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Understanding the Effect of Arginine and the Freeze Concentrate on Antibody Lyophilisates

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General Table of Content

Chapter 1

Introduction - Established and Novel Excipients for Freeze Drying of Proteins

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1. Introduction

About 90 lyophilised protein drug products are currently marketed in the United States or Europe. These biopharmaceuticals cover monoclonal antibodies, hormones, enzymes, vaccines, and fusion proteins [1, 2]. Proteins are commonly freeze-dried to overcome or reduce instabilities that may occur in liquid state. Stabilisers and excipients are added to form an amorphous matrix, in which the protein molecules are embedded and show enhanced stability [3]. Sucrose is the classically used amorphous matrix former. Two theories can explain its stabilising mechanism: the water replacement theory and the vitrification concept. The water replacement theory relies upon hydrogen bonding between the sugar and the protein molecules, thus replacing the water molecules at the protein surface. Thereby, the sugar molecules stabilise the native conformation of the protein [4]. According to the vitrification concept, protein mobility is substantially reduced in the glassy matrix, and reaction rates between protein molecules and between a protein molecule and, for example, water, oxygen, or other reactive species are decreased [5, 6]. In order to obtain a pharmaceutically acceptable parenteral product, excipients are required to provide protein stability but also isotonicity, physiologically compatible pH, and elegant appearance. In the following we discuss the typically used excipients as well as the potential of new excipients in freeze-drying of proteins. Some excipients fulfil multiple functions; for example, histidine acts as protein stabiliser, reducing aggregation and at the same time acting as buffer, and surfactants can stabilise the protein against freezing stress but also may accelerate reconstitution. In the following, the excipients are structured in a matrix by chemical nature and functionality to improve understanding, which is shown in Figure 1-1.

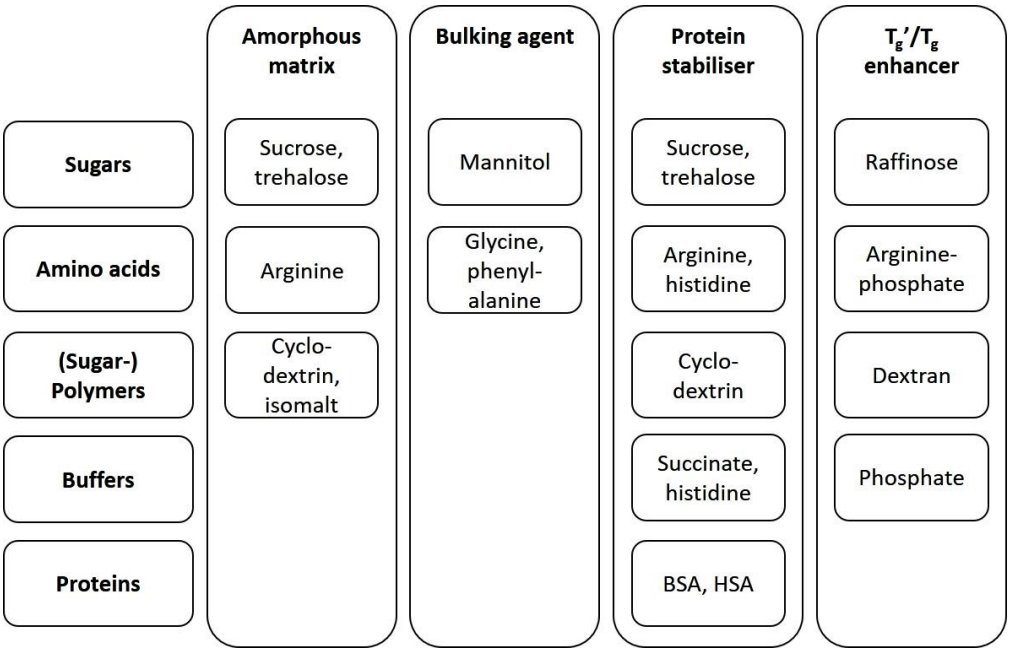


Figure 1-1: Exemplary excipients arranged in a matrix system according their chemical nature and functionality.

2. Sugars and polyols

2.1 The commonly used: sucrose, trehalose, and mannitol

The two disaccharides sucrose and trehalose are most commonly used in lyophilisation as protein stabilisers. They are approved for various parenteral routes. The small and flexible sugar molecules are able to cover the protein surface, forming hydrogen bonds with the protein molecules. Both form amorphous matrices, which are key for protein stabilisation. Thus, they act as both cryo- and lyoprotectant.

Sucrose is the most frequently used sugar in the freeze drying of biopharmaceuticals. Besides the cryo- and lyoprotective effects of sucrose, a key feature is that it is a non-reducing disaccharide. A T_g' of -32°C and a T_g of 74°C make sucrose favourable for both the lyophilisation process and the storage stability [7]. Solid contents of up to 10% (w/w) are suitable to form elegant cakes and to achieve isotonic products after reconstitution [8]. Nevertheless, the glycosidic bond of sucrose may be cleaved, forming the reducing monosaccharides glucose and fructose, which results in Maillard reactions with protein or amino group-carrying excipients [9]. Thus, even more stable stabilisers are pursued. Furthermore, there is interest in sugar matrices with a higher T_g' or T_c to achieve faster primary drying processes [10]. Recently, the pharmaceutical grade of sucrose was found to contain some nanoparticulate impurities [11].

Trehalose has emerged as an alternative to sucrose in lyophilisation after the approval of the first lyophilisate stabilised with trehalose Herceptin®, which contains trastuzumab. The glycosidic bond of this non-reducing disaccharide is more stable than that of sucrose [9]. Additionally, trehalose shows higher T_g' and T_g values than sucrose, with -28°C and 119°C, respectively [7]. Trehalose can crystallise as dihydrate during freezing, which can negatively impact protein stability. Crystallisation can be inhibited by a combination with sucrose, for example [12]. Furthermore, trehalose dihydrate formation occurs mainly during annealing, but upon drying, the dihydrate converts into the amorphous anhydrate [13].

The sugar alcohol mannitol is used on regular basis as bulking excipient for protein lyophilisates. Amorphous mannitol can help to stabilise proteins, such as LDH, β-galactosidase, and l-asparaginase, but has low T_g' values of −32°C and −25°C and a very low T_g of 12.6°C with a high tendency to crystallise [14, 15]. Upon crystallisation mannitol loses its ability to stabilise proteins. Mannitol forms different polymorphs as well as a hemihydrate depending on the crystallisation temperature, rate, and the annealing step during the lyophilisation process [16]. The crystallisation leads to a scaffold, which not only provides pharmaceutically elegant cake appearance but also enables process temperatures during

primary drying above the T_g' or the collapse temperature T_c of the amorphous matrix. Exemplarily, at a mannitol to sucrose ratio of 4:1, primary drying can be run at −10°C product temperature [17]. The amorphous sucrose matrix at this ratio stabilises proteins sufficiently [18].

2.2 Other small saccharides and polyols

Sucrose is chosen widely for freeze drying formulations because of its protein stabilising efficiency. However, amorphous sucrose-based formulations show often relatively low T_g values and can show stability problems when stored at higher temperatures or elevated humidities [19]. Various saccharides are under investigation because of their different physical properties and protein-stabilising effects [20].

