniken för torra biologiska läkemedel. En lösning nedkyls tills vattnet fryser till is. Därefter sublimeras isen bort under vakuum och genererar en torr produkt med ca 1 % kvarvarande vatten. Processen är välbeprövad (har funnits sedan 60-talet), har stor spridning inom läkemedelsindustrin, samt att regulatoriska myndigheter har mycket god kännedom kring frystorkning. Frystorkning har dock vissa nackdelar, som en lång process (som kan ta många dagar upp till någon vecka), hög energiförbrukning och därmed höga kostnader. Vid sidan av frystorkning är spraytorkning den näst-vanligaste torktekniken för biologiska läkemedel. Att spraytorka är en sammanhängande process, vilket innebär att sprayade droppar torkas till ett pulver utan ett mellansteg. Jämfört med frystorkning är spraytorkning mer gynnsam ur tids- och energisynpunkt. Tyvärr har även spraytorkning begränsningar, framförallt av att mera kvarvarande vatten återfinns i pulvret. För mycket kvarvarande vatten kan omvandla matrisen från amorf till kristallin och därigenom inte längre bädda in det biologiska läkemedlet. Så frågan är, finns det andra torktekniker vi kan använda oss av?

Målsättningen med denna avhandling är att undersöka alternativa torktekniker för biologiska läkemedel. Vidare önskas en förståelse utvecklas för hur formulerings-

och processegenskaper påverkar det biologiska läkemedlets stabilitet och produktens egenskaper. Slutligen ämnas att utforska pålitliga, förutsägbara och reproducerbara torktekniker för biologiska läkemedel.

Denna avhandling har undersökt vakuumskumtorkning som en alternativ torkteknik för biologiska läkemedel. Vakuumskumtorkning avlägsnar lösningsmedlet (vatten) under sänkt tryck (vakuum), vilket möjliggör kokning vid rumstemperaturer och utan ett infrysningssteg. Processen är snabb och inom 30 min erhålles ett stabilt skum. Genom att möjliggöra kokning vid rumstemperatur, är vakuumskumtorkning en alldeles utmärkt torkteknik för värmekänsliga material, såsom biologiska läkemedel. Proteiner har uteslutande använts som modell för biologiska läkemedel.

Resultaten visade att vakuumskumtorkning kan användas för att torka biologiska läkemedel. Materialet uppvisas som ett torrt, stabilt skum, med en tjocklek på 20–40 µm, Figur 1. Just tjockleken på det torra, stabila skummet kan visa sig vara oerhört viktig när större biologiska läkemedel används. Som jämförelse är ett protein i storleksordning 1–10 nm, medan en bakterie är omkring 1–3 µm, alltså ungefär 1000 gånger skillnad(!). En annan fördelaktig egenskap hos det vakuumskumtorkade materialet är att upplösning sker ungefär lika snabbt som för frystorkat material.



Figur 1. Representativt fotografi på ett torrt, stabilt skum och tjockleken hos skumlamellen.

Avslutningsvis har denna avhandling bidragit med avsevärd kunskap kring vakuumskumtorkning som en alternativ torkteknik för biologiska läkemedel. Tack vare den här forskningen finns en förhoppning om att forskande läkemedelsföretag, istället för en konventionell torkteknik, väljer just vakuumskumtorkning för utveckling av nya biologiska läkemedel.

List of publications

I. Osanloo D.T., Fransson J., Bergenståhl B. and Millqvist Fureby A. (2023), **Effects of drying methods on physical properties and morphology of trehalose/mannitol mixtures**, *Drying Technology*, 43; 503-522.

II. Osanloo D.T., Mahlin D., Bjerregaard S., Bergenståhl B. and Millqvist Fureby A. (2024), **Formulation factors affecting foam properties during vacuum foam-drying**, *International Journal of Pharmaceutics*, 652.

III. Osanloo D.T., Mahlin D., Bjerregaard S., Bergenståhl B. and Millqvist Fureby A. **Exploring vacuum foam-drying as an alternative, to already existing drying techniques, for biologics**. *Manuscript.*

IV. Osanloo D.T., Mahlin D., Bjerregaard S., Dobryden I., Sjövall P., Bergenståhl B. and Millqvist Fureby A. **Mapping phase distributions of vacuum foam-dried biologic in a matrix system**. *Manuscript.*

Publications not included in this thesis

I. Tyagi N., Gidlöf Z., Osanloo D.T., Collier S.E., Kadekar S., Ringstad L., Millqvist Fureby A. and Roos S. (2023), **The impact of formulation and freeze-drying properties and performance of freeze-dried *Limosilactobacillu reuteri* R2LC**, *Applied Microbiology*, 3; 1370-1387.

The author's contribution to the papers

I. The author designed the study with the co-authors. The author performed all the experimental work. The author performed the data analysis together with the co-authors and wrote a first draft which was revised by the co-authors.

II. The author designed the study with the support of the co-authors. The author performed most of the experimental work. X-ray Photoelectron Spectroscopy (XPS) was performed as an analytical service at RISE, Stockholm. The author performed the data analysis together with the co-authors and wrote a first draft which was revised by the co-authors.

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Abbreviations

aC12G2	α-dodecyl maltoside
APG	Alkyl polyglycosides
BSA	Bovine serum albumin
D40	Dextran with an average molecular weight of 40 kDa
DSC	Differential scanning calorimetry
FD	Freeze-drying
Lipase	Recombinant human bile-salt stimulated lipase
MHH	Mannitol hemihydrate
MTS	Matrix system
PS20	Polysorbate 20
RWC	Residual water content
SEM	Scanning electron microscopy
SD	Spray drying
SFD	Spray-freeze drying
SUC	Sucrose
VFD	Vacuum foam-drying
T_g	Glass transition temperature
TGA	Thermal gravimetric analysis
T_m	Melting temperature
ToF-SIMS	Time of flight secondary ion mass spectrometry
t.s.	Total solids of the dry matter
XPS	X-ray photoelectron spectroscopy

Introduction to drying technologies of biologics

The global pharmaceutical industry valued the biopharmaceutics or "biologics" market in 2013 at 140 billion USD (Ohtake et al., 2020). Biologics is a collective term for molecules that are made from a living organism or its products and are utilized in the prevention, diagnosis, or treatment of diseases. The family of biologics includes proteins, enzymes, antibodies, growth factors, cells, and live bacteria. The formulation of biologics typically starts as a solution. A solution is cost-effective and does not require advanced formulations. However, due to storage stability issues (chemical and physical) and strict transport conditions, biologics may need to be converted into a dried state (Krishnamurthy and Manning, 2002). This thesis aims to explore alternative drying technologies for biologics. Moreover, the thesis aims to create an understanding of how formulation factors and process properties influence protein stability and product properties. Finally, this thesis aims to investigate reliable, predictable, and reproducible drying technologies for biologics.

Freeze-drying

Freeze-drying (FD) is considered the "gold standard" to which other drying technologies must be compared for dry biologics. FD is well established in the industry, it is suitable for aseptic production, the regulatory agencies are well familiar with the concept, and it is possible to generate a product in bulk (trays) or single units (liquid filling into vials) (Tang and Pikal, 2004, Carpenter et al., 1997). Moreover, a freeze-dried product generally contains a residual water content (RWC) of approximately 1 % and dissolves rapidly during reconstitution (Carpenter et al., 2002, Walters et al., 2014)

The process of FD can be divided into a freezing stage and a drying stage. Figure 1 describes a typical FD process used in this thesis, how the drying trajectory affects the solvent (typically water) and solutes, and the generated final dry material. Prior to freeze-drying, a homogenous solution is prepared and filled into vials (1). The freezing stage is initiated, and the product is frozen below its glass transition of the

freeze-concentrate (T_g') (2). The mixture displays a two-phase system, with ice crystals co-existing with a freeze-concentrate and including unfrozen water. The drying is initiated by applying a vacuum. The pressure is decreased below the vapour point at -10 °C (10 Pa) and the ice crystals are removed by sublimation (3). This part of the drying is referred to as the primary drying stage and removes all the ice crystals but not the trapped water inside the freeze concentrate. If the process ended after the primary drying stage, the high RWC would imminently render product collapse (Tang and Pikal, 2004, Pikal et al., 1990). In order to refrain from collapse, a secondary drying stage is applied. The pressure is decreased even further (0.5 Pa), and the temperature slowly rises to 20 °C (4). This drives the removal of trapped water by diffusion and desorption, generating a dry, porous matrix with an RWC of about 1 % and complete reconstitution within a couple of seconds (Tang and Pikal, 2004)(**Paper I, III**). The established freeze-dried material demonstrates a porous network juxtaposed with a solid material, Figure 1a. The wall thickness of the solid material is thin, about 1–5 µm, Figure 1b, and will be discussed later in Chapter 1 *"Vacuum foam-drying"*.

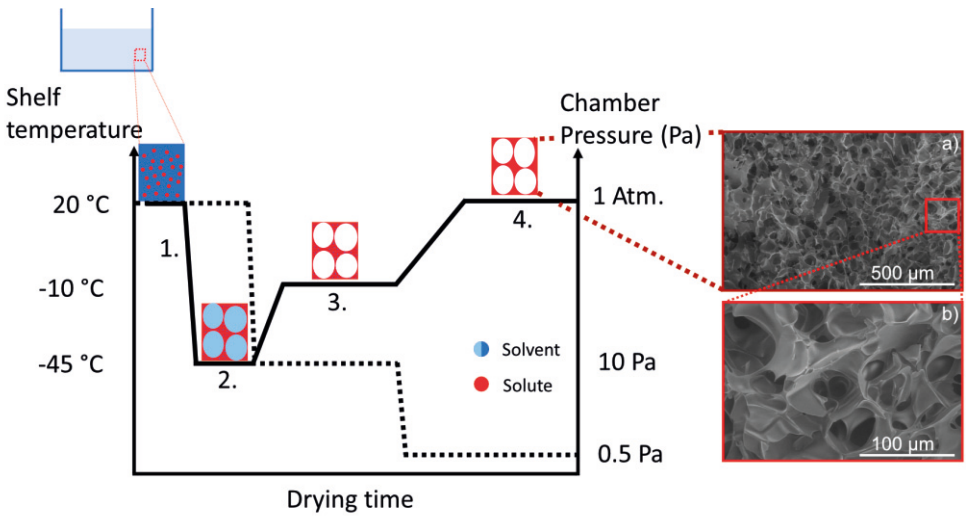

Figure 1. Conceptual drawing illustrating the drying trajectory of the freeze-dried material influenced by shelf temperature (full lines) and chamber pressure (dotted lines), the state of solvent and solute, and the subsequent pore network (a) and pore wall thickness (b) of freeze-dried materials (modified after **Paper I**).

Spray drying

After freeze-drying, spray drying (SD) is the second most utilized technique for dry biologics. The process is continuous, which means that the manufacture takes place in one step and delivers a fine powder, suitable for pulmonary or nasal delivery (Vehring, 2008, Vehring et al., 2007). Depending on batch size, the process finalizes within minutes to hours.

Figure 2 explains the formation of an SD particle, as the droplet dries, and how the drying trajectory affects the states of solvent (water) and solids. A homogenous solution is prepared and pumped into the instrument. The atomizer sprays the solution into droplets (1). In the SD chamber, hot gas renders evaporation of water which presents a decrease in droplet diameter (2 and 3). During this period, the temperature at the droplet surface equates to the wet bulb temperature (T_{wb}) which depends on the process conditions used during spray drying (Masters, 1991, Santos et al., 2017). In this thesis, the inlet temperature (T_{in}) and outlet temperature (T_{out}) were 150 °C and 80 °C, respectively, and correspond to a T_{wb} of about 40 °C. Dissolved solutes are transported via surface adsorption, diffusion and convection to the droplet surface. T_{wb} lasts until the formation of a solid crust at the droplet surface (4) occurs. At this point, the size does not undergo any further reduction and water diffuses through the crust before evaporation. Diffusion of solvent is slower than the heat transfer and, thus, generates a rapid increase in the droplet temperature (5). This proceeds until the temperature reaches the dry bulb temperature of the air (T_{db}) (6). T_{db} corresponds to the surface temperature of the dried particle after almost all water has evaporated and is close to T_{out} (80 °C). The interior temperature of the established particle will remain at T_{db} before collection. In comparison to a freeze-dried material, the RWC is a bit higher (3–4 %) and reconstitution slower (Abdul-Fattah et al., 2007a, Abdul-Fattah et al., 2007b) (**Paper I, III**).

As the droplet solidifies into a particle, the morphology is, hence, determined by what constituent remains at the surface. Disaccharides and other small molecular entities typically generate a smooth surface with little to no variations, Figure 2a. In contrast, larger molecular entities such as proteins and polymers may highly affect the surface with the presence of dents and ridges, Figure 2b.

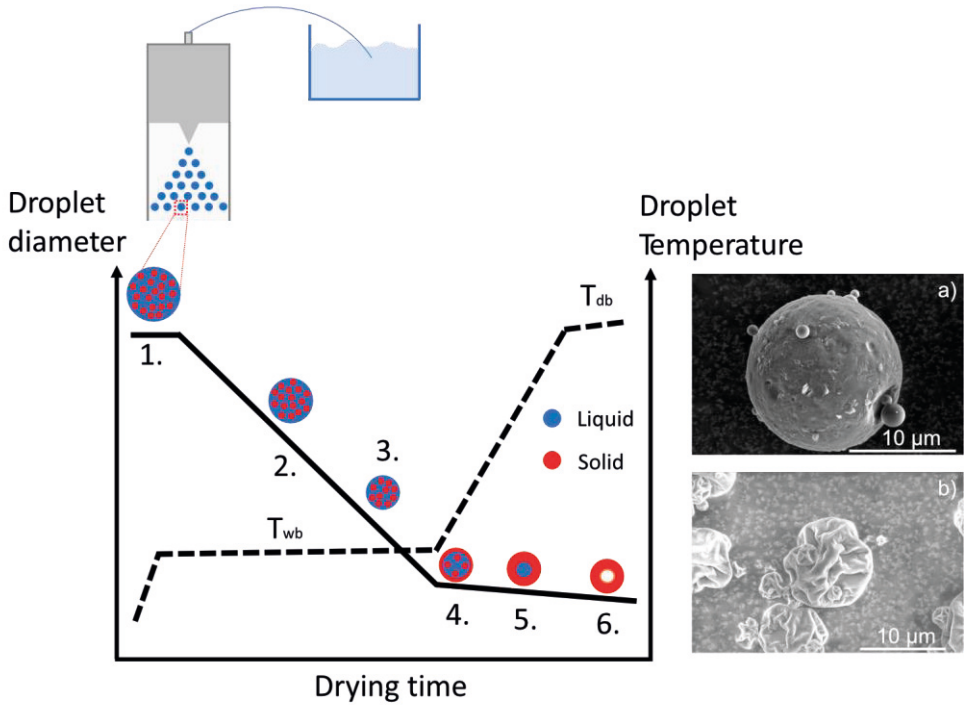

Figure 2. Conceptual drawing demonstrating the drying trajectory of a spray dried material, the state of liquid (blue) and solid (red), and droplet size (full lines) and temperature of the drying droplet (dashed lines) (modified after Elversson et al. (Elversson, 2005) and **Paper I**).