Maltose showed the ability to maintain BSA and ovalbumin conformation during freeze-drying, whereas maltooligosaccharides had decreased stabilising effects with increasing saccharide units [21].

Screening different disaccharides regarding their stabilising effect of freeze dried β-galactosidase resulted in about 60% remaining activity after 90 days at 45°C for the following sugars: sucrose, trehalose, cellobiose (glucose and glucose), isomaltulose (glucose and fructose), and melibiose (galactose and glucose). However, cellobiose, isomaltulose, and melibiose all belong to the group of reducing sugars and may therefore induce the Maillard reaction [7].

Owing to their higher molecular weight, trisaccharides require more solid content to reach isotonicity in freeze-drying formulations. This, on the one hand, may provide more amorphous matrix mass in which protein molecules are embedded and could be considered beneficial especially at high protein concentration to reduce interactions. On the other hand, a higher total solid content may slow down drying and delay reconstitution. Furthermore, large saccharides can provide high T_g values [21], but are reported to be less effective in protein stabilisation due to steric hinderance with increasing size [22]. Melezitose, a non-reducing trisaccharide based on two glucose and one fructose unit and produced by plant eating insects, which shows a high T_g of 160°C, was evaluated for lyophilisation of blood and plasma proteins. The stabilising effect for factor VIII was better than trehalose during processing and after storage at 40°C for 4 weeks. Furthermore, melezitose provided good stability of factor IX, rFVIII, pFIX, and rHES-G-CSF lyophilisates over 6 months [23-25].

Raffinose is composed of galactose, glucose, and fructose, but it is a nonreducing sugar owing to its chemical stability. It forms amorphous lyophilisates with a T_g' of −26°C and a T_g of 109°C. Annealing at −10°C results in the crystalline raffinose pentahydrate form, which was dehydrated during primary drying and eventually became amorphous. LDH activity was reduced in the annealed samples, although

the final product containing 5% to 14% raffinose was amorphous [7, 26]. The stabilising potential of raffinose via water replacement appears to be inferior to sucrose. Whereas 100% raffinose resulted in a markedly higher T_g of 37°C at approximately 5% residual moisture than sucrose and raffinose/ sucrose mixtures, the remaining LDH activity was higher with higher sucrose content upon storage at 44°C for 45 days [27].

While mannitol is used as a crystalline bulking agent, other polyols, which form an amorphous phase could act as cryo- and lyoprotectors. Furthermore, small polyols like glycerol and sorbitol can act as plasticisers, excellent hydrogen-bond formers, and void fillers in glasses [28-31].

The plasticising effect of glycerol leads to a T_g decrease and a molecular mobility increase of a sugar matrix [28, 32]. Adding a small amount of glycerol to a trehalose formulation decreased the protective effect of trehalose to lysozyme during primary drying owing to the plasticiser behaviour. In secondary drying, glycerol showed an antiplasticising effect by forming strong hydrogen bonds with trehalose, thereby suppressing fast local dynamics of trehalose [33]. Interestingly, adding small amounts of the plasticiser sorbitol to a sucrose formulation for an IgG1 resulted in a decrease in subvisible particle levels after lyophilisation as well as after subsequent storage at 40°C for 4 weeks, even at a relatively high sucrose to protein ratio of 10:1 [34].

The sugar alcohols maltitol, lactitol, and maltotriitol were able to form amorphous glasses during freeze-drying and prevented activity loss of LDH upon storage even at 50°C. They also protected bovine serum albumin from lyophilisation-induced secondary structure perturbation. In contrast, xylitol, a pentitol, and the hexitols sorbitol and mannitol collapsed or resulted in crystalline solids [35].

The sweetening agent and tabletting excipient isomalt consists of two stereoisomers, 6-O-α-d-glucopyranosyl-d-sorbitol and 1-O-α-d-glucopranosyl-d-mannitoldihydrate. Isomalt forms amorphous lyophilisates, which did not crystallise even at up to 16% residual moisture. Stereoisomer mixtures at four different ratios could not prevent a loss of LDH activity during freeze-drying, but preserved the enzymatic activity better than sucrose during long term stability testing [36, 37]. Overall, the polyols could be options for protein stabilisation, potentially in mixture with sucrose.

Meso-erythritol was identified as potential bulking agent for lyophilisation due to its high crystallisation propensity [38], but it has not been tested with biopharmaceuticals.

2.3 Oligo- and polysaccharides

Dextrans are oligo-glucosides, which stay amorphous during lyophilisation [39, 40]. Pure 70 kDa and 6 kDa dextran show in solution T_g' values of −11°C and −14°C and T_g values of 167°C and 144°C, respectively. Adding 70 kDa dextran to trehalose at a 1:1 ratio led to an increase of T_g' from −28°C to −20°C and of T_g from 88°C to 110°C. However, pure 70 kDa dextran and dextran/trehalose combinations did not improve the stability of insulin, LDH, β-galactosidase, and HBsAg upon storage at 60°C (<10% r.h.) for 4 weeks as compared to trehalose [41]. Along with other polymers, dextrans can cause liquid-liquid phase separation during freeze concentration when used as cryoprotectant [42, 43].

The 4 kDa fructane inulin also results in higher T_g' and T_g values of −17°C and 154°C, as compared to −28°C and 121°C of trehalose, respectively. However, inulin did not improve storage stability of proteins at 60°C for 4 weeks as compared to trehalose, although it performed better than 70 kDA dextran [41].

Another polyglucoside, which can be used as amorphous T_g' and T_g modifier, is maltodextrin, which is obtained by partial hydrolysis of starch. Five-percent maltodextrin with a dextrose equivalent (DE) of 5 or 8 showed a T_g' of -10°C and enables faster drying cycles [18]. Formulations containing maltodextrin DE 5 or 8 and Polysorbate 80 or polyethyleneglycol PEG ensured stability of two *Clostridium difficile* toxins at ambient temperature [18]. In general, the dextrans, inulin, and maltodextrin have chemically reactive end-groups and should therefore be used with care in a protein formulation.

Whereas hydroxyethyl cellulose (HEC) 90 kDa, known as a gelling and thickening agent, provided better protection of LDH against freeze-thaw stress than sucrose, less activity was found after freeze-drying than with sucrose. The HEC lyophilisates had a high T_g of 160°C, but reconstitution took 40 min for 1% HEC [44].

Hydroxyethyl starch (HES) shows similar benefits as dextran on the physical properties of lyophilised proteins (see 3.2.3). Whereas interleukin-11 (rhIL-11) lyophilisates with 2.5% 200 kDa HES as sole excipient showed high T_g, indicating potentially high storage stability, HES failed to inhibit lyophilisation and storage-induced unfolding of rhIL-11. The combination of 2.5% HES with 2.5% or 5% sucrose or trehalose formulation of rhIL-11 resulted in good storage stability through water replacement by the sugar molecules and the increased T_g values arising from HES [45]. The stability of HESylated interferon α-2b was superior to PEGylated, indicating a beneficial effect of HES also in conjugated form [46].

Cyclodextrins (CDs) are cyclic, nonreducing oligoglucosides and can be differentiated into three main classes, α-, β-, and γ-CDs, which are composed of six, seven, and eight glucose molecules, respectively. They are hydrophilic on the outside, have a rather hydrophobic core, and can be used to solubilise and

stabilise poorly water-soluble compounds. Owing to systemic toxicity concerns and to increase water solubility, CDs are functionalised [47, 48] and hydroxypropyl- and sulfobutyl-CDs are approved for parenteral products [49, 50].

With their hydrophobic pocket, CDs can interact with lipophilic amino acid side chains of proteins, ultimately reducing hydrophobic interactions. Thus, CD-derivates can reduce protein aggregation in liquid and could stabilise proteins during cooling, freezing of solutions and upon rehydration of lyophilisates [51].

CD derivates can increase T_g values; for example, freeze-dried β-CD with interleukin-2 (IL-2) had an increased T_g up to 108°C with 2.5%–3.5% RM, which might enhance protein stability during storage [52, 53]. Furthermore, HP-β-CD provides a high collapse temperature T_c of –9°C [54, 55].

At higher concentrations, HP-ß-CD stabilises proteins via water-replacement and vitrification [56]. Addition of HP-β-CD to trehalose reduced the aggregation of an IgG with an optimal mass ratio of trehalose to HP-β-CD of approximately 3.3 to 1 [57]. Both pure trehalose and pure HP-β-CD kept the IgG aggregation level low in formulations containing 80% carbohydrate and 20% IgG [58]. HP-α-CD, HP-β-CD, PM-β-CD, and HP-γ-CD preserved LDH activity during freeze-drying with more than 80% preserved, compared to trehalose with 70% [56].

Low HP-β-CD concentrations of about 0.1% provide a surfactant-like prevention of protein aggregation at interfaces. HP-β-CD may thus be an alternative to polysorbate or poloxamer in protein drug products, especially in case of a low specific surface area of the product [59].

3. Amino acids

Amino acids are commonly used in protein liquid formulations as buffer systems, to stabilise the native protein structure, to enhance protein solubility, or to reduce viscosity at high concentration [60-62].

With respect to protein lyophilisates it is suggested that charged amino acids may be good void fillers of amorphous sucrose matrices. In this respect positively charged amino acids had an advantageous stabilising effect on human serum albumin (rHSA), potentially related to their larger molar volume, as compared to negatively charged amino acids [32]. Amino acids are known for their low T_g' values, for example −62°C for glycine [63] or −42°C for l-arginine and −47°C for l-arginine·HCl [64], which can be critical for the lyophilisation process [65, 66]. Nevertheless, freeze-dried amino acids resulted often in high T_g values, for example about 90°C for l-arginine with citrate or about 100°C for l-histidine citrate, both around pH 6 [67]. Furthermore, amino acids like glycine and phenylalanine crystallise during

freeze drying and can therefore act as bulking agents [66, 68-70]. A glycine to raffinose or trehalose ratio of at least approximately 1.2 to 1.5 is required to render crystalline glycine, which enables manufacturing of elegant lyophilisates even at a primary drying temperature 10°C above T_g'. The combination of sugar and bulking agent prevented loss of LDH activity during freeze drying [71]. At low glycine to sugar ratios, glycine can be present in the amorphous state and is able to improve protein stability. Being a low-molecular-weight plasticiser, glycine can increase solid-state stability of mAbs despite lowering T_g [72]. Amorphous glycine was found to increase the activity of freeze-dried factor V and factor VIII formulations [73].

Arginine has weak affinity to the protein surface, which indicates that the stabilisation effect of arginine is not due to preferential exclusion. It is able to form hydrogen bonds with protein molecules upon lyophilisation, and in addition ion dipole interactions are possible owing to its positive charge [62, 74, 75]. The mechanisms on the molecular level are still discussed, but protein-protein interactions and aggregation are prevented mainly because of weak binding of arginine to the proteins [62].

In mixtures with a hydroxy di- or tricarboxylic acids, arginine and histidine show bell-shaped changes in the T_g' profiles, with low T_g' values for low and high carboxylic acid concentrations. For example, l-arginine and pure citric acid have T_g' values of -44°C and -55°C, respectively, but in combination at a 1:1 ratio for pH adjustment the T_g' is increased up to a maximum of around -25°C [67]. The T_g' of histidine increases from -47°C at pH 4.0 to -31°C upon pH adjustment to 7.7 with hydrochloric acid. Addition of sucrose to the histidine-containing formulation leads to an additional increase in T_g' [76]. Stärtzel et al. freeze dried 50 mg/ml mAb in ratios 16:64 and 64:16 arginine to sucrose, testing chloride, citrate, phosphate, and succinate as counter ions. Major cake defects were observed with chloride, whereas all other formulations rendered only minor defects. The T_g values were between 58°C and 86°C [77]. Addition of arginine hydrochloride to sugar-based BSA lyophilisates reduced protein aggregation during manufacturing and upon storage [78]. Arginine exhibits a stabilising effect also if the product is collapsed, as shown for the aggregation propensity of an IgG1 in arginine phosphate pH 7.3 [79]. Albumin used to stabilise recombinant factor VIII during lyophilisation can be replaced by l-arginine, l-glutamic acid, and l-isoleucine. The mixture of 36 mM arginine, 57 mM glutamic acid, and 7 mM isoleucine was as effective as albumin [80].

Adding histidine to sucrose formulations resulted in decreased aggregation levels of an IgG but a perturbed secondary structure. Solid-state hydrogen-deuterium exchange and Fourier-transform infrared spectroscopy did not predict protection through histidine, and ionic interactions or suppressed dynamics might be the reason for the perturbation [81]. Freeze drying LDH in pure histidine buffer (10 – 150 mM pH 7.3), which resulted in increased enzyme activity with higher histidine

concentration, was found optimal at pH 6, higher than citrate and phosphate buffers [82]. Both for fibroblast growth factor 21 and catalase, testing different formulations demonstrated stabilising effects of various amino acids. Regarding aggregation and bioactivity of fibroblast growth factor 21, a formulation containing 2% mannitol, 2% trehalose, 0.05% glycine, and 0.1% Poloxamer 188 preserved the protein best [83]. Alanine, glycine, serine, arginine, histidine, lysine, 4-hydroxy proline, and threonine showed a stabilising effect on catalase activity, while the stabilising effect other amino acids was weaker and concentration dependent [84].

Phenylalanine, isoleucine, and leucine crystallise during lyophilisation and can therefore be used as bulking agents. Starting at low ratios of 2.5:47.5 amino acid to sucrose, the bulking efficiency improved, and a pharmaceutical elegant cake was obtained at a ratio of 5:45. Neither 2 mg/ml nor 50 mg/ml mAb suppressed crystallization of leucine and isoleucine [68]. Thus, compared to mannitol and glycine, less amino acid is required to form a crystalline scaffold. Consequently, more sugar can be used in the formulation to generate a higher stabiliser to protein ratio.

l-Glutathione can be used as antioxidant to increase the storage stability of proteins, for example, factor VIII [85]. An antioxidative effect can also be achieved with methionine already at rather low concentrations [2, 86, 87]. Exemplarily, some commercial hormone (Pergoveris®, Gonal-f®) and coagulation factor (NovoEight®, NovoSeven®) products contain methionine as antioxidant.

Glycylglycine is used as buffering agent for stabilisation of freeze-dried factor VIIa (NovoSeven®), which showed, along with glycine, stabilising effects during freeze-thaw experiments [88], but a specific stabilising effect in lyophilisation has not been reported yet [89, 90].