Alternative drying technologies

Another (alternative) drying technology used in this thesis is spray-freeze drying (SFD). SFD is a drying process that involves elements of both spray drying (atomization of droplets) and freeze-drying (drying by sublimation).

Figure 3 depicts the SFD process used in this thesis and how the drying trajectory impacts the state of the solvent (water), the solutes, and the formation of a final particle. A homogenous solution is prepared and sprayed into a beaker pre-filled with a very cold liquid, this thesis used liquid nitrogen (T_m of -196 °C) (1). At contact with the liquid nitrogen, the droplet displays many but small ice crystals co-existing with a freeze-concentrate and trapped water (2). The beaker was transferred into a -86 °C freezer which ripens the crystal's size (3). Instant freezing generates smaller ice crystals and more water trapped in the freeze concentrate, compared to using a controlled freezing rate. The increase in temperature relaxes the system and causes trapped water in the freeze-concentrate to diffuse and ripen the ice crystals.

The beaker was transferred from the -86 °C to a pre-cooled freeze-dryer with a shelf temperature of -45 °C, which ripens the ice crystals a second time (4). Again, the system relaxes, and further releases trapped water in the freeze-concentrate that diffuses and ripens the ice crystals. Similarly to freeze-drying, drying takes place by a decrease in pressure (10 Pa) which removes the ice crystals by sublimation (5). Further decrease in pressure (0.5 Pa) and slow rise of temperature (20 °C) removes trapped water, inside the material, by diffusion and desorption (6). The generated SFD particles are highly porous, free-flowing, and with low RWC.

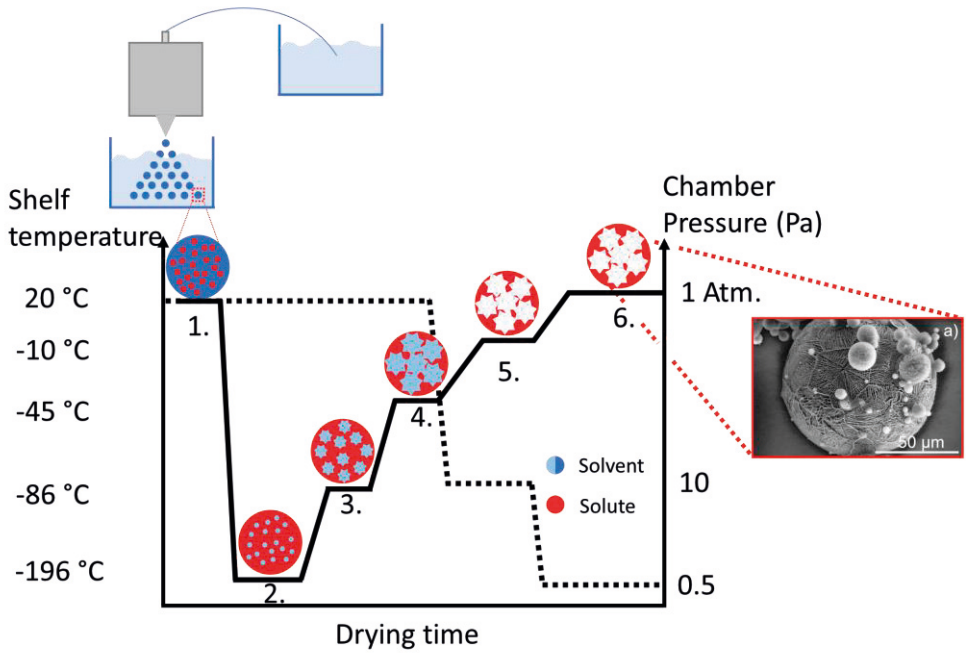

Figure 3. Conceptual drawing illustrating the drying trajectory of a spray-freeze dried material influenced by shelf temperature (full lines) and chamber pressure (dotted lines), the state of solute and water, and the final formation of a solid particle (a).

The presented drying technologies all subject the biologics, either through freezing (FD and SFD) or heating (SD). Both freezing and heating are two notorious parameters that frequently jeopardize the stability of biologics (Ohtake et al., 2020, Lovalenti et al., 2016). Therefore, a question arises if it is possible to dry biologics without the application of freezing or elevated temperature but still attain similar product characteristics from a freeze-dried or a spray dried material. Vacuum foam-drying is a drying technology that uses similar equipment as freeze-drying. In vacuum foam-drying the solvent is evaporated at reduced pressure which allows a shelf temperature closer to ambient temperature. The formation of a solid foam is rapid without a freezing step (Bronshtein, 2004). Hence, by avoiding freezing and elevated temperatures, vacuum foam-drying can offer milder conditions for biologics and has been proposed as an alternative drying technology for thermosensitive materials such as proteins, enzymes, antibodies, cells, and live bacteria.

The aim of this thesis

This thesis focuses on the drying of biologics by use of traditional drying techniques (freeze-drying and spray drying) and explores alternative drying techniques (spray-freeze drying and vacuum foam-drying). It is hypothesized that an amorphous matrix benefits the stability of dry biologics. The drying techniques' impact on the final structure and dimensions in relation to product properties were also of interest. This thesis also investigates the surface formation, upon drying, and its impact on the presence of stability of biologics.

The research project has identified vacuum foam-drying as an alternative drying technology for biologics. Vacuum foam-drying is a quite unexplored drying technique, and the aim is to highlight the generation of a solid material. Furthermore, this thesis will also investigate how formulation parameters and process conditions affect the formation of a solid material and the stability of the biologic. Additionally, this thesis scrutinizes the distribution of the components upon the formation of a surface and the internal distribution of components of the dry material.

By fulfilling the mentioned aims, the ambition of this thesis is to present vacuum foam-drying as an alternative drying technology for biologics.

1. Vacuum foam-drying

Introduction: An ambiguous literature

Foam drying (FD), cavitation foam drying (CFD), preservation by foam formation (PFF), and vacuum foam drying (VFD) are some variations mentioned in the literature that involve the creation of a dry foam by the utility of vacuum. Table 1 summarizes them and highlights the processing conditions used and the generated impact on the foam.

Table 1. A summary of known processes that use a vacuum to generate a dry, glassy foam.

Method	FD (Annear, 1970)	FD (Abdul-Fattah et al., 2007a)	CFD (Ohtake et al., 2011)	PFF (Bronshtein, 2004)	VFD (Hajare et al., 2010)
Primary drying conditions	T[1]: 25 °C P[2]: pump PT[3]: N/A[4] T[5]: 1 h	T: 15 °C P: 6.6 Pa PT: -20–5 °C t: 1 h	T: 15 °C P: 13 Pa PT: >10 °C t: 45 h	T: Ambient[6] P: 1.01 hPa PT: -4–10 °C t: 2.5 h	T: 10–30 °C P: 100–0.06 hPa PT: t: 24 h
State of the solution prior to foaming	Boiling	Freezing	Boiling	Evaporation / boiling	Evaporation / boiling

[1] Shelf temperature

[2] Chamber pressure

[3] Product temperature during drying

[4] Not available from the literature

[5] time of drying.

[6] It is assumed that ambient temperature refers to about 20 °C.

Even though different process conditions, all examples generated a glassy foam that preserved the biologics' stability. Nevertheless, most of the examples use similar process conditions but adapt unique names, or similar names with different process conditions. Others employ rather complex and long primary drying conditions. This leaves the reader with ambiguity regarding what and how a vacuum foam-dried process works. For all sense of clarification, this thesis will refer to the process of water removal by reduced pressure at ambient temperatures as vacuum foam-drying (VFD).

Appearance

When developing a VFD product, the most important parameter should be to preserve the stability of the biologics, i.e. the function. However, product aesthetics are also important during manufacture. Studies have shown that FD cakes that show signs of collapse, shrinkage, and/or damage are rejected due to failed quality standards. The appearance of a vacuum foam-dried product should be of no exception. Figure 4a-c demonstrates three types of unsuccessful VFD attempts that shall be deemed as failures of fulfilled product aesthetics. 1) If the target chamber pressure is too high or the rate of decrease in pressure is too low, foaming never proceeds to occur. Instead, slow evaporation of water settles the material like a film along the edges and bottom of the vial. Slow evaporation of water can also render crystallization of excipients which jeopardizes the biologic's stability, Figure 4a. 2) The target shelf temperature is too low, or the rate of pressure decreases too high. The product temperature proceeds below 0 °C and the water crystallizes into ice, Figure 4b. Even though the process ends up with a solid foam with glassy characteristics, exposure to ice has been proposed to jeopardize the biologic's stability due to protein aggregation (Kasper and Friess, 2011). 3) The VFD process induces heavy boiling, Figure 4c. This type of mechanism is a bit fickle, as it relates to the formulation component's interaction with water (**Paper II**). Figure 4d illustrates the preferable aesthetics for a product manufactured by VFD and should demonstrate a solid, homogenous foam, with a slightly raised centre, and preferably little or no staining on the vial's walls and neck.

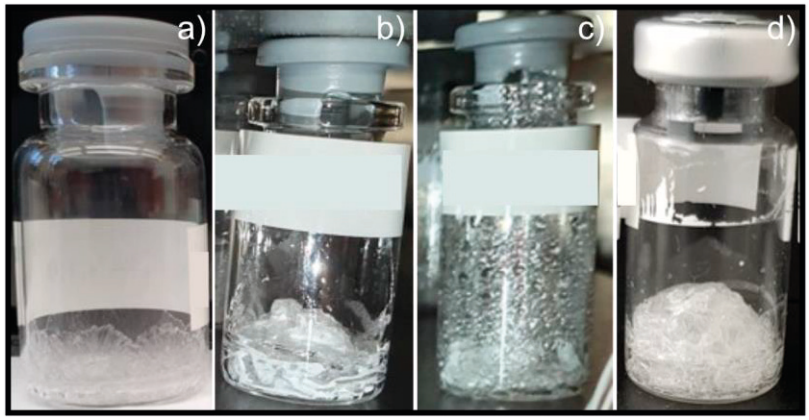

Figure 4. Photographic images demonstrating attempts of VFD, resulting in a film (a), freeze (b), and heavy boiling (c). A vacuum foam-drying process is deemed successful when the produced solid foam contains a homogenous solid foam, with a slightly raised centre, and no staining (d).

Figure 5 illustrates the established vacuum foam-dried material (5a) and the microstructure (5b). This thesis used scanning electron microscopy (SEM), a qualitative method that with high resolution presents information on the surface structure and will be further discussed in Chapter 3 *"Phase separations and molecular mapping"*. Observed by SEM, the microstructure of examined vacuum foam-dried material is quite similar and typically illustrates longer straight ridges juxtaposed to smoother, regular domains.

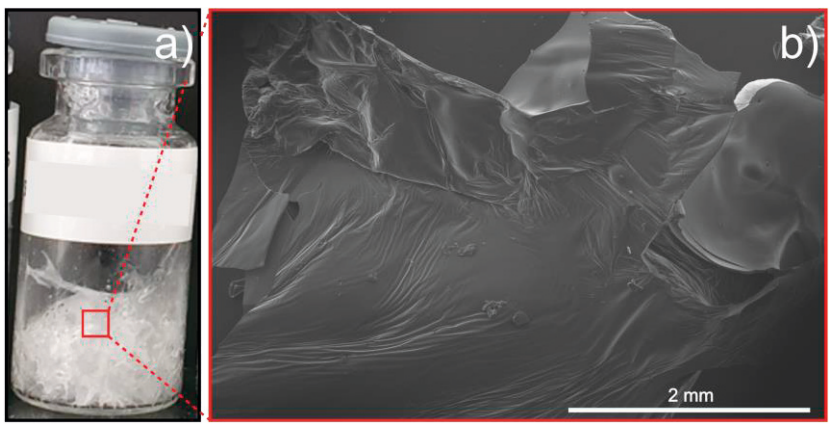

Figure 5. Representative photograph of a vacuum foam-dried material in a vial (a) and scanning electron microscopy (SEM) image of the solid foam lamellae (b).

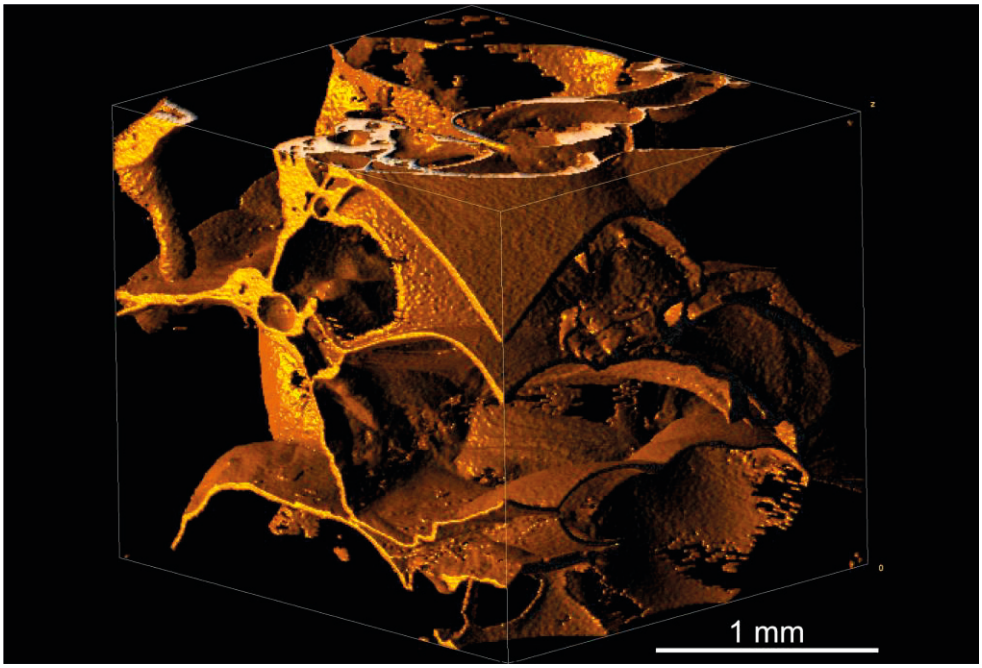

Figure 6. Tomographic 3D image of VFD 4/96 (% w/w) lipase/sucrose, 30 % total solids.