4. Polymers

Dry amorphous polymer matrices may stabilise proteins similarly to sugar matrices (see also 3.2.3). They were thought to even be superior owing to their high T_g values, as already described above in sugar polymers (see 3.2.3). However, they are less capable of water replacement, and therefore combinations with small-molecule excipients are typically required to achieve adequate protein stability. Polymers may also prevent proteins from aggregating owing to their surface active properties, through sterical hindrance of protein-protein interactions as well as via increased viscosity, reducing protein structural movement [91, 92].

However, polymers may trigger phase separation, which can have a detrimental effect on protein stability [93].

Adding up to 20% PEG decreased the T_g' of 10% sucrose to −48°C. Upon annealing at −25°C, PEG crystallised [94]. Higher PEG concentrations show the risk of phase separation, and the protein may concentrate in one phase, increasing the risk of aggregation [93, 95]. Initially amorphous PEG is prone to crystallise during primary drying, which could induce protein aggregation. Interestingly, this crystallisation could be inhibited by higher sucrose amounts [96]. A collapsed lyophilisate containing LDH with sucrose or trehalose to PEG at a ratio of 3:2 showed the same monomer content and subvisible particle level as a noncollapsed cake [79]. On long-term stability at 40°C for 26 weeks, the collapsed cakes remained the same or showed higher monomer content and less subvisible particle level as noncollapsed cake [97]. Interestingly, trehalose glycopolymers (2–50 kDa) resulted in higher remaining activity than polyethylene glycol after freeze-drying and thermal stress [98].

Adding copovidone, a copolymer of 1-vinl-2-pyrrolidone (60%) and vinyl acetate (40%) to a lysozyme formulation without further excipients maintained biological activity and conformation integrity during freeze-drying and subsequent storage [99]. Similar results were found for PVA on the stability of β-galactosidase [100].

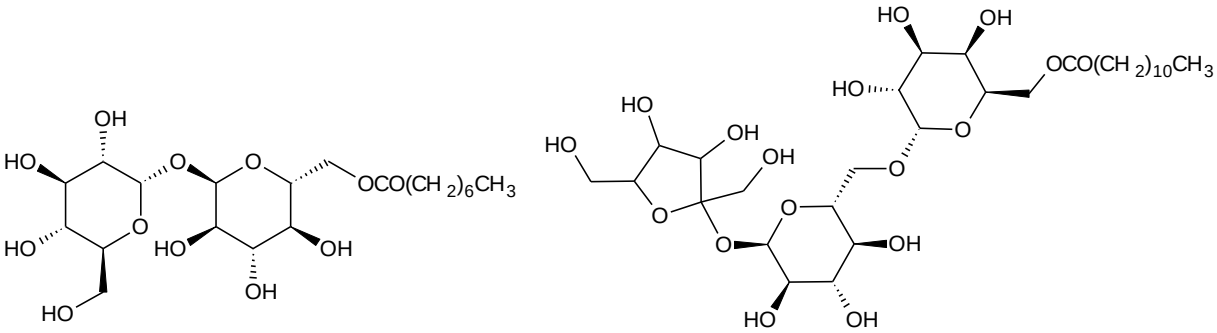
5. Surfactants

During freezing, proteins are exposed to the ice-water interface, which can contribute to freezing-induced protein denaturation and aggregation. By addition of surfactants, proteins can be protected from both freezing- and surface-induced denaturation [101]. Surfactants may also potentially foster protein refolding, reducing aggregation in the reconstitution step [31, 102]. The most commonly used surfactants in commercial lyophilized protein products are polysorbate 20 and 80. Exemplarily, polysorbate 80 is used for Benlysta®, Inflectra®/Flixabi®, and Empliciti®, and polysorbate 20 for Xolair® and Herceptin® products [2]. Polysorbate is prone to degradation, such as oxidation, autoxidation, and hydrolysis, which are influenced by various factors including pH, temperature, and oxygen level. Degradation is more pronounced in liquid than in lyophilised state [103-105].

Poloxamer 188 is a water-soluble surface-active polyoxyethylene-polyoxypropylene triblock copolymer. It can also compete with the protein for interfaces, preventing adsorption-induced conformational changes and aggregation [75], and is exemplarily used for Gazyvaro®.

Trehalolipids are reported to have surfactant-like abilities [106, 107]. They are of interest as stabilisers for lyophilisation processes owing to their glass-forming ability [108]. Sugar-based surfactants can be used as alternative to polysorbate eliminating the instability-prone polyoxyethylene moiety [107]. 6-*O*-octanoyl trehalose and 6-*O*-lauroyl raffinose, illustrated in Figure 1-2, showed a better efficiency

in maintaining LDH activity to pure trehalose. The T_g of both sugar-based lipid formulations was as approximately 85°C [109]. Trehalolipids did prevent aggregation of IL-11, similarly to polysorbate 80, during freeze-drying and upon short term storage at 50°C for 1 week [107].



6-*O*-Octanoyl trehalose

6-*O*-Lauroyl raffinose

Figure 1-2: Structures 6-O-octanoyl trehalose, and 6-O-lauroyl raffinose.

6. Buffer

Protein injectables typically require pH adjustment and stabilisation with buffers. Exemplary well-known buffer systems are histidine (see amino acids in buffer systems in section 3) and phosphate, succinate, or citrate [110]. Buffers used in commercially available freeze-dried antibody drugs are shown in Table 1-1. Some buffer species can selectively crystallise, causing pH changes. Disodium phosphate has lower solubility than monosodium phosphate, potentially leading to earlier crystallisation upon concentration during freezing and causing a pH shift of up to 3 pH units [111-114]. This effect is less pronounced for potassium phosphate buffers [115]. Furthermore, succinate buffer is prone to crystallisation and therefore to pH shifts, which can be inhibited by amorphous sucrose, trehalose, glycine, and mannitol [116]. Higher antibody concentrations show self-buffering characteristics as well as inhibition of the beforementioned pH shifts. At 50 mg/ml, antibody can act similar to 6 mM citrate or 14 mM histidine buffer [117].

Buffers not only affect protein stability via the pH but also by potential direct interaction or changing the mobility in the glassy matrix. During freezing protein stability can be increased by phosphate and citrate, which do not act as strong hydrogen-bonds formers, in formulations with stabilisers such as sucrose, which is preferentially excluded in solution [118]. It is often proposed to use low buffer salt concentrations owing to their ability to act as a plasticiser in sugar-based systems and thereby diminish

protein stability. Since buffer salts as well as sodium chloride added as tonicity agent or resulting from pH-adjustment with acid or base decrease the T_g' of the formulations, the collapse tendency is increased [119]. However, the dibasic phosphate ion HPO_4^{2-} is also able to increase the T_g of sugar formulations at higher pH values. While trehalose showed a T_g of 94°C at different pH values, the T_g was increased by phosphate to 119°C at pH 7.5 [120]. Through self-aggregation of HPO_4^{2-} ions, a hydrogen network with trehalose is built, whereas the monobasic phosphate ion $H_2PO_4^-$ does not form such network and decreases the T_g, acting as a plasticiser [121].

Carboxylic acids and their sodium salts, such as sodium citrate, protected the secondary structure of BSA and IgG better with increasing concentrations. This suggested stabilisation through direct interactions by substitution of water molecules [122]. Furthermore, addition of sodium citrate to a sucrose formulation led to an increase in T_g from 70°C up to 150°C at a sodium citrate to sucrose ratio of 3:1 [123]. The stability of highly concentrated mAb (40 – 160 mg/ml) freeze-dried with sucrose or trehalose was substantially improved in the presence of succinate buffer as compared to a buffer-free system [124]. Furthermore, sodium tetraborate increased T_g' and T_g of BSA / sugar formulations [21]. The buffering agent dimethyl-succinate was found to stabilise lignin peroxidase as effectively as sucrose and enabled fast primary drying [125].

Table 1-1: Commonly used buffers for freeze-dried antibody drugs commercially available, adapted from Gervasi et al. [2].

Buffer	International Nonproprietary Name (INN)	Trade Name
Citrate	belimumab	Benlysta®
	blinatumomab	Blincyto®
	brentuximab vedotin	Adcetris®
	etoluzumab	Empliciti®
Histidine	canakinumab	Cosentyx®
	omalizumab	Xolair®
	pembrolizumab	Keytruda®
	secukinumab	Cosentyx®
	siltuximab	Sylvant®
	trastuzumab	Herceptin®; Herzuma®; Kanjinti®; Ontruzant®
	vedolizumab	Entyvio®
Lysine	blinatumomab	Blicyto®
Phosphate	basiliximab	Simulect®
	infliximab	Flixabi®; Inflectra®; Remicade®; Remsima®
	gemtuzumab ozogamicin	Mylotarg®
	mepolizumab	Nucala®
Succinate	trastuzumab emtansine	Kadcyla®
TRIS	inotuzumab ozogamicin	Besponsa®

7. Others

7.1 Proteins

HSA, also in the recombinant form, can prevent protein adsorption to surfaces and act as water replacement molecules, maintaining protein activity [111, 126]. HSA is used mostly for solubilising and protecting low-dosed and hydrophobic proteins such as cytokines, coagulation factors, or botulinum toxin [87, 126]. The utilisation of HSA in parenteral drug products comes along with immunogenicity concerns because of the proteinaceous nature of the excipient and the potential formation of mixed

aggregates. Additionally, HSA impacts protein analytics. Replacement of HSA in lyophilised products can be achieved by combining surfactants with an amino acid like glycine, arginine, or histidine [126-128].

Other protein excipients that are not approved for use in human parenteral drug products are intrinsically disorders proteins (IDPs). IDPs can act as cryoprotectants, and an IDP to LDH ratio of 10:1 resulted in similar activity levels after freeze-drying as BSA [129]. Comparing different proteinaceous additives, only the weakly acidic proteins BSA and ovalbumin stabilised LDH as well as other enzymes; however, the effectiveness was limited as compared to sugars [130].

7.2 Alcohols

In the past, cosolvent systems have been used to improve dissolution characteristics and drying times of lyophilised products [131, 132]. The addition of 0.5% and more TBA to a albiglutide (GLP-1 agonist) formulation resulted in a reduction of reconstitution time [133].

7.3 Preservatives

Multidose protein formulations require preservation. Intron A® (Interferon α-2b) is a commercially available multidose product that contains benzyl alcohol as preservative in the solid. In other cases, benzyl alcohol is part of the reconstitution medium, for example, in Novarel®, Pregnyl®, and Profasi®, all three containing human chorionic gonadotropin, and Nutropic®, containing human growth hormone [89]. Benzyl alcohol can substantially decrease the protein aggregation temperature and can increase protein aggregation in liquid [134]. Reconstitution of rhIL-1ra with 0.9% benzyl alcohol in water resulted in a higher level of aggregation than reconstitution with water for injection. Nevertheless, once in a liquid state, benzyl alcohol did not accelerate aggregation of rhIL-1ra at room temperature [135]. CroFab® (Crotalidae polyvalent immune Fab) contains thiomersal as preservative in the lyophilisate [89].

8. Expert opinion

Excipients are crucial for providing the chemical and physical stability of protein drugs in lyophilisates. The excipients serve different functions and sometimes combine several roles. A substantial list of protein-stabilising excipients has been identified. However, in most cases formulators stick to the good

old approved sucrose, or potentially trehalose, combine it with surfactant and buffer selected from a small portfolio, if necessary, complement this with crystalline-bulking agent, typically mannitol, and specific stabilisers, which usually function both in the liquid and the dry state.

Sucrose is the gold standard for providing an amorphous matrix and water replacement. Only a few sugars and amino acids are good alternatives, specifically trehalose, HP-β-CD, or arginine. Bulking agents provide pharmaceutically elegant cakes and potentially enable faster primary drying. Mannitol plays the leading role, with glycine, phenylalanine, leucine, and isoleucine as potential other options. Cake elegance also comes with high protein drug content and increased total solid content. Overall, the need for new bulking agents is low, except for bulking agents that can be used at lower concentration in order to keep the sugar stabiliser content high considering isotonicity. In this context reconstitution time, which can become very long at high protein drug concentration, may need to be considered upon excipient selection, as total solid content and cake structure affect the water penetration into the lyophilized cake.

Some excipients under evaluation do not lead to improved protein stability. Instead they provide higher T_g' for more robust primary drying; higher T_g is not necessarily correlated with stability and lyophilisate elegance. A few larger saccharides and polysaccharides like melezitose, raffinose, inulin, dextran, maltodextrin, HEC, and HES serve this function.

The most suitable pH is often identified already in a liquid screening. The buffer should be selected considering its crystallisation tendency and potential pH shift upon freezing, which can harm the protein and may lead to a discrepancy between the pH value adjusted in the liquid state and the theoretical pH in the solid state.

In most cases, surfactant is added, again selected from a small group consisting of polysorbate 20, polysorbate 80, and poloxamer 188, although many more surface-active molecules would be available. Addition of an antioxidant is advisable in the case of highly oxidation-sensitive protein molecules. The need for a preservative is rare and it may also be a part of the reconstitution medium.

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Chapter 2

Objectives of the Thesis

Lyophilisation is a commonly used method to prolong the shelf life of biopharmaceutics [1]. Understanding the process and its parameters during the three phases freezing, primary drying, and secondary drying, are key. The freezing step gained special interest amongst researchers as it impacts the entire process and the final product. This first step affects intra- and inter-vial uniformity [2], primary and secondary drying rate and product temperature [3], physical state of the sample [4], residual moisture [5], reconstitution time [6] and also protein stability [5, 7].

During freezing, ice crystallises leading to an upconcentration of solutes in the unfrozen aqueous phase forming the freeze concentrate (FC) [8]. The question arises what is the exact composition of the FC, specifically how much water is unfrozen and how is the protein stability in the FC. On the one hand side the FC is extremely viscous leading to an increased colloidal protein stability due to decreased mobility and reaction rates [9, 10]. On the other hand side instabilities can be triggered in the FC due to several reasons. A potential phase separation rendering a protein-rich phase with a lower stabilising excipient to protein ratio can occur [11-13]. Furthermore the concentration of charged solutes is very high, corresponding to a high ionic strength which can result in shielding of repulsive charges at the protein surface which are important for colloidal stability [14, 15]. In addition, a pH shift due to the temperature change and crystallising buffer components can occur altering the protein stability [16-19]. Independent of the protein environment in the FC phase, the ice-FC interface is prone for protein unfolding [20] and the freezing process impacts the ice-FC interface area [21-26]. Dependent on the pH, excipients and protein concentrations, proteins can undergo cold denaturation [24].