As the generated vacuum foam-dried material is brittle, the difficulty lies in observing the inside of the material. Neither photographs nor SEM can provide this information. X-ray microtomography (µCT) was utilized to gain knowledge on the distribution of pores and solid material, Figure 6. µCT is a technique that can be used to acquire 3D images of solid materials and previous studies have used µCT to determine the distribution of pores in freeze-dried materials (Palmkron et al., 2023, Palmkron, 2024)

Primary drying

The primary drying stage of VFD is responsible for transitioning a solution into a solid foam in the glassy state. Figure 7 demonstrates the general principle of VFD (temperature, chamber pressure, time, and water content) used throughout this thesis. A solution is prepared, and 1 mL filled into vials with 30 % t.s. dry matter (70 % water) (1). The process is initiated by a controlled rate of pressure reduction to 7 hPa, which evaporates water (60 %) (2). Evaporative cooling induces a temperature reduction, however, kept above 0 °C by balancing the heat input from the shelf. Vapour bubbles are formed at the bottom of the vials and induce boiling. The oscillating temperature results from the boiling which fluctuates the contact

between the probe and solution. As the boiling proceeds and water is removed (50 %), the system generates unstable foams characterized by the formation and breakage of bubbles (3). Due to water removal and an increase in solutes, the system is exposed to a rapid increase in viscosity. Once enough water is removed from the system, the lifespan of the bubbles is extended so that a soft, but stable (rubbery) foam (about 20 % water) is formed (4). Further drying removes trapped water from the matrix (about 10 % water left) and the soft (rubbery) foam transitions into a solid (glassy) foam (5).

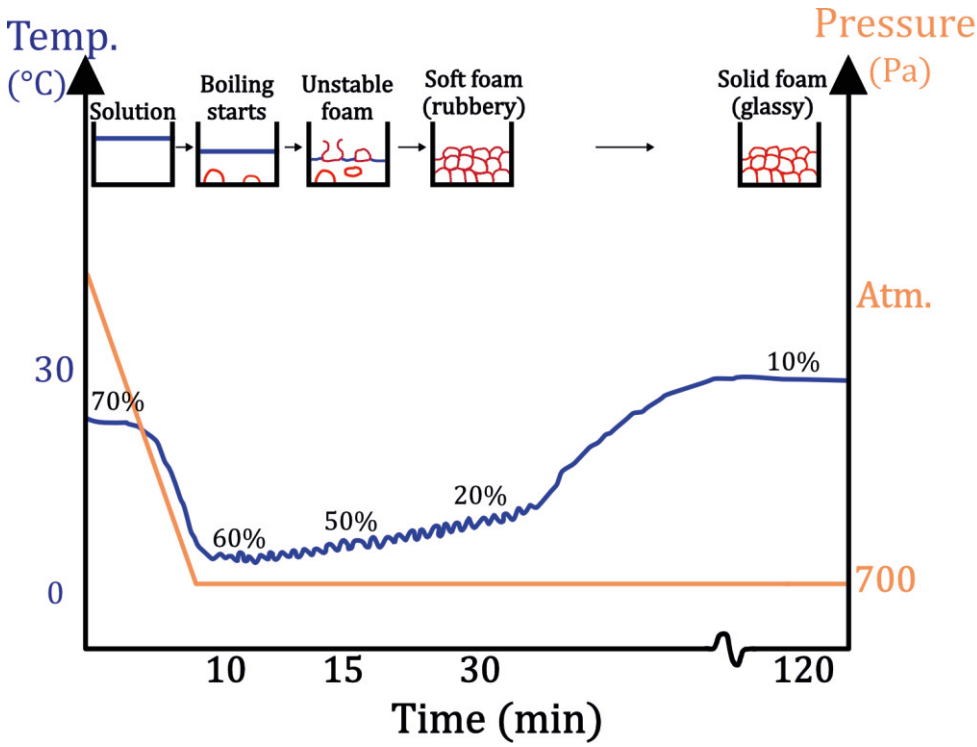

Figure 7. A schematic overview aimed to exhibit the drying trajectory by formation from a solution to a solid (glassy) foam, during the primary drying stage of vacuum foam-drying. The blue lines illustrate the product temperature (drying trajectory), and the orange lines show the chamber pressure. The percentages just above the drying trajectory present the water content for each stage (modified from **Paper II**).

Once a solid (glassy) foam was created, the underlying assumption from **Paper II** was that viscosity was the most important parameter to generate a solid foam. Figure 8 displays the dynamic viscosity as a function of the total solids of four solutions. Two of the solutions consisted of bovine serum albumin (BSA) and sucrose (SUC), in two different BSA concentrations (1 % and 4 %), with the t.s. kept at 30 %. The

other two used a fixed concentration of BSA (1 %) and a matrix system (MTS) comprised of sucrose and dextran with an average molecular weight of 40 kDa (D40) at 75/25 (% w/w) and 50/50 (% w/w), with the t.s. kept at 10 %. BSA (and other proteins) and D40 are prone to enhance the viscosity of a system (Tirtaatmadja et al., 2001). The extrapolated sample viscosity (dotted lines) as a function of t.s. demonstrates that increased protein and D40 concentrations affect the dynamic viscosity of the system. In comparison, to generate a dynamic viscosity of 700 mPas requires about 80 % t.s. of BSA/SUC, 1/99 (% w/w) and about 60 % t.s. of BSA/MTS:SUC/D40, 1/99:50/50 (% w/w). However, irrespective of viscosity enhancers, above 85 % t.s. was required to transition the unstable foam (beige area) into a soft (rubbery) foam (pink area).

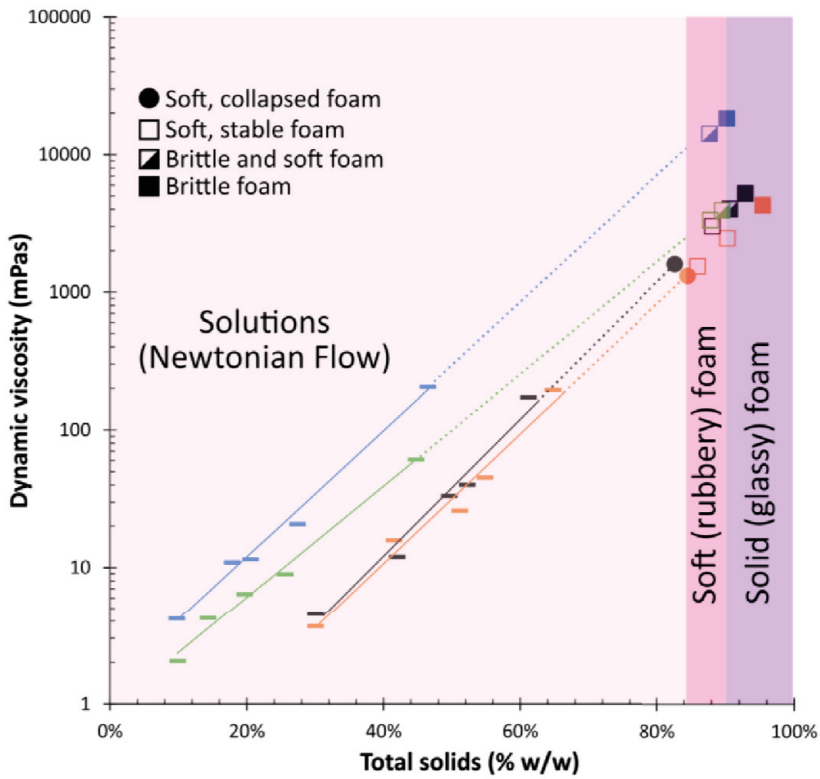

Figure 8. Dynamic viscosity as a function of % total solids (w/w) for 1/99, BSA/SUC (%, w/w) (orange), 4/96, BSA/SUC (%, w/w) (black), 1/99:75/25, BSA/MTS:SUC/D40 (%, w/w) (green), and 1/99:50/50, BSA/MTS:SUC/D40 (%, w/w) (blue) during the primary drying stage of vacuum foam-drying. The starting concentration for the BSA/SUC and BSA/MTS:SUC/D40 was 30 % and 10 % (w/w, dry weight), respectively. The dashed lines demonstrate the logarithmic extrapolations of the measured viscosity. The regions display the transition from solutions (beige), soft (rubbery) foam (pink), and stable (glassy) foam (purple).

Even though important to solidify the unstable foams into a stable foam, the results revealed that the viscosity of the solutions, prior to the transition into a soft (rubbery) foam, was not the most important parameter. All the solutions generated a stable foam (irrespective of viscosity enhancer), but the process conditions (shelf temperature and chamber pressure) remained unaltered (30 °C and 7 hPa). Therefore, a set of shelf temperatures, chamber pressures, two concentrations (1 and 4 % (% w/w dry weight), respectively) of the protein bovine serum albumin (BSA), and two starting total solids content (15 and 30 % t.s. respectively) was utilized, and the resulting foamability monitored after 2 hours of primary drying. The foamability was monitored in terms of the percentage of foamed vials (% FV), state of the foam (SoF), foam overrun (FO), the rate of evaporation, and the time until a stable foam was observed (t_{sf}). Sucrose was used as a matrix former throughout the experiments. Figure 9 demonstrates the most interesting results, where the shelf temperature was set to 30 °C, and the chamber pressure varied from 7, 10, 12, and 15 hPa, for BSA/SUC with ratios 1/99 and 4/96 at 30 % t.s. The foam overrun of the solid foams (the method is further described in **Paper II**) and the rate of evaporation were assessed using Equations 1 and 2, respectively.

$$FO = \left(\frac{v_d - v_s}{v_s}\right) \times 100 \ [\%\ v/v] \qquad [1]$$

$$\Delta T = T_{shelf} - T_{prod} \ [°C] \qquad [2]$$

where T_{shelf} and T_{prod} are the shelf temperature and product temperatures at the start of the primary drying, respectively.

The % FV and ΔT were rather consistent at 100 % and 19–22 °C, respectively. However, as the chamber pressure increased, noticeable changes in the foamability were revealed.

• % FV • SoF • FO (%) • ΔT • t_{sf}	 • 100 % • Brittle • 1100 ± 50 • 21 °C • 40 min  • 100 % • Brittle • 1670 ± 50 • 19 °C • 35 min	 • 100 % • Soft • 850 ± 50 • 21 °C • 52 min  • 100 % • Soft • 1210 ± 40 • 22 °C • 49 min	 • 100 % • Soft • 480 ± 90 • 19 °C • 64 min  • 100 % • Soft • 820 ± 50 • 19 °C • 59 min	 • 0 % • Solution • 0 % • 12 °C • N/D  • 0 % • Solution • 0 % • 12 °C • N/D
Chamber pressure	7	10	12	15

Figure 9. Representative photographic images and a summary of the process properties for 1/99 and 4/96, BSA/sucrose (% w/w), 30 % t.s. after 2 h of primary drying. The shelf temperature was set to 30 °C, whilst the chamber pressure varied from 7, 10, 12, and 15 hPa. The bulletins represent, in ascending order: the percentage of foamed vials (FV); the state of the foam (SoF) (soft or brittle); the foam overrun (FO) (%) calculated by Equation [1]; ΔT is the rate of evaporation and was calculated by Equation [2]; and the time until a stable foam was observed. Measurements were performed in ten replicates and, when presented, the error bar is represented as the standard error of the mean (SEM).

As the chamber pressure increased (from 7 to 10 to 12 hPa, respectively), both protein concentrations, exhibited a gradual decrease in FO-values, stable foams that progressed from brittle (glassy) to soft (rubbery), and a longer time to transition the unstable foam into a stable foam. At a chamber pressure of 15 hPa and after 2 hours of primary drying, the solutions remained as viscous solution. In comparison, ΔT decreased from 19–22 °C (100 % foamed vials) to 12 °C (no foaming of vials). This implies that the initial evaporative cooling, at the start of primary drying, was not sufficiently high (low ΔT) to induce boiling.

Secondary drying

Once the solution solidifies into a solid foam, the RWC is still high (about 10 %), and the end of the process would jeopardize the stability of the vacuum foam-dried material. Even 5 % RWC in an amorphous sucrose matrix is enough to imperil the stability of the material (Roos, 1993, Roos and Drusch, 2015). The role of the matrix former will be discussed in Chapter 2 *"Formulation components"*. Therefore, to avoid perils of the material, immediately after the end of primary drying a secondary drying step is implemented. Much similar to freeze-drying, the goal of secondary drying is the remove residual water trapped inside the solid matrix (Pikal et al., 2005). In freeze-drying, the rate-limiting factor, of removing trapped water from the solid matrix, can be ascribed to the surface area (size of pores) and the dimensions of the solid material (wall thickness) and generally requires 3–6 hours of drying (Tang and Pikal, 2004, Pikal et al., 1990). The thickness of a typical freeze-dried material is in the range of 1–5 µm, Figure 10b (Palmkron et al., 2023) (**Paper I,**

III). Consequently, a process that develops larger pores (decreased surface area) generates a thicker material that takes a longer time to dry. In comparison to the wall thickness of a freeze-dried material, the wall thickness of a vacuum foam-dried material is 20–40 µm, at least four times thicker, Figure 10d (**Paper II, III**). After 90 hours of secondary drying, the RWC of a vacuum foam-dried material is high (1–4 %), Figure 11a. This means that the distance trapped water travels through the lamellae before desorption is much longer, Figure 11b.