After ice crystallisation is completed at the end of the freezing step [27], the FC formed has an immensely high total solid content of around 70% to 80% (w/w) and 20% to 30% unfrozen water [28-31]. Comprehensive understanding of the FC composition and analysis of the protein stability in the FC will help to improve lyophilisation processes.

Thus, the first aim of this thesis was to establish an easy DSC method to determine the composition of the FC. Subsequently, the solid content in different antibody containing formulations was to be analysed (Chapter 3). In a next step, a methodology needed to be established to prepare the FC. With the FC at hand, the influence of the upconcentrated solutes on protein stability with a focus on protein aggregation and protein self-interaction was to be studied (Chapter 4).

Sucrose is most frequently used gold standard as amorphous matrix former in lyophilisation of biopharmaceuticals due to its cryo- and lyoprotective effect [1, 32]. Despite being a non-reducing sugar with a moderate T_g' of -32°C and high T_g of 75°C, which makes sucrose favourable for both the freeze-drying process and storage stability, long-term storage at elevated temperature may lead to breaking of the glycosidic bond, formation of reducing glucose and fructose units ultimately leading to protein glycation [32, 33].

Next to disaccharides such as sucrose and trehalose some amino acids are used in freeze-drying in combination with sugars due to their buffering capacities sugars, specifically histidine [34], their ability to form a crystalline bulking matrix, specifically glycine [35, 36], the formation of an amorphous matrix with void filling effect e.g. lysine [37], or a specific positive effect on protein self-interaction and aggregation as in case of arginine [38].

Among the glass forming amino acids, arginine stands out as protein stabiliser due to its ability to reduce protein-protein interactions and aggregation in both liquid formulations and lyophilisates, depending on protein, further excipients, and concentrations [38-40]. Arginine is a basic amino acid resulting in a high pH in aqueous solution above 10 and exhibits a low T_g' of -42.7°C [38, 41]. Typically, the hydrochloride form is used in protein formulation. Its T_g' of -46°C is well below that of sucrose, but the lyophilisates show a high T_g of 74°C. Other counter ions such as citric acid, phosphoric acid, and succinic acid result in a substantially increased T_g' and T_g [41].

The second objective of the thesis was on freeze-dried protein formulations without the need for sugars based on arginine. At first the effect of different counter ions, citrate, hydrochloride, lactobionate, phosphate, and succinate on cake properties and antibody stability was studied in detail considering effects of e.g. pH, antibody concentration and sucrose addition (Chapter 5). As the arginine lyophilisates show high T_g values also at higher residual moisture and due to the known risk of overdrying [42], the influence of the residual moisture content on protein stability in arginine lyophilisates was to be studied (Chapter 6). Arginine hydrochloride shows the best protein stabilisation amongst the arginine salts. But it comes with low T_g' and product collapse. Finally, to combine the excellent stabilising with good cake properties different approaches, e.g. high protein concentration, combination with sucrose as excipient providing a higher T_g' and mixtures with the crystallizing bulking agents mannitol and phenylalanine were evaluated (Chapter 7).

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Chapter 3

Method Development and Analysis of the Water Content of the Maximally Freeze Concentrated Solution Suitable for Protein Lyophilisation

The following chapter has been published as research article in the European Journal of Pharmaceutics and Biopharmaceutics and appears in this thesis with the journal's permission:

Ivonne Seifert, Alessandro Bregolin, Davide Fissore, and Wolfgang Frieß
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The supplementary data was inserted into this chapter.

Abstract

During freeze-drying of a liquid formulation, a freeze-concentrate is formed in the first phase, the freezing step. Understanding the composition of the maximally freeze concentrated solution can help to judge the process stability of biopharmaceuticals during lyophilisation. Our objective was to develop a suitable method to determine the water content of the maximally freeze concentrated solution using differential scanning calorimetry (DSC). Three different methods were compared: (i) the intercept of the glass transition temperature of the maximally freeze concentrated solution T_g' and the melting temperature T_m for a concentration series, (ii) the linear regression of the melting enthalpy starting from the onset of T_g' until the end of the melting event for a concentration series, and (iii) a one-point determination of the amount of unfrozen water. While Method 1 is accurate but requires the analysis of a high number of samples, Method 3 requires only one single sample, with a loss of accuracy. Method 2 works best taking sample preparation and accuracy into account. Various systems containing sugar (sucrose, trehalose) and other excipients (histidine buffer, phosphate buffer, sodium

chloride, arginine hydrochloride, arginine citrate) were evaluated with different antibody concentrations to evaluate the composition of the maximally freeze concentrated solution. The freeze concentrates exhibited a water content of 20 - 30%, slightly dependent on the excipients, but independent of the antibody concentration. The methodology we developed is broadly applicable for the analysis of the composition of maximally freeze concentrated solutions and can help to elucidate protein stability during lyophilisation.

Keywords

T_g', Maximally freeze concentrated solution, Unfrozen water, DSC, mAb, Protein

Table of Contents

1. Introduction

Cooling of aqueous solutions below the freezing temperature leads to formation of ice. The crystallisation of ice corresponds to a removal of water from the system and an up-concentration of all solutes remaining in the unfrozen phase. This process is known as freeze concentration. During freeze drying of biological material or biopharmaceuticals, freeze concentration affects the behaviour of the proteins through solute concentration, potential crystallisation of buffer components accompanied by a pH shift, phase separation, increased viscosity, increased ionic strength, or interfacial stress [1-7]. An increased viscosity during freezing can lead for example to phase separation with a protein-rich phase, potentially lacking stabilising excipients [2, 4, 8]. On the other hand, reaction rates can be reduced with increased viscosity [9, 10]. A pH shift through buffer component crystallisation and increased ionic strength can change the protein-protein interactions [11, 12]. Additionally, freeze concentration is accompanied with both thermodynamic and kinetic changes causing either stabilisation or destabilisation of a protein. It also need to be kept in mind that since freeze concentration continues until ice formation is complete and that therefore protein perturbation occurs over a longer time frame [13].

The freeze concentrated solution is defined by the mass fraction of solids w_g' and the mass fraction of unfrozen water w_{uf} [14]. W_{uf} and w_g' can be determined by techniques such as cryoscopy and differential scanning calorimetry (DSC). Another approach is to follow the phase change state via the product temperature during ice nucleation within the freeze drying itself [14-20].

Different methods are described in literature to calculate w_g' using DSC. One approach is to measure a single sample. The amount of frozen water in the sample can be calculated from the ice melting endotherm by taking reference to the ice melting endotherm of pure water. Finally, w_g' can be obtained based on the total solid content of the solution via the amount of unfrozen water [21, 22]. Typically, the melting enthalpy of pure water is compared with the melting enthalpy of a 20% w/w solution. For sucrose, trehalose, and glycerol w_g' can be 64%, 83%, and 54%, respectively [21]. A w_g' of 68% for glycerol results if using a stepwise evaluation of the apparent melting heat and calculating the ice fraction. Thereby, the sample temperature is alternated in a series of heating and isothermal steps [23]. Different w_g' values for different solid contents are observed for this type of one point determination [24]. Since ice vitrification might not be completed after a simple freezing step, annealing should be applied. By this a higher sucrose w_g' of 81% (w/w) is obtained [25].

In contrast to a one point determination method, two approaches using intercepts of fits are possible to determine w_g' based on a concentration dependent phase diagram (Figure 3-1). Literature is divided over the glass transition temperature of the maximally freeze concentrated solution, stating two

transitions as T_g' and T_g''. The Gordon-Taylor equation can be used to fit exemplarily the glass transition of sucrose-water mixtures at different ratios. The intercept of the T_g curve as a function of sucrose concentration and T_g'' at approx. -45°C for sucrose renders w_g'. Alternatively, the intercept of the melting temperature (T_m) as a function of sucrose concentration curve and T_g' at approx. -34°C for sucrose leads to w_g'. For sucrose a w_g' of 81 - 83% (w/w) results with both methods [26-31]. For trehalose different w_g' values, ranging from 70% to 83% (w/w), are reported [32, 33].

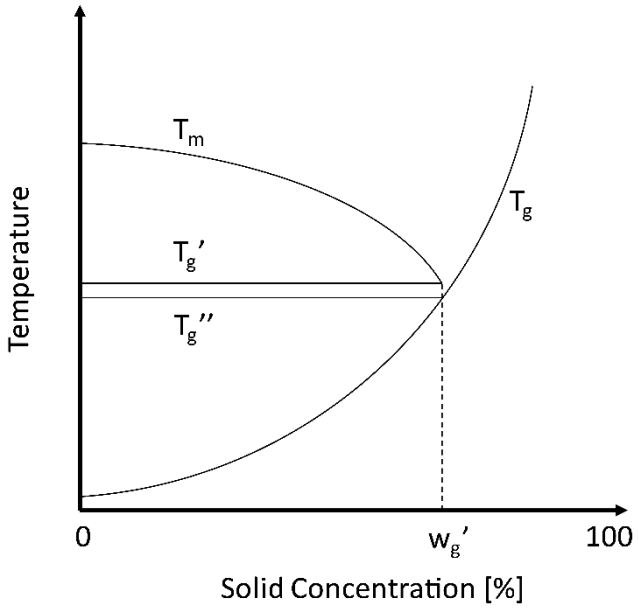

Figure 3-1: Schematic phase diagram, adapted from [14].

Furthermore, a linear function of the ice melting enthalpy against the solid concentration can be extrapolated to ΔH = 0 J/g to obtain w_g' [34-36]. This is a simple method without the need for major mathematical theories. Thereby, a w_g' of sucrose of 79% (w/w) results [34]. Thus, overall rather different w_g' values are reported for sucrose ranging from 64% [21, 37], 77% [29], up to 82% [25, 26, 38] and for trehalose ranging from 71 to 83% [32, 33]. Other reported w_g' values of excipients relevant for freeze-drying are 46% for polyethylene glycol 40000, which increased with the addition of sucrose at a 1:1 ratio to 60% [39], and 81% (w/w) for citric acid [40].

W_g' is currently not considered, when it comes to freeze drying of proteins. Knowing the exact composition can help to prepare the freeze concentrates and study e.g. protein-protein interactions or protein stability excluding ice-freeze concentrate interface effects. To answer these questions and due to the substantial deviation of w_g' values stated in literature, the aim of this study was to evaluate and compare three different DSC based approaches to determine w_g' of sucrose as model substance:

(i) using the intercept of the T_g' and T_m curves; (ii) using a linear regression of the melting endotherm; and (iii) using a one point measurement of the frozen water based on the melting endotherm. The methods were to be evaluated with respect to accuracy and effort. Subsequently the w_g' of different formulations relevant for lyophilisates of biopharmaceuticals was to be analysed. Besides sugar and protein also buffers, amino acids, and NaCl as excipients were evaluated.

2. Materials and Methods

2.1 Materials

Sucrose, L-histidine base, L-histidine monohydrochloride, L-arginine base, and L-arginine hydrochloride monohydrate (ArgHCl) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dihydrate, citric acid anhydrous, and sodium chloride were obtained from VWR International, Ismaning, Germany. All samples were prepared with highly purified water (HPW; Sartorius Arium Pro, Sartorius, Göttingen, Germany). A monoclonal IgG_1 antibody (mAb) in 15 mM histidine buffer pH 5.3 was used as standard protein.

2.2 Methods

2.2.1 Determination of w_g'

2.2.1.1 Differential scanning calorimetry (DSC)

T_g' and T_m were determined with a Netzsch Polyma 214 (Netzsch, Selb, Germany), a DSC 821^e (Mettler Toledo, Gießen, Germany), a TA Discovery (TA Instruments, New Castle, DE, USA), and a TA Q2000 (TA Instruments) under N_2 atmosphere. Low concentration samples are difficult to determine due to a high requirement for sensitivity and therefore different instruments were explored. 15 - 25 µL solution with 0% up to 65% (w/w) solid content was weighted into aluminium crucibles and sealed hermetically. The samples were cooled from 20°C to -60°C (-40°C only for Netzsch) at 5°C/min, reheated to -30°C at 2°C/min, held for 1 h to ensure complete ice formation [29], cooled to -60°C (-40°C only for Netzsch) at 5°C/min, and finally heated to 20°C at 2°C/min. Calibration was performed with indium as provided by the manufacturer. Netzsch Proteus Analysis, Mettler STAR^e, TRIOS, and TA Universal Analysis software were used for data analysis. The specific heat capacity of water (ΔC_p) was evaluated with a tangential step before and after the ice melting in a pure HPW sample. The melting enthalpy (ΔH) was evaluated by an integration starting from the beginning of the T_g' endothermal shift and ending at the

return to baseline after the melting event. Sigmoidal or spline baseline settings were used for the integration.

2.2.1.2 Calculation of w_g'

2.2.1.2.1 Method 1: Intercept of T_g' and T_m curves

Values of T_m vs. the relative solid content (x) were fitted to the Chen model (Equation (1)), an extension of the Clausius-Clapeyron equation [31] with T_m and T_w being the melting temperature of sample and HPW, respectively, β the molar freezing temperature constant for water (1860 kg·K/mol), M the molar mass of water. B and E were fitted to the experimental data with OriginPro 2018. The onset temperature of T_g' was fitted as horizontal line. W_g' was determined as the intercept of the T_m curve and the onset temperature of T_g' line.

Equation (1)

$$T_m = T_w + \frac{\beta}{M} \cdot \ln \frac{1 - x - Bx}{1 - x - Bx + Ex}$$

2.2.1.2.2 Method 2: Linear regression

The obtained DSC thermograms were integrated from the onset temperature of T_g' until the end of the melting peak with a sigmoidal baseline type to obtain the melting enthalpy ΔH. A linear regression function of the ice melting enthalpy against the solid concentration was extrapolated to ΔH = 0 J/g to calculate w_g'.