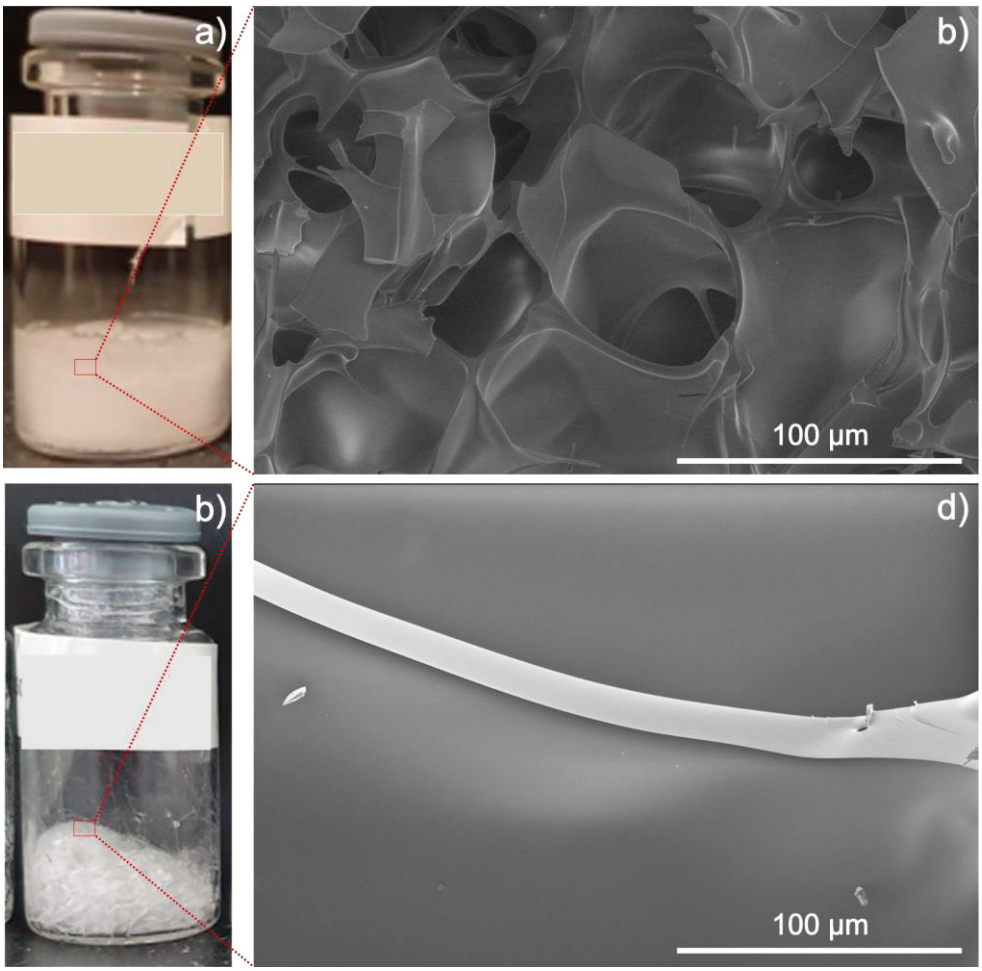

Figure 10. Representative photographs and SEM images of freeze-dried (a and c) and vacuum foam-dried (b and b) materials, respectively. The SEM images depict the typical wall thickness of the freeze-dried (b) and the vacuum foam-dried (d) material.

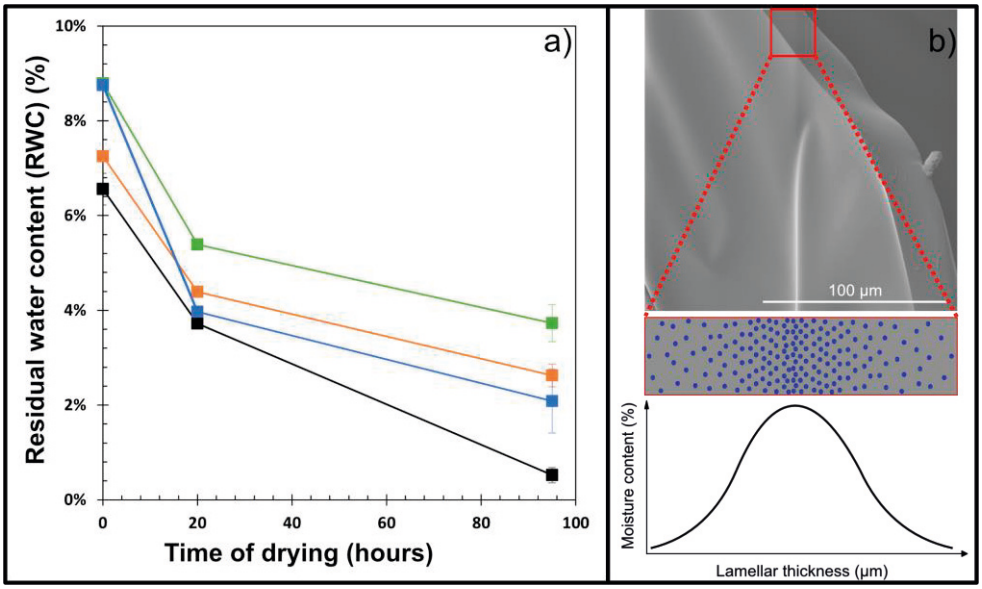

Figure 11. Residual water content as a function of secondary drying time (a) for 1/99 BSA/SUC (% w/w), 30 % t.s. (orange), 4/96, BSA/SUC (% w/w), 30 % t.s. (black), 1/99:75/25, BSA/MTS:SUC/D40 (% w/w) (green), and 1/99:50/50, BSA/MTS:SUC/D40 (% w/w) (blue), and a hypothetical drawing of water distribution trapped inside the lamellae (b).

2. Formulation components

Introduction: Formulation of dry biologics

The stability of biologics can be categorized as chemical (breakage of covalent bonds) or physical (aggregation and denaturation) and is important to maintain during any type of production, such as drying (Manning et al., 2010). However, drying imposes great stress on the biologics and more often jeopardizes their stability. Stress factors from drying that drive the instability of biologics include: temperature fluctuations; freezing; elevated temperatures; mechanical stress; and interfaces (Hawe et al., 2012). During the drying process, changes in the biologic formulation can also induce instability, mainly through changes in pH, ionic strength, and protein concentration (Tamizi and Jouyban, 2016). To avoid instability issues during drying, it is considered prudent to include excipients in the biologic formulations. In this thesis, the excipients used for dry biologic formulations, are referred to as matrix formers and surface competitors.

Matrix formers

Matrix formers are used to increase the stability of dry biologic formulations. This takes place mainly through two mechanisms. The first mechanism takes place by replacing the hydrogen-bonding interaction between the solvent (water) and the biologic, referred to as the "water replacement theory" (Carpenter and Crowe, 1989). Studies have shown that matrix formers' ability to interact through hydrogen bonds increases the stability of biologics (Cleland et al., 2001, Andya et al., 1999). The second mechanism is by embedding the biologic in an amorphous, dry glass which limits the biologics' mobility (Wang, 1999). Embedded in an amorphous glass, the biologic's mobility is severely reduced which is correlated to an enhanced stability (functionality) (Yoshioka and Aso, 2007, Carpenter et al., 2002) which will be discussed later in the subsection *"Amorphous state"*.

Amorphous state

Compared to their crystalline counterpart, amorphous materials are quite different. The main characteristic of amorphous materials is the lack of a crystal lattice. This means that amorphous materials exhibit no melting temperature (T_m) i.e., since melting by definition is the breakage of the crystal lattice. However, what is known about the amorphous state is that it is highly energetic and resembles that of a liquid. Another aspect unique to amorphous materials is the characteristic temperature known as the glass transition temperature (T_g). The theoretical aspects of the T_g are limited, but the transitions involve dramatically increased viscosity, lowered molecular mobility, thermal expansion, and heat capacity, Figure 12.

Figure 12 demonstrates that cooling of a liquid below its T_m generates a rubbery state. The rubbery state can also be referred to as a supercooled liquid. To avoid any confusion, this thesis will refer to it as the rubbery state. From the rubbery state, it is thermodynamically favourable for the material to crystallize. The heat released at recrystallization corresponds to the melting enthalpy (ΔH_m). However, if the cooling is sufficiently fast and below the T_g, the molecules will never have time to form a crystal and instead enter the glassy (amorphous) state. It is called the glassy state due to the material's resemblance to glass, which is distinguished as rigid and brittle.

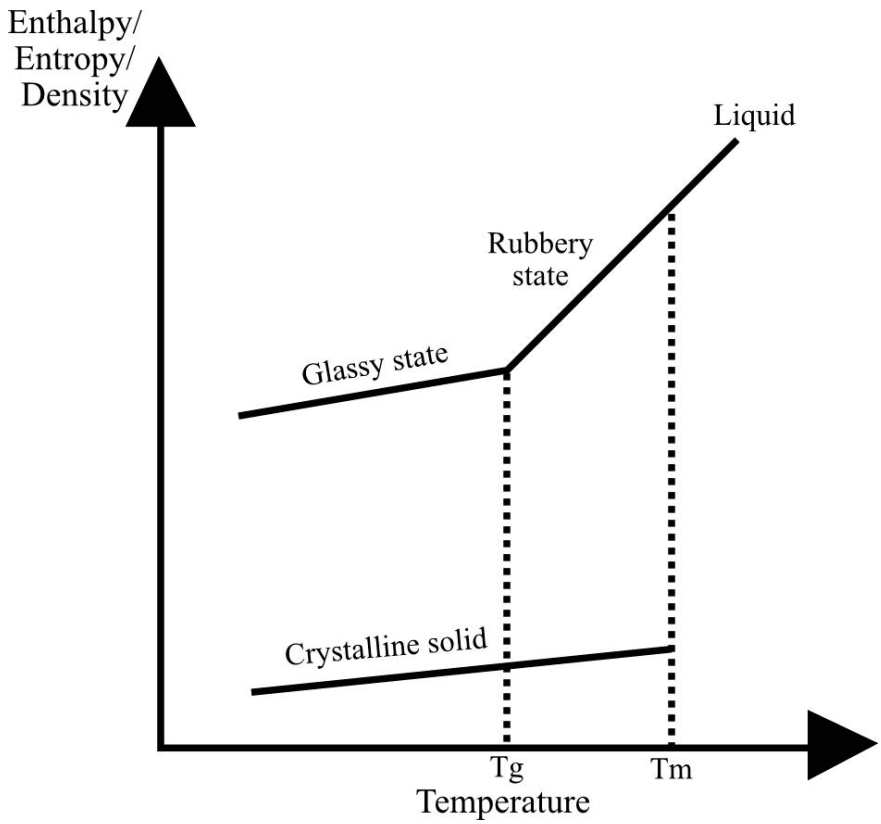

Figure 12. State diagram of Enthalpy/entropy/density as a function of temperature. T_m is the melting temperature and T_g is the glass transition temperature. Modified after *(Kawakami and Pikal, 2005)*.

For dry biologic formulations, a T_g 50 °C higher than the storage temperature is enough to prevent crystallization and to provide sufficient stability to the biologic (Hancock and Zografi, 1997). The T_g depends on the formulation components and the residual water content in the dry product. Unfortunately, amorphous materials are notoriously hygroscopic. This results in an increased amount of residual water in the product. Water with a T_g of -136 °C, reduces the T_g of the matrix former and increases the molecular mobility, thus, acting as a plasticizer (Roos and Drusch, 2015). 5 % of water in amorphous sucrose is sufficient to reduce the T_g to 20 °C (Roos, 1993). Hence, if stored at room temperature, the molecular mobility increases, and the material enters the rubbery state, where crystallization proceeds, and jeopardizes the stability of the biologic.

The T_g of a formulation (mixture) can be predicted by use of the Gordon-Taylor Equation (GT-Eq), Equation 3 (Roos, 1993).

$$T_{g,mix} = \frac{w_1 T_{g1} + k(1-w_1) T_{g2}}{w_1 + k(1-w_1)} \quad [3]$$

Where T_{g1} and T_{g2} are the glass transition temperatures, w_1 is the weight fraction of component 1, and k is an empirical constant. The GT-Eq is fairly accurate and can be used to predict the T_g of amorphous mixtures (**Paper I, II**).

Disaccharides

Matrix formers typically used in biologic formulations are sugars. All sugar molecules contain hydroxyl groups, which provide hydrogen bonding opportunity, and can generally be converted into the amorphous state. Some sugars (such as glucose, fructose and galactose) have a low T_g and are avoided as they can compromise the matrix former's stability during storage (Roos, 1993). Others have a high T_g (lactose and maltose) and seem suitable, but upon storage, these reducing sugars take part in Maillard reactions and are also avoided (Zhou et al., 2013, Carpenter et al., 2002). Disaccharides such as sucrose and trehalose fulfil all aforementioned requirements. They can hydrogen bond, turn amorphous upon drying, and are non-reducing sugars. Dry sucrose and trehalose have a reported T_g in the range of 60–70 °C and 100–130 °C, respectively (Roe and Labuza, 2005, Roos, 1993, Crowe et al., 1996). Compared to trehalose, sucrose possesses a lower T_g which can result in stickiness during the SD process (Imtiaz-Ul-Islam and Langrish, 2009). SD of protein formulations with trehalose is preferred as the higher T_g offers better drying (less sticky powder) which improves the protein stability (Haque et al., 2015).

At the beginning of this thesis, trehalose was used as a matrix former in placebo formulations dried by FD, SD, and SFD (**Paper I**). Mannitol was also present in the trehalose placebo formulations, although, the results highlighted that mannitol jeopardized the stability of the matrix former. The role of mannitol on the matrix former will be discussed later in the subsection *"mannitol"*.

Trehalose was used as a matrix former and compared with sucrose for a protein (enzyme) formulation, without and with surfactants dried by vacuum foam-drying (VFD) (**Paper II**). The role of the surfactants will also be discussed later, in the section *"Surface competitors"*. Once the VFD was finalized, clear differences were observed between trehalose and sucrose as matrix formers, Figure 13.

Figure 13. Representative images of vacuum foam-dried formulations in vials of 1/99 (% w/w) lipase/sucrose (a-c), 1/99 (% w/w) lipase/trehalose (d-f), 4/96 (% w/w) lipase/sucrose (g-i) and 4/96 (% w/w) lipase/trehalose (j-l), respectively (modified after **Paper II**).

Even though very similar in molecular structure (sucrose and trehalose are composed of the molecules glucose 1,2-glycosidic bound to fructose and glucose 1,1-glycosidic bound to glucose, respectively), the way sucrose and trehalose interact with water differs somewhat. Compared to sucrose, trehalose exhibits greater interaction with water (Olsson and Swenson, 2020, Lerbret et al., 2005, Branca et al., 2005, Branca et al., 2001, Gharsallaoui et al., 2008). Some studies indicate that trehalose interacts with more water molecules at a given concentration (Lerbret et al., 2005). Other studies imply that trehalose interacts more with water at concentrations below 50 wt%, whilst the interaction is similar at concentrations above 50 wt% (Gharsallaoui et al., 2008). However, the mechanism(s) behind the effects of sugar-water interactions on the foam formation, under vacuum, is elusive and remains yet to be concluded. What can be concluded is that the sucrose-based foams were expanded into the centre of the vial, with almost no material at the walls above the upper surface of the dry foam. In comparison, the trehalose-based foams were more dispersed over the walls, even above the upper surface of the solid foam (**Paper II**). As described in Chapter 1, section *"Appearance"*, the spattering tendency is connected to the physical appearance of the solid foams. Since all the other parameters (lipase activity, matrix crystallinity, RWC) were comparable, the

spattering tendency was the decisive parameter and sucrose was chosen as the matrix former for later studies.

Dextran

Despite sucrose providing good characteristics as a matrix former for biologics dried by VFD, the RWC is about 3–4 % (**Paper II**). In comparison to a freeze-dried material, VFD generates higher RWC (Abdul-Fattah et al., 2007a, Abdul-Fattah et al., 2007b)(**Paper I, II**). Since water already has been established as a plasticizer questions arise regarding the long-term stability at 3–4 % RWC trapped in a sugar matrix former. One way to overcome this issue is by including a high Tg stabilizer in the matrix former, such as dextran (Li et al., 2022).

Dextrans are polysaccharide polymers comprised of (1–6) linked α-D-glucose moieties and some (1–3) branched α-D-glucose moieties (Shukla et al., 2014, Abid et al., 2018). Depending on the size, dextran with a molecular weight of 1 kDa to 500 kDa attain a T_g of 152–226 °C (Larsen et al., 2019). This thesis used dextran with an average molecular weight of 40 kDa (D40).

However, dextran alone interacts insufficiently with proteins (and other biologics) (Thakral et al., 2021, Chang and Pikal, 2009, Allison et al., 2000). Paradoxically, an increase in the chain length of dextran generates better stabilizers (higher T_g) but jeopardizes the protein structure (higher degree of aggregation) (Pikal et al., 1991, Tanaka et al., 1991). Due to steric hindrance in the dextran molecule, the amorphous matrix is expected to contain dead spaces, which are free of intermolecular and intramolecular interactions, such as hydrogen bonds (Imamura et al., 2002). This might explain why dextran alone provides poor protection for proteins. To solve this problem, it has been proposed that a matrix former comprised of a mixture of a disaccharide (sucrose or trehalose) and dextran can provide better stability for dry proteins (and other biologics). In the amorphous matrix, the disaccharide interacts favourably with the protein, whilst dextran enhances the stability (increased T_g) of the matrix former (Allison et al., 2000, Imamura et al., 2002). Another concern is crystallization in the matrix former, which can induce loss of protein activity (Millqvist-Fureby et al., 1999a, Millqvist-Fureby et al., 1999b) (**Paper II**). The inclusion of dextran typically eradicates crystallization in the matrix former (sucrose/dextran) and preserves the biologics' stability (Li et al., 2022). This thesis used a mixture of sucrose/D40 in a weight ratio of 75/25 (% w/w, dry weight) as a matrix former for biologics (**Paper III, IV**).

Mannitol

Bulking agents, such as sorbitol and mannitol, may crystallize during manufacture, and act as structuring components (scaffolders) in freeze-dried formulations. Mannitol is commonly used as a scaffolding agent that acts by crystallizing within the amorphous matrix, thereby providing resistance towards macroscopic collapse during freeze-drying (Wang and Pikal, 2012). In the crystalline form, mannitol exists in four known polymorphs, namely anhydrous α-, β-, and δ-mannitol and mannitol hemihydrate (MHH) (Burger et al., 2000, Nunes et al., 2004). The preferred polymorph is β-mannitol, as it is the most stable polymorph (highest T_m) at ambient temperatures (Takada et al., 2009). Nevertheless, the literature has illustrated that drying technique, processing conditions, and presence of other materials can influence the crystalline form of mannitol and also lead to polymorphic mixtures of mannitol in the final dried material (Liao et al., 2007, Liao et al., 2005, Grohganz et al., 2013)(**Paper I**). Polymorphic mixtures of mannitol can present heterogeneous structural and material properties, which are unfavourable in terms of product quality and stability (Andrieu and Vessot, 2018). Of all the polymorphs mannitol appears in, the least desirable is the metastable MHH as it contains water bound in the crystal. During storage, MHH tends to convert into a more stable polymorph and by doing so releases crystal-bound water, which compromises the stability of the final product (Yu et al., 1999). Slow freezing (freeze-drying and annealing freeze-drying) in combination with the presence of other excipients can promote the formation of the troublesome MHH (Liao et al., 2007, Liao et al., 2005, Badal Tejedor et al., 2020)(**Paper I**). However, the formation of MHH does not have to be the only concern, as another issue may manifest. Mannitol can be converted into an amorphous state, with a reported T_g of 13 °C (Kim et al., 1998).

Figure 14 displays the RWC as a function of fraction amorphous mannitol in the trehalose/mannitol mixtures and the presence of MHH. All trehalose/mannitol mixtures (75/25–25/75, % w/w) present amorphous mannitol solubilized in amorphous trehalose co-existing with crystalline mannitol. Moreover, the trehalose/mannitol mixtures (75/25–25/75, % w/w), generally displayed two T_g (phase separation): the higher T_g represents that of amorphous trehalose; the lower exhibit the mixtures with amorphous mannitol solubilized in amorphous trehalose (**Paper I**). This knowledge on how the drying technique and mixtures of stabilizer (trehalose) and scaffolder (mannitol) influence the matrix former must be taken with caution, as phase separation, formation of multiple polymorphs, formation of amorphous mannitol, and manufacture of MHH all are relevant for the quality of the final protein formulation.

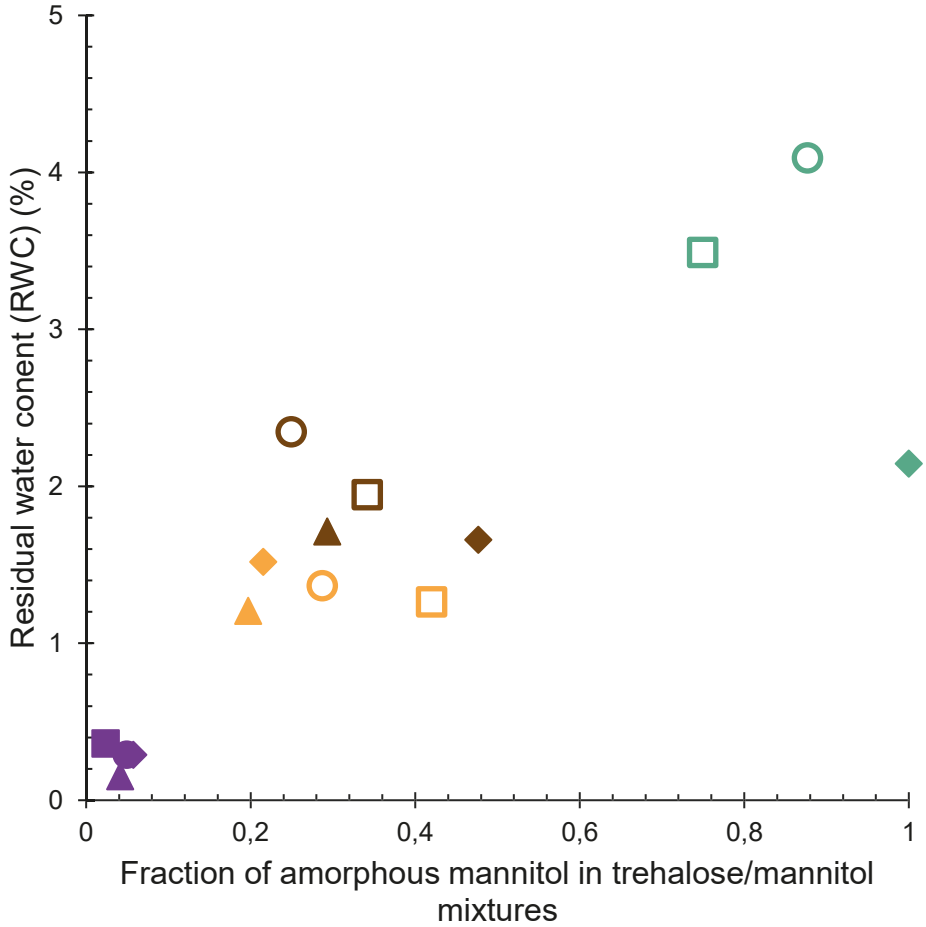

Figure 14. The residual water content of freeze-dried (squares), annealing freeze-dried (circles) spray-freeze dried (diamond) and spray dried (triangles) of trehalose/mannitol with solid content (% w/w) 75/25 (green), 50/50 (brown), 25/75 (orange), 0/100 (grey) as a function of the fraction of amorphous mannitol in trehalose/mannitol mixtures. Open symbols indicate the presence of MHH whilst closed symbols indicate no presence of MHH in the mannitol crystalline structure (modified after **Paper I**).

Surface competitors

Some biologics such as proteins, enzymes and antibodies are amphiphilic and are prone to adsorb to interfaces (vapour/liquid or liquid/solid). Previous studies have connected that the overrepresentation of surface-active proteins at the air/water interface of SD droplets determines the surface composition of the final powder

(Fäldt et al, 1993, Fäldt and Bergenståhl, 1994, Fäldt and Bergenståhl, 1996). The surface adsorption of proteins is noticeable and even at low protein contents (0.1 % w/w) demonstrates prominent overrepresentation (approximately 20 % surface coverage) (Fäldt and Bergenståhl, 1994). Moreover, proteins seem to have a higher affinity to adsorb to the air/liquid interface compared to the ice/liquid interface (Millqvist-Fureby et al., 1999b, Webb et al., 2002, Fäldt and Bergenståhl, 1996). Interactions with the surface can induce conformational change of the protein which can induce aggregation and ultimately loss of activity (Amin et al., 2014, Webb et al., 2002, Arsiccio et al., 2018). The matrix former cannot prevent the protein from interfacial adsorption. Hence, the inclusion of a surface competitor that rapidly adsorbs to the interface can reduce the surface adsorption of proteins and may improve the protein's stability during storage (Millqvist-Fureby et al., 1999a, Nuzzo et al., 2015b). This thesis used two types of non-ionic surfactants: ethoxy ethylene sorbitan ester (polysorbate 20); and alkyl polyglycosides (APG) (dodecyl maltoside). The alkyl chain length was kept constant to only investigate the impact of the surfactant head group, in the presence of the lipase, on the foamability and surface coverage of the lipase.

Polysorbate 20

Polysorbates are amphiphilic, non-ionic surfactants composed of fatty acid esters of polyoxyethylene (POE) sorbitan. This thesis used polysorbate 20 (PS20). PS20 is constituted of a hydrophilic POE sorbitan ring headgroup and a hydrophobic alkyl chain of 12 carbons in length. The sorbitan ring is comprised of three ethylene oxide (EO) polymers attached at three different hydroxyl positions, with a total number of 20 EO subunits. One of the EO subunits is attached through an ester linkage to the alkyl chain. PS20 is one of the most frequently used surfactants for biologic formulations (Gervasi et al., 2018). The adsorption of polysorbates to the air/water interface is fast and has been shown to reduce the surface exposure on the proteins for FD, SD, and VFD (Millqvist-Fureby et al., 1999a, Millqvist-Fureby et al., 1999b, Adler et al., 2000)(**Paper II**). Polysorbates would be ideal surface competitors for protein formulations would not be for a couple of drawbacks. The manufacture of PS20 can result in a heterogeneous distribution of POE with different alkyl chain lengths (Ayorinde et al., 2000). This led to the development of polysorbates with homogenous alkyl chain length qualities, named super-refined polysorbates (Doshi et al., 2020). Even though super-refined, polysorbates need to be stored in a dark and cool environment as light and heat can induce oxidation. Moreover, degradation of polysorbates can also occur by hydrolysis on the fatty acid ester bound which, again, results in heterogenous distribution and quality drawbacks (Kerwin, 2008). However, the most serious drawback is the manufacture of polysorbates as it requires the use of oleochemicals (Nagtode et al., 2023). The cost is low, and the yield is high, but the cost is on the environment. Even though more environmentally friendly than petroleum-based surfactants, PS20 and other

oleochemical-based surfactants have been shown to severely damage wastewater treatment, aquatic life (microbial, fish, and other aquatic lifeforms), and plant photochemical energy conversion efficiency (Sarubbo et al., 2022). As the pharmaceutical industry is moving to a more environmentally friendly approach, this thesis also approached the use of green surfactant options, such as APGs.

Alkyl polyglycosides

APGs are also amphiphilic, non-ionic surfactants, however, composed of a saccharide headgroup linked through an ether-binding to an alkyl chain. APGs have been used in formulated products since the late 1990s and are recognized as environmentally friendly, nontoxic, and cost-effective surfactants (Hill and Ach, 2008, Chatterjee et al., 2015, Nagtode et al., 2023, Sarubbo et al., 2022). This thesis used dodecyl maltoside of α-anomeric composition (aC12G2) (**Paper II, III, IV**).

The surface competition of surfactants and proteins can be investigated through the interfacial adsorption tendency, Figure 15. In comparison to without the presence of surfactants, the interfacial tension of a model protein (lipase) with the presence of either PS20 or aC12G2 reduces and shows promise as an alternative surface competitor to proteins.

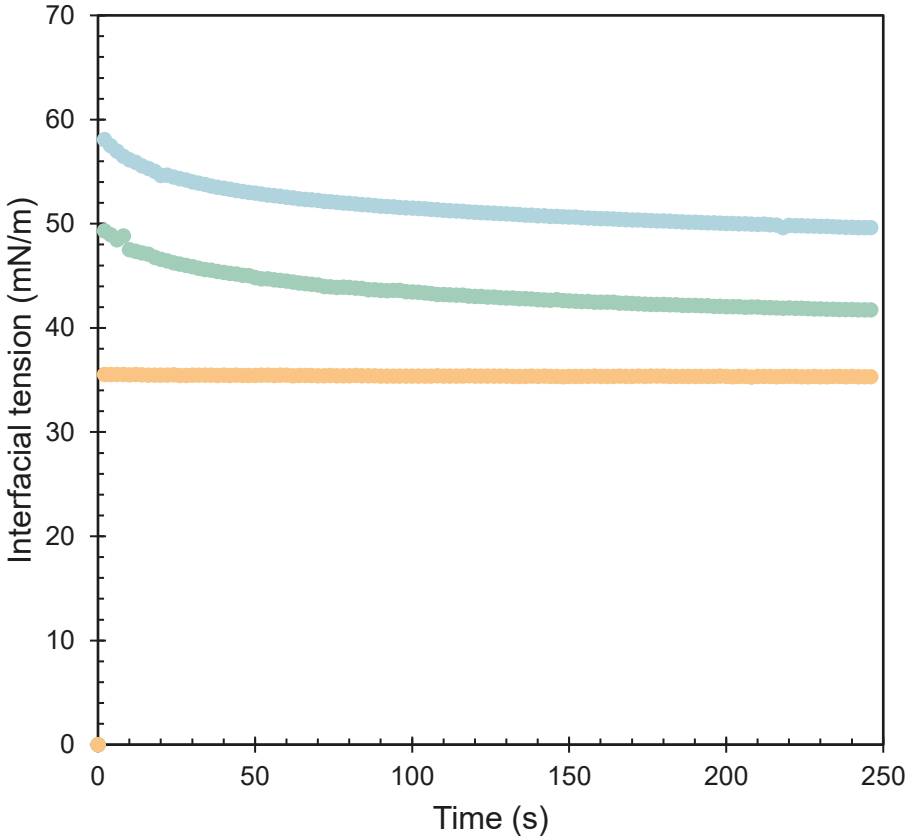

Figure 15. The interfacial tension as a function of time for water solutions consisting of 0.3 % lipase (blue), 0.3 % lipase & 0.03 % PS20 (green), and 0.3 % lipase & 0.03 % aC12G2 (brown), respectively.

Even though the same concentration of aC12G2 is competitive with PS20 on lipase interfacial tension reduction, the same reduction of protein from the surface was not seen for the solid foams (**Paper II**). The surface coverage of the lipase was quantitatively estimated by the use of X-ray photoelectron spectroscopy (XPS), a technique that will be covered later, in Chapter 3 *"Molecular mapping"*. Nevertheless, whilst PS20 effectively reduced the lipase surface coverage, the surface coverage of the lipase remained unaffected in the presence of aC12G2 (**Paper II**). The surfactants differ regarding the size of the head group. PS20 and aC12G2 occupy approximately 60 $Å^2$ and 40 $Å^2$ surface area, respectively (Kanduč et al., 2021, Szymczyk et al., 2018). This implies that the size of the head group may impact the surface coverage of the lipase. In addition, the head group of PS20 is large and flexible, while the head group of aC12G2 is smaller and rigid (**Paper II**). The surfactant concentration was low, 0.1 % (w/w), but well above the critical

micelle concentration. In comparison to 0.1 % (w/w), a ten-fold increase of the surfactant (aC12G2) concentration can and does effectively exclude the proteins from surface adsorption (Adler et al., 2000)(**Paper III**).

Comparison of drying technologies on biologics

This thesis has explored and compared four drying techniques, their corresponding associated time scales, and temperature ranges on placebo formulations and biological formulations. Table 2 summarizes and compares the four used drying techniques' influence on the drying and the product characteristics (**Paper I, II, III**). Note, that all drying techniques produced amorphous matrix formers. In comparison, VFD benefits by generating a material produced from milder conditions (no freezing or elevated temperatures) and shows quite rapid reconstitution. Moreover, the thicker walls can also benefit the encapsulation of larger biologics. However, VFD generates higher RWC values (3–5 % compared to 1 % from FD and SFD). As mentioned earlier, water is a plasticizer which jeopardizes the long-term stability of amorphous materials. A longer secondary drying time can reduce the RWC for VFD materials to as low as 1 %, however, only achieved by use of 90 hours which consumes much energy. For FD materials, an increase in shelf temperature can enhance the rate of water desorption and effectively reduce the secondary drying time (Pikal et al., 1990). Perhaps this applies to VFD materials as well.

Table 2. An overview of included drying techniques influence on drying and product characteristics (**Paper I, II, III**).

	Freeze-drying	Spray drying	Spray-freeze drying	Vacuum foam-drying
Drying time	Days	Minutes	Days	Hours to days
Temperature range	-45–20 °C	80–150 °C	-196–20 °C	10–30 °C
Potential process-induced stress (denaturation)	Cold denaturation, ice/liquid interfacial denaturation	Thermal denaturation, air/liquid interfacial denaturation, shear stress from spray nozzle	Cold denaturation, air/liquid interfacial denaturation, shear stress from spray nozzle	Air/liquid interfacial denaturation
Residual water	1–2 %	3–4 %	1 %	3–5 %
Microstructure	Continuous network, highly porous	Dense particles with some vacuoles	Highly porous particles	Continuous network, large pores
(Particle) Size	N/A[1]	5–10 µm	10–50 µm	N/A
Wall thickness	1–5 µm	N/A	0.1–1 µm	20–40 µm
Dissolution properties	1–5 s	5–10 min	N/D[2]	10–40 s

[1] Not applicable

[2] Not determined

Figure 16 outlines and compares the principal drying pathways (temperature, solute concentration, and water content) of the FD, SD, SFD, and VFD solutions. All solutions start at room temperature (approximately 20 °C).

The (vial) FD solution is cooled below the ice melting temperature (T_m) and continued cooling below T_m induces ice formation. Release of heat increases the system's temperature (Fa_1), whereafter continuous cooling of the sample to the desired final temperature (-45°C). This develops the formation of a metastable two-phase system consisting of solid ice co-existing with a viscous liquid freeze-concentrate that surrounds the ice crystals (Fb_1). The primary drying procedure takes place by lowering the pressure to 10 Pa (Fc). This lowers the product temperature to about -42 °C (the dew point at 10 Pa partial water pressure). As this is below the glass transition temperature of the freeze-concentrate (T_g'), the formulations can be expected to remain as a stable glassy structure. Simultaneously, sublimation is initiated, i.e., the solid ice is removed as water vapour. When all the solid ice has

sublimated, the primary drying is considered finalized. At this point, the remaining water is trapped inside the non-frozen structure. Therefore, the pressure is decreased even further to 0.5 Pa and the temperature is slowly raised to speed up the desorption of water (Fd). The FD process is finalized with an RWC of 1–1.5 % (**Paper I, III**).

The SFD solution is sprayed in liquid nitrogen (about -196 °C) and subjected to rapid (quench) freezing. Quench cooling generates a non-equilibrium freezing. Hence, water is unable to diffuse to the ice crystals before the temperature has decreased below the T_g. This results in a very low T_g, and a droplet temperature that is below the T_g line of water-solutes (Fa_2). As the droplets are transported into a -45 °C pre-cooled shelf (explained in Figure 3) and held for 4 hours, the quench frozen droplets relax and trapped water from the freeze-concentrate diffuses into the ice crystals. This increases the T_g, and the system ends up with a T_g close to the shelf temperature (about -45 °C) (Fb_2). The primary drying and secondary drying follow the same trajectory as for the vial FD. The SFD process is finalized with an RWC of 1–1.5 % (**Paper I**)

The SD solution is pumped and sprayed from the nozzle into the chamber, whereupon heat supplied by the hot gas renders solvent evaporation (removal of water) (Sa). Water is continuously removed which decreases the size of the droplet. Due to evaporative cooling, the temperature of the droplet is maintained at the wet bulb temperature (about 40 °C in the present processing conditions). Heat in the drying air is consumed by the evaporation process. Thus, the temperature of the gas phase is reduced along the spray dryer to the outlet temperature (80 °C). The droplet size proceeds to reduce until a solid crust at the droplet surface is formed (Sb). Consequently, mass transfer limitations slow the evaporation rate, and the temperature of the now solid particle begins to rise and approach the temperature of the surrounding gas (Sc). During powder collection, the temperature cools back close to room temperature. The spray drying process is finalized with an RWC of 3.5–4 % (**Paper I, III**).

The vacuum foam-drying process is initiated by a controlled rate of decrease in pressure to 7 hPa. Water is evaporated at the reduced pressure. Due to evaporative cooling, the temperature is reduced whilst kept above 0 °C by balancing the heat input from the shelf. Vapor bubbles are formed at the bottom of the vials and induce boiling (Va). The boiling proceeds and generates unstable foams, characterized by the formation and breakage of bubbles (Vb). As water is evaporated the viscosity of the system increases. Once the solute concentration is high (at least 80 % w/w) the lifespan of the bubbles is increased so that a stable, but soft (rubbery) foam (Vc) is formed. Further removal of water forms a solid (glassy) foam (Vd). The role of secondary drying in vacuum foam-drying is similar to that of freeze-drying i.e., removal of trapped water inside the matrix structure. The vacuum foam-drying process is finalized with an RWC of 3–5 %, (**Paper II, III**).

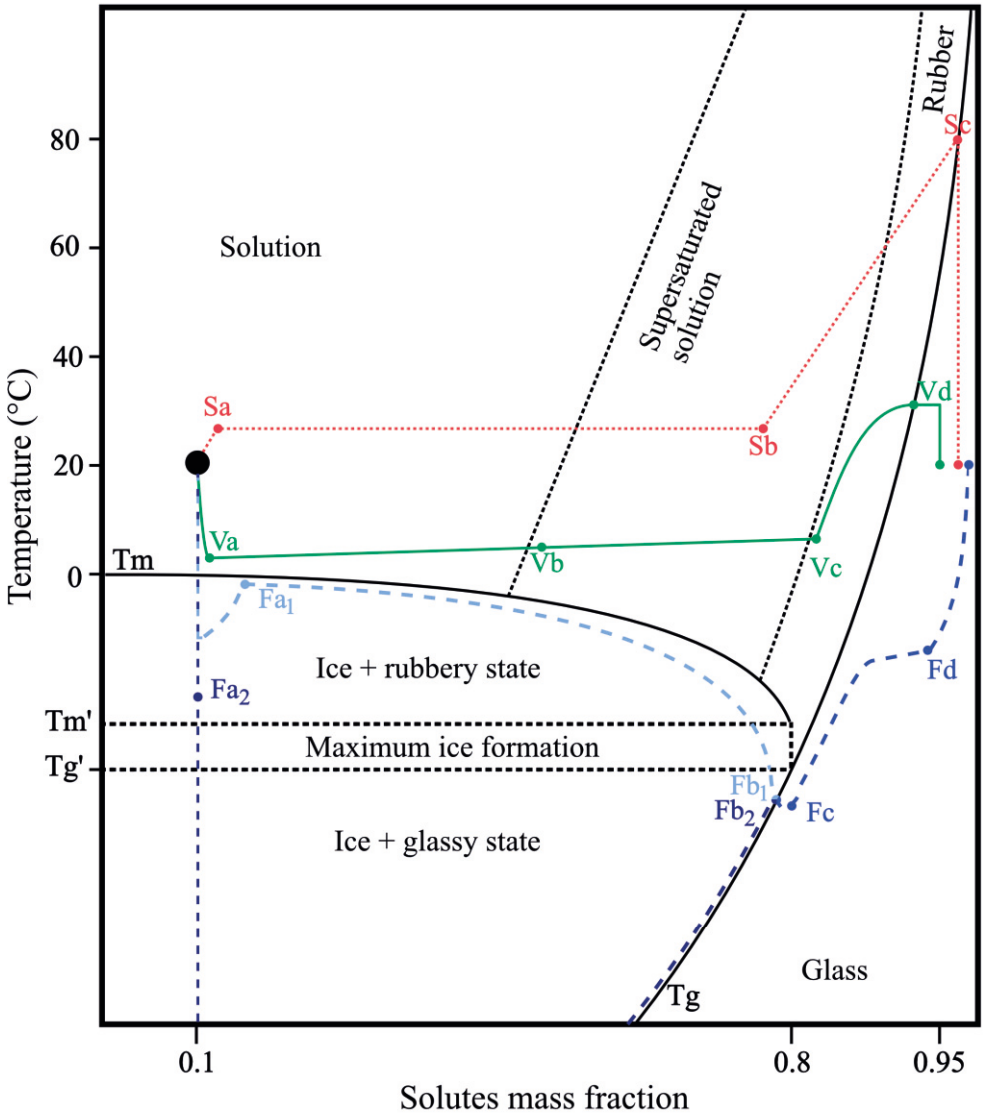

Figure 16. A conceptual state diagram comparing the principal drying pathways for freeze-dried (blue, dashed) with the use of conventional freeze rate (light blue) and fast, spray-freeze (dark blue), spray dried (red, dotted lines) and vacuum foam-dried (green, full lines). T_m is the melt temperature, T_g' is the glass transition of the freeze-concentrate, T_m' is the melt temperature of the freeze-concentrate, and T_g is the glass transition temperature (modified after **Paper III**).

The effect of the drying pathways for the drying techniques, used in this thesis, on the formation of a solid material has been explained and compared. But how does it affect the biologic stability? For this, a recombinant human bile salt stimulated lipase (lipase) was used, and the stability was examined through its activity (**Paper II, III**). The lipase is an enzyme that catalyses the hydrolysis of triglycerides to monoglycerides and free fatty acids. The catalysis occurs through surface adsorption of the lipase at the oil/water interface (Bläckberg and Hernell, 1981). Human lipases possess a small flexible loop that partly covers the active site (Salhi et al., 2021). Contact with hydrophobic domains (oil or vapour) opens the loop and enhances the lipase activity (Salhi et al., 2021, Bläckberg and Hernell, 1981, O'Connor and Wallace, 1985)(**Paper II, III**).

Figure 17 demonstrates the activity of the lipase as a function of lipase content, without or with the presence of the surfactant aC12G2, dried by FD, SD, and VFD (**Paper III**).

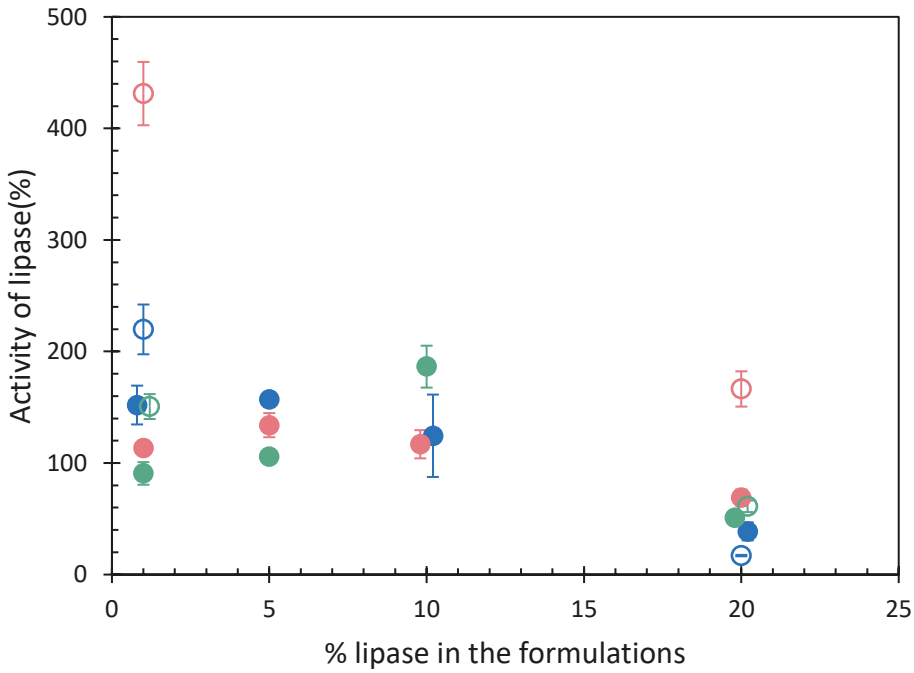

Figure 17. Activity measurements for lipase content 1-20 %/matrix freeze-dried (blue), spray dried (orange), and vacuum foam-dried (grey). Filled circles display without aC12G2 whilst empty circles exhibit with aC12G2. Measurements were performed in triplicates and values were presented as mean values with standard error of the mean (SEM). Overlapping points have been notched in the horizontal direction to improve clarity (modified after **Paper III**).

The lipase displays preserved or enhanced activity, without or with the presence of aC12G2, dried by all drying technologies, up to 10 % lipase content. 20 % lipase content generally demonstrated a loss of activity. The crystallinity of the matrix former is a typical culprit that induces loss of activity on proteins (Millqvist-Fureby et al., 1999a, Millqvist-Fureby et al., 1999b)(**Paper II**). The inclusion of D40 effectively eradicates crystallinity and provides enhanced stability of the matrix former (Li et al., 2022)(**Paper III**). Another culprit that can damage the activity of biologics, as mentioned previously in this chapter *"Surface competitors"*, is the interfacial adsorption of proteins (and other surface-active biologics) (Webb et al., 2002). Loss of activity was demonstrated for all 20 % lipase content without aC12G2. Previous studies have demonstrated that the addition of surfactants can exclude the protein from surface adsorption and thereby preserve the protein stability (Arsiccio et al., 2018). Even though the presence of aC12G2 induced a reduction in 20 % lipase surface adsorption (SD and VFD), the lipase still presented a majority loss of activity (FD and VFD). Consequently, crystallization and surface adsorption were excluded as reasons for the loss of lipase activity. Another phenomenon that might induce the loss of stability of proteins is phase separations (Bogdanova et al., 2023). Instead of stabilizing protein-matrix former interactions, phase separations promote protein-protein interactions, which can induce aggregation and may result in loss of protein stability (**Paper III, IV**).

Differential scanning calorimetry (DSC) is a type of calorimeter that detects phase transformations, by measuring the heat flow difference between a sample and a reference as a function of temperature and/or time. In this thesis, DSC was used to observe the T_m of crystalline material (**Paper I, II**) but more concretely to detect the T_g of the matrix former (**Paper I–IV**). It has been proposed that amorphous materials can exhibit multiple T_g, separated by clear phase transitions (Hancock et al., 2002). Figure 19 displays the DSC curves of 1 % and 20 % lipase/matrix former without or with 1 % aC12G2, dried by VFD.

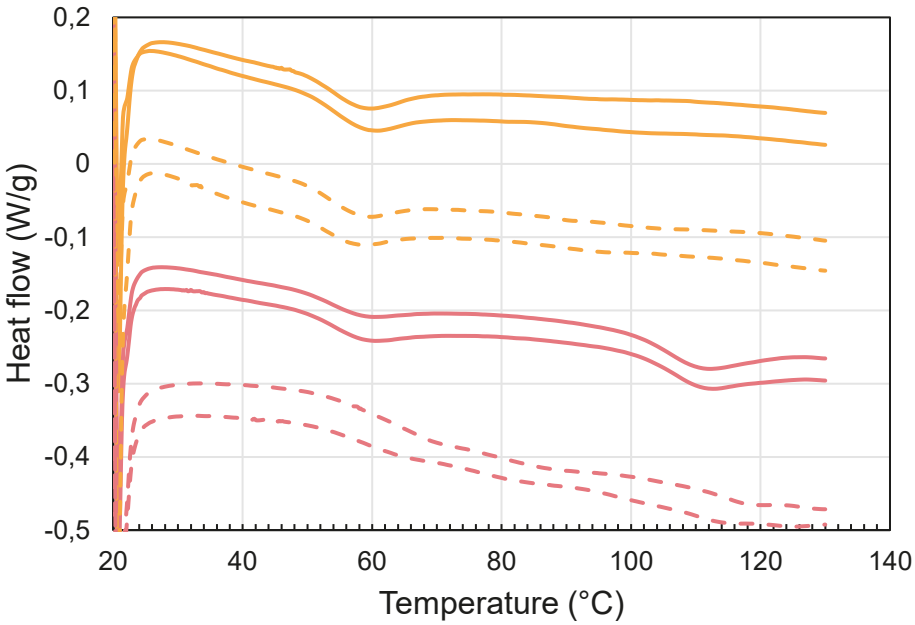

Figure 19. Differential scanning calorimetry (DSC) curves of VFD lipase/matrix (% w/w), 1/99 (brown, full lines), 1/99 & aC12G2 (brown lines, dashed), 20/80 (red, full lines), 20/80 & aC12G2 (red, dashed lines) (modified after **Paper IV**).

The T_g of dry sucrose and D40 present values of 67 °C and 223 °C, respectively (Larsen et al., 2019, Roe and Labuza, 2005). Even though Figure 19 only displays VFD materials, all formulations (1 % without and with aC12G, 5 %, 10 %, and 20 without and with aC12G % lipase content) dried by all drying techniques (FD, SD, VFD) exhibited a distinctive T_g in the region of 44–92 °C. This first region is denoted as T_{g1} (a sucrose-rich phase). As the lipase content increased, another higher T_g was discovered in the region of 105–129 °C. This second region is referred to as T_{g2} (a dextran-rich phase). T_{g2} would be higher if not some of the sucrose was solubilized in the amorphous dextran-rich phase, thereby, plasticizing T_{g2}. As mentioned previously, dextran is are excellent glass formers (high Tg) but poor protein stabilizers (steric hindrance prevents good interactions) (Imamura et al., 2002). Subsequently, for 20 % lipase content (without and with aC12G2) either phase separates into a dextran-rich phase or forms more lipase-lipase interactions. Nevertheless, both situations facilitate lipase-lipase interactions, which may render aggregation, which promotes loss of activity (**Paper III**). Phase separations and how to map the molecular components will be discussed in chapter 3 *"Phase separations and molecular mapping"*.

3. Phase separations and molecular mapping

Introduction: The implications of phase separations and how to map them

Throughout this thesis, in principle, multiple ways (different drying techniques, different matrix formers, and different surface competitors) were utilized, and the product properties were assessed after drying. One major (if not the main) goal of drying is to preserve the stability of the biologics and this was demonstrated through the use of different drying technologies, biologic contents, matrix former, and surface competitors (**Paper II, III**). As discussed in Chapter 2 *"Formulation components"* and specifically Figure 19, a formulation comprised of excipients that presumably should benefit the biologic (amorphous matrix former, surface competitors, mild drying conditions) might be circumvented by the biologic itself. DSC could be used to identify inhomogeneities in the solid materials and successfully identify the protein's inclination to phase separate from the matrix former (Nguyen et al., 2020, Bogdanova et al., 2023) (**Paper III, IV**). However, the sensitivity of DSC is rather insufficient and cannot highlight phase separations at a smaller scale (surface sensitivity) or in a spatial scale (internal composition) (Nguyen et al., 2020). SEM is also a valuable tool for capturing surface morphological events for solid materials. Coupled with an energy dispersive X-ray spectroscopy (EDX), SEM-EDX can measure the elemental composition within a material. However, SEM-EDX lacks surface sensitivity and the ability to quantify and image the surface elemental compositions is poor.

As demonstrated by previous articles, proteins have a propensity to interfacial adsorption and overrepresent to surfaces, affecting the final particle morphology, reconstitution abilities, and aggregation tendency (Fäldt and Bergenståhl, 1994, Landström et al., 2000, Nuzzo et al., 2015a, Andersson et al., 2018). Techniques such as X-ray photoelectron spectroscopy and time of flight secondary ion mass spectroscopy are highly surface sensitive techniques, and combined, quantify and map the elemental composition. (Nguyen et al., 2020). Moreover, proteins can also phase separate internally, within the material. This type of phase separation can occur either for 1) two macromolecular components; or 2) at high concentrations

for macromolecular components from smaller carbohydrates (Nuzzo et al., 2015b, Nuzzo et al., 2015c). Confocal Raman microscopy has been proposed as a useful technique to map the component instances of phase segregation within the material (Nuzzo et al., 2015c). Combined, the use of these techniques allows for a detailed component map (surface and internal composition) that will highlight the occurrence and location of any phase separations.

Techniques used to monitor the surface and internal composition of the VFD materials

As the literature predominantly covers biologics dried by FD or SD, it is no surprise that most of the techniques mentioned in the previous paragraph have been used to monitor the elemental composition of FD and SD materials (Nguyen et al., 2020, Millqvist-Fureby et al., 1999a, Millqvist-Fureby et al., 1999b, Nuzzo et al., 2015b, Nuzzo et al., 2015c). Figure 20 schematically illustrates how each technique is used to map the elemental composition of the VFD material.

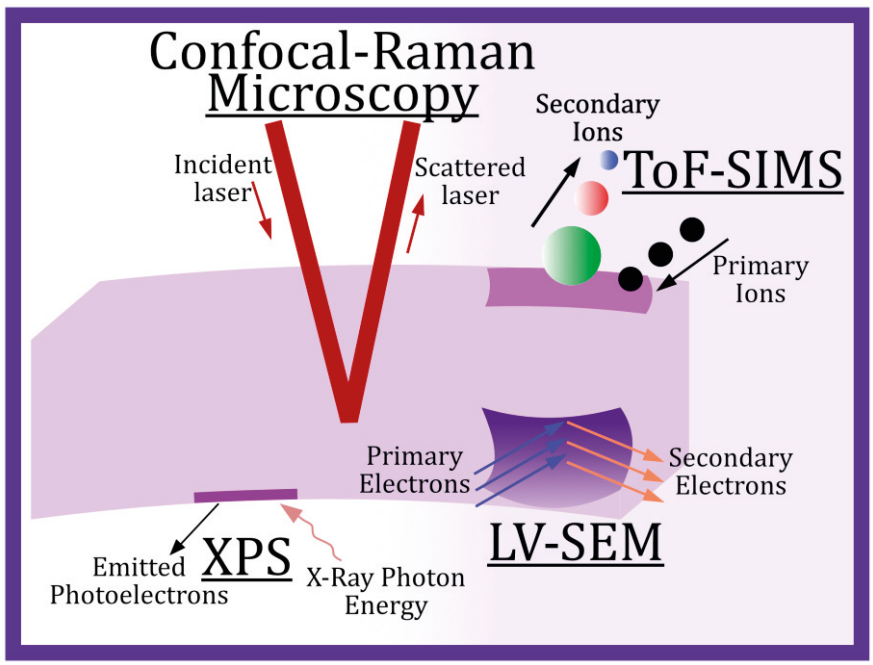

Figure 20. Schematic drawing illustrating the techniques used to map the molecular compositions of the vacuum foam-dried materials.

As mentioned previously in this thesis, the thickness of the VFD material is about 20–40 μm and it might be suitable to obtain the elemental information (surface and internal) by combining the mentioned techniques.

Scanning Electron Microscopy (SEM)

As mentioned earlier in this thesis, SEM was used throughout this thesis to acquire qualitative information with high resolution (**Paper I–IV**). SEM functions by irradiating a material with an electron source under vacuum. At contact with the material, electrons (backscattered electrons and secondary electrons) are released, obtained by a detector and, thus, generating an image of the material morphology. Backscattered electrons show high sensitivity to differences in atomic numbers, however, this thesis provided less useful as the atomic numbers of the used components were quite similar. In contrast, secondary electrons are released from the material surface and offer detailed information on the surface morphology. This thesis used secondary electrons to acquire the SEM images. SEM is a valuable tool for describing the material's morphology but also for estimating the length scales of morphological attributes (walls, pores, dents, ridges) (**Paper I–IV**).

X-Ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), which can also be referred to as electron spectroscopy for chemical analysis (ESCA), provides elemental analysis of solid materials. XPS is highly surface sensitive and quantifies all elements (except hydrogen and helium) with a signal that decays exponentially over an approximate penetration depth of 1–5 nm of the material surface. In order to not mix the electrons emitted from the material with the elements naturally occurring in the air, XPS requires to be performed under a high vacuum (4×10^{-7} Pa). Once under high vacuum, an X-ray source irradiates the material with monochromatic X-ray photons. As the X-ray photons hit the material, core electrons are emitted. Due to inelastic scattering during the electrons' path through the material, most of the electrons lose energy and remain in the material. Only the electrons very close to the surface emit and reach the detector. These emitted electrons contain a specific kinetic energy (E_k). The kinetic energy corresponds to the difference between the photon energy ($h\nu$) and the binding energy (E_b) of the emitted electron and is described through Equation 4:

$$E_k = h\nu - E_b - \phi \qquad [4]$$

where ϕ is the correction constant for the spectrometer work function. This means that the binding energy of each emitted electron is attributed to a specific element

and orbital. Thus, these emitted electrons are used for quantification of the surface composition.

In this thesis, all matrix formers and surface competitors used in the lipase formulations are comprised of carbon (C) and oxygen (O). Besides C and O (and much lower amounts of some other elements), the lipase contains nitrogen (N). Thus, N is seen as a confident indicator to quickly investigate the surface adsorption of the lipase (**Paper II**). While this strategy easily quantifies the lipase composition at the material surface, limitations occur regarding the possibility of quantifying combinations of matrix former and surface competitors at the material surface. The use of the matrix-based Equation 5 can be used to overcome this issue and is explained as:

$$I_{sample} = \gamma \cdot I_{comp.} \quad [5]$$

Where I_{sample} is the vector that contributes the relative signal of each element in the sample, γ is the vector that describes the relative coverage of each element in the material, and $I_{comp.}$ is the matrix containing the elemental composition of the pure components. The methodology is further explained elsewhere (Fäldt et al, 1993). Figure 21 shows the surface composition for VFD lipase/matrix former at ratio (% w/w) 1/99 and 20/80 without and with 1 % aC12G2, respectively.

Notice the variation between duplicate analyses of the same sample. Compared to FD and SD, VFD shows a larger component variability at the surface (**Paper III**). In multiple SD formulations comprised of proteins and carbohydrates mixtures, the frequency of dents and ridges, analysed from SEM images, is an indicator of the presence of protein at the surface (Fäldt and Bergenståhl, 1994, Landström et al., 1999, Elversson and Millqvist-Fureby, 2005, Nuzzo et al., 2015a, Andersson et al., 2018). SEM images of the VFD materials depict domains as completely smooth juxtaposed to domains as highly irregular, Figure 6 (**Paper II**). SEM indicates the formation of domains at the lamellae surface. XPS exhibit a variability of the quantified elemental components at the surface. Complementary information on the formation of domains at the surface might be provided by ToF-SIMS.

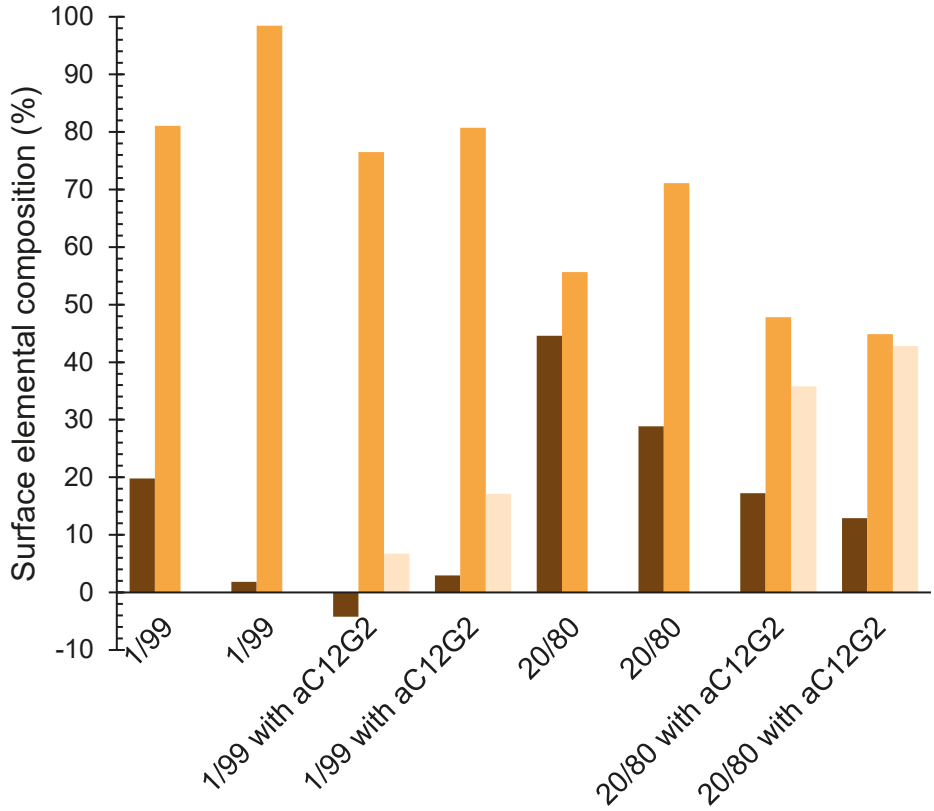

Figure 21. The surface elemental composition of lipase (dark brown), matrix former (brown), and aC12G2 (beige) for VFD (% w/w, dry weight) 1/99 and 20/80 lipase/matrix former without and with aC12G2, respectively. Each measurement was performed in duplicates and all data points were demonstrated in the diagram (modified after **Paper IV**).

Time of flight secondary ion mass spectrometry (ToF-SIMS)

Time of flight secondary ion mass spectrometry (ToF-SIMS) was used for the vacuum foam-dried material and provided imaging of the components at the material surface. Similar to XPS, ToF-SIMS is highly surface sensitive, penetrates depths of 1–2 nm of the material surface and can be used to differentiate between the components at the surface (Rafati et al., 2009). Figure 22 depicts surface images and the elemental distributions of the components (lipase and sucrose) dried by VFD.

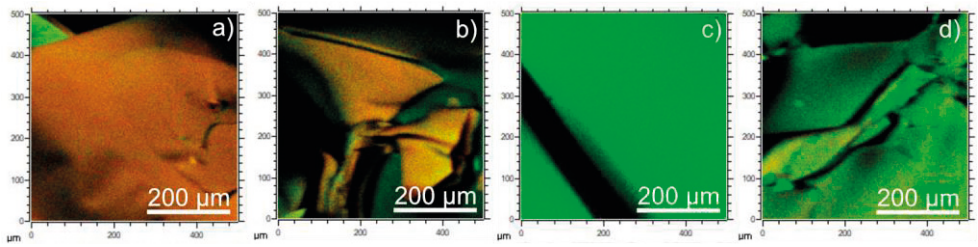

Figure 22. ToF-SIMS surface imaging of vacuum foam-dried lipase/matrix (% w/w), 1/99 (a), 1/99 with aC12G2 (b), 20/80 (c), and 20/80 with aC12G2 (d), respectively. The image demonstrates the overlaying secondary ion image from the diagnostic cations within the ToF-SIMS spectra for lipase (green) and sucrose (red) (modified after **Paper IV**).

During the initial stages of the VFD process (Figure 7, "boiling starts"), boiling generates bubbles that rise to the surface. The bubble wall is thin (less than 1 µm) and seen as unstable (Gramlich and Homsy, 2006). The lipase propensity to adsorb to the vapour/liquid interface is fast, without major diffusive hindrance (narrow film thickness and low viscosity). Removal of water up-concentrates the solutes which enhances the solution's viscosity. During the unstable foam stage, the water content is about 50 % and extends the lifespan of the bubbles before collapse. At high solute concentrations (about 80 %), the viscosity rises to the extent that the bubble lifespan extends until solidification to a soft (rubbery) foam. Provided by SEM, the wall thickness of the lipase formulations is quite large, 20-40 µm (**Paper II, III**). This may reflect the thickness of the last unstable bubbles before solidification into a stable foam. Once stable, the last bubble might age differently. This may impede surface adsorption and might explain why the lamellae morphology shows domains as completely smooth and other domains as highly irregular. Now that the surface composition has been covered in detail, let us delve into the internal composition of the VFD material.

Confocal Raman microscopy

Raman spectroscopy analyses the vibrational modes of elements by scrutinizing the inelastic scattering of photons after interactions with a material. This occurs through the detection of energy changes between incident and scattered photons associated with the stokes and anti-stokes transitions. Thus, identifying structural information about a material. Equipped with an optical microscope (confocal Raman microscopy), enables mapping of the internal composition of materials with a vertical resolution of about 500 nm. Figure 23 maps the internal composition of a VFD system comprised of 1/99 and 20/80 (% w/w, dry weight) lipase/matrix former (matrix former composed of sucrose/D40 at 75/25 % w/w), without or with aC12G2, respectively.

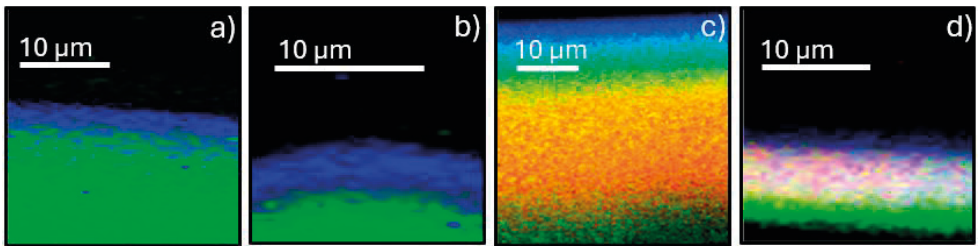

Figure 23. Confocal Raman microscopy mapping of a vertical slide of lipase/matrix (% w/w), 1/99 (a), 1/99 with 1 % aC12G2 (b), 20/80 (c), and 20/80 with 1 % aC12G2 (d), respectively. The image shows the overlaying spectra of sucrose (green), D40 (blue) and lipase (red) (modified after **Paper IV**). Note the different scale bars.

For all formulations, confocal Raman microscopy depicts that the dextran (blue) phase seems to separate and enrich at the top 2–3 µm layer (but also with less intensity below the top 2–3 µm layer), while amorphous sucrose (green) can be detected all across the lamellae except for the top 2–3 µm layer (**Paper IV**). Interestingly, the results by confocal Raman microscopy on all lipase formulations seem to indicate there is a segregation between dextran at lipase, although to a different degree. Moreover, for 20 % (without or with aC12G2), the lipase and amorphous sucrose can be seen in the same domain, indicating a homogeneous mixture. Consequently, confocal Raman microscopy confirms that it is possible to investigate and depict the components' phase separation (Nuzzo et al., 2015c) (**Paper IV**).

Components' impact on the phase separation

Comparisons of all materials (lipase/MTS without or with aC12G2) demonstrate instances of phase separation in different length scales. Figure 24 illustrates the distribution of components in the VFD lamellae: as the solution dries from a homogenous solution, it enters the two-phase region and forms domains (in non-equilibrium), prior to solidification into stable foam (Figure 24a); and how the (internal) components are distributed across the material (Figure 24b). Initially, the components (lipase, sucrose, and D40) are expected to form a one-phase solution. Once the VFD process starts, water is successively removed, and the components (dextran and lipase) pass the binodal curve. At this point, dextran and lipase are gradually subjected to phase separation in the fluid. As more water is removed, the phase separation of lipase and dextran progresses, and the phase-separated regions grow in size. Once the solution viscosity is high enough and the solution solidifies into a stable foam (about 80 % solutes t.s.), the phase-separated regions transition into an arrested state in non-equilibrium. The phase separation is in non-equilibrium, as the lipase and dextran still exhibit some overlap in some regions and complete

absence in others. Partial phase separation also occurs between dextran and amorphous sucrose which explains the occurrence of two T_g (Figure 19).

Notice that phase separation also occurs at the surface. As demonstrated by ToF-SIMS and XPS, the surface shows heterogeneity with lipase-rich domains and matrix former-rich domains. Although the matrix former partly covers the surface, the surface is still predominantly covered by surface-active materials (lipase and aC12G2) (**Paper III, IV**). Consequently, combinations of the utilized characterization techniques are important to depict partial or complete phase separation in different regions of the VFD lamellae.

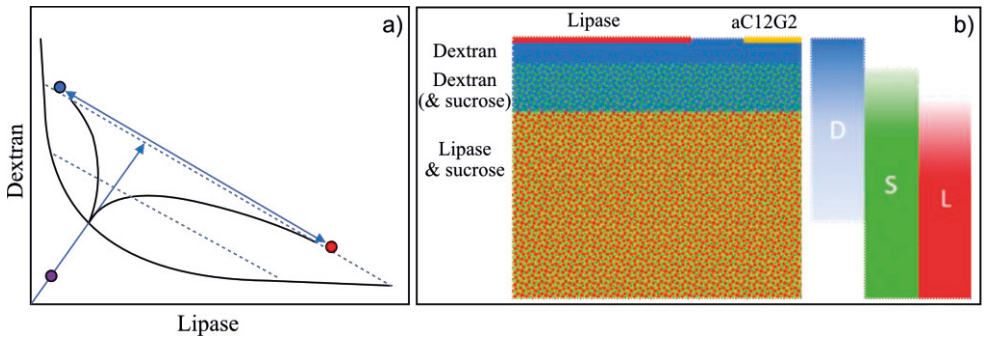

Figure 24. A schematic illustration of the components distribution in the VFD lamellae that depicts a) the phase separation during VFD and b) domains enriched with dextran (D, blue), or lipase (L, red), while sucrose (S, green) distributed in between these two regions, provided by the combined information from XPS, ToF-SIMS, and confocal Raman microscopy. The shading in the bars represents the three components and corresponds to the relative signal intensities. The surface is enriched with the surface-active components lipase (red) and aC12G2 (yellow) (modified after **Paper IV**).

4. Conclusions

This thesis has compared conventional drying techniques (freeze-drying and spray drying) to alternative drying technologies (spray-freeze drying and vacuum foam-drying). The drying techniques' thermal pathways can be described from the liquid state to the dried state. Depending on the material properties, the matrix former can be generated as a glass (completely amorphous) or partial glass (amorphous with crystalline domains).

- Excipients can affect the stability of the matrix former. Scaffolders (such as mannitol) can act as jeopardizers by solubilizing into an amorphous continuous phase **(Paper I).**

It is hypothesized that the presence of a glassy matrix former is essential to preserve the stability of the biologics. In this thesis, a bioactive enzyme (recombinant human bile salt stimulated lipase) was used as a model protein drug to assess the stability (activity) after drying.

- Freeze-drying, spray drying, and vacuum foam-drying processes can be designed to preserve the stability of the lipase in a mainly amorphous matrix (**Paper II, III**).

It is assumed that these results can be extrapolated to most enzymes and most likely to a majority of potential protein drugs.

The process and the formulation parameters have been related to the product properties and the morphological structures and dimensions.

- The wall thickness of the dry, solid foam-dried material is thicker, at least 4 times thicker than the wall thickness of freeze-dried materials (**Paper II and III)**.
- Comparisons of the reconstitution characteristics between freeze-drying, spray drying, and vacuum foam-drying showed that the structures and dimensions determine the reconstitution time (**Paper III**).

The components' distribution upon surface formation was different for the assessed drying techniques. It is assumed that the surface activity can decide the surface coverage after drying.

- Spray dried material showed a predominant surface coverage of the surface-active components (lipase and aC12G2). Vacuum foam-dried exhibited partial surface coverage of the surface-active components (lipase and aC12G2). Freeze-drying demonstrated a low degree of overrepresentation of the surface-active compounds (lipase and aC12G2) (**Paper III**).

The process of vacuum foam-drying has been further developed by investigating the heat conditions, pressure conditions, effect of total load and initial solids content. The surface and internal distribution of the components in the vacuum foam-dried lamellae were mapped in detail and connected to phase separations.

- The critical factor that drives the stabilisation of the foam during the final stage of the primary drying of vacuum foam-drying is the establishment of a rubbery state, rather than viscosity.
- The vacuum foam-dried surface is heterogeneously distributed, and forms domains enriched in lipase and domains enriched in carbohydrate **(Paper II and IV)**. The internal composition highlights the phase separation of macromolecular components, lipase and dextran **(Paper IV)**.

Vacuum foam-drying may show promise as a reliable, predictable, and reproducible drying technology for biologics. It can be expected that if used correctly, vacuum foam-drying can be used as a concept for most dry biologics.

5. Future aspects

This thesis has presented vacuum foam-drying as an alternative drying technology for biologics and enhanced the understanding of how process and formulation parameters affect the stability of biologics and product properties. The results indicate that a successfully vacuum foam-dried formulation presents a matrix former that remains amorphous and a surface competitor that rapidly adsorbs to the surface. Thus, remaining embedded in a glassy matrix and avoiding the dangers from the surface enhances the biologics' functionality. However, this thesis has only studied a protein as a model of active biologics. Therefore, it would be interesting to investigate other types of larger biologics such as antibodies, mRNA, DNA, cells, and live bacteria. The thickness of a vacuum foam-dried material is approximately 4–40 times thicker than a freeze-dried material and may provide full encapsulation of larger biologics such as live bacteria.

As established, the results demonstrate that the residual water content for a vacuum foam-dried product is higher than for a freeze-dried product. For future research, it is suggested to investigate how the biologics activity and matrix formers stability are affected during long-term storage.

Another interesting phenomenon is the rate of surface adsorption on the foaming ability. Even though not discussed, early results indicate that the foaming ability can be predicted from the utilized surface adsorbing component. This can in turn provide further information about the foam's appearance and spattering tendencies.

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