2.2.1.2.3 Method 3: Single sample determination

The effective fusion enthalpy of ice in solution L_e was calculated from the specific heat coefficient ΔC_p, T_m and T_w (Equation (2)). The amount of frozen water w_f (Equation (3)) is obtained from the melting enthalpy of the sample ΔH_s, the sample mass m_s filled in the pan, the melting enthalpy of water ΔH_w and L_e (adapted from [24] including the assumptions of neglectable effects of the ice melting enthalpy on the temperature and heat dilution). Finally, w_g' is calculated sample dependent with the solid content (solute concentration c_s and sample mass m_s), the total water content w_{total} and w_f (Equation (4)).

Equation (2)

$$L_e = \Delta C_p \cdot (T_m - T_w)$$

Equation (3)

$$w_f = \frac{\Delta H_s \cdot m_s}{\Delta H_w + L_e}$$

Equation (4)

$$w_g' = \frac{c_s \cdot m_s}{(W_{total} - W_f) + c_s \cdot m_s}$$

2.2.2 Determination of w_g' in ternary and higher systems

The compositions tested are listed in Table 3-1. Arginine citrate (ArgCitr) powder (residual moisture 5.35%) was produced by spray drying (Mini Spray Dryer B-290, Buchi, Essen, Germany) an arginine citrate solution pH 6.0. An 8% (w/w) solution was spray dried at 140°C inlet temperature, 81°C outlet temperature, 70% aspiration, 600 L flow, and 10% pump speed. mAb was dialysed against the individual buffers or HPW with a 10-fold buffer exchange in Vivaspin® 20 Ultrafiltration Uni (30 kDa, Sartorius, Goettingen, Germany) and upconcentrated if necessary.

The formulations were prepared at consistent solute ratios but different total solid content ranging from 10% up to 50% w/w.

Table 3-1: Formulations used for DSC experiments to evaluate w_g'. The ratios of different excipients including sugar and buffer to mAb are stated in italic. The formulations were prepared at consistent solute ratios ranging fom 10% up to 50% w/w solid content.

Abbreviation	**Formulation**	**Sugar**	**Buffer**	**Further excipient**	**mAb**
Suc	Sucrose	*7*	--	--	*0, 0.2, 1, 5, 20*
Suc+His 5.3	Sucrose + Histidine pH 5.3	*7*	*0.23*	--	*0, 0.2, 1*
Suc+His 7.4	Sucrose + Histidine pH 7.4	*7*	*0.23*	--	*0, 0.2, 1*
Suc+Phos 5.3	Sucrose + Phosphate pH 5.3	*7*	*0.15*	--	*0, 0.2, 1*
Suc+Phos 7.4	Sucrose + Phosphate pH 7.4	*7*	*0.15*	--	*0, 0.2, 1*
Suc+0.4NaCl	Sucrose + NaCl	*7*	--	*0.4*	*0, 0.2, 1*
Suc+0.8NaCl	Sucrose + NaCl	*7*	--	*0.8*	*0, 0.2, 1*
Tre	Trehalose	*7*	--	--	*0, 0.2, 1, 10*
ArgCitr	Arginine citrate	--	--	*5*	--
ArgHCl	Arginine*HCl	--	--	*5*	*0, 0.2*
NaCl	NaCl	--	--	*0.4*	*1*
mAb	mAb	--	--	--	*pure*

3. Results and Discussion

3.1 Evaluation of methods to determine a maximally freeze concentrated system

The first aim of this study was to identify the most suitable method to determine w_g' of a maximally freeze concentrated system balancing accuracy and sample and time consumption. 1.5% up to 65% (w/w) sucrose solutions were analysed using three methods.

The first method makes use of the intercept of two curves. The first curve represents the linear fit of the onset of T_g'. The second curve reflecting the T_m peak is fitted with the Chen model, adapted from the Clausius-Clapeyron equation [31, 41] resulting in B and E of 0.056 ± 0.0063 and $8.19 \cdot 10^{-5} \pm 9.84 \cdot 10^{-6}$, respectively. The T_m curve and T_g' onset temperature (around -34°C) complies with literature data [42, 43]. The intercept of the two curves reflects w_g' (Figure 3-2). Method 1 results in 77.4 ± 1.7% sucrose in the maximally freeze concentrated system.

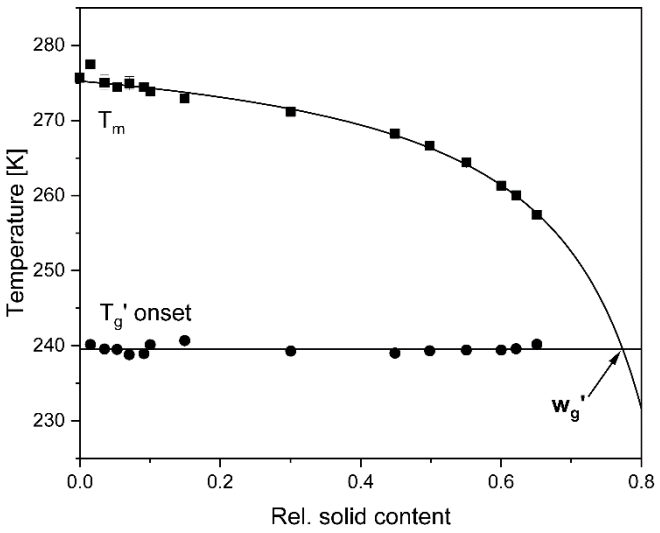

Figure 3-2: Determination of w_g' in a sucrose-water mixture using the intercept of the experimental evaluated T_m peaks (squares) fitted with the Chen model corresponding to Equation (1) and the onset of the corresponding T_g' (dots) fitted as horizontal line.

Method 2 utilises the solute concentration dependent change in the melting enthalpy to obtain w_g'. The DSC thermogram is integrated for ΔH starting at the onset of T_g' until the end of the melting event (Figure 3-3). The linear fit of ΔH is extrapolated to 0 J/g, which reflects the freeze concentrated state (Figure 3-4) [35]. This method 2 results in a w_g' of 75.2 ± 0.8% sucrose in this binary freeze concentrate.

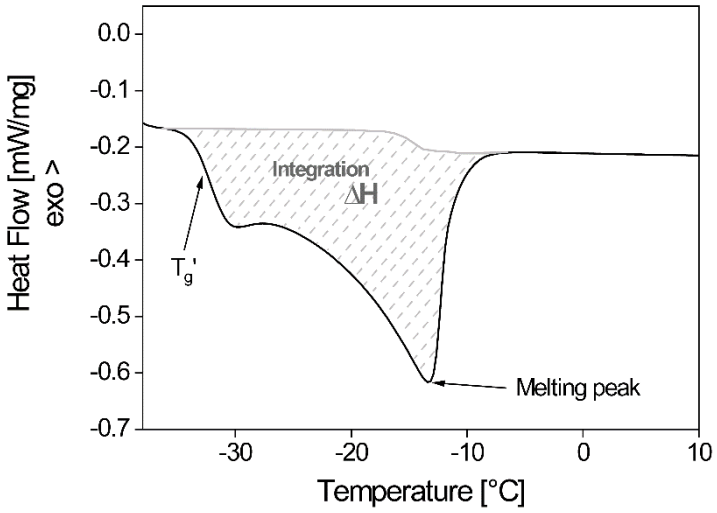

Figure 3-3: Exemplary DSC trace of 62% sucrose with ΔH integrated starting from the onset of T_g' until the end of the melting event.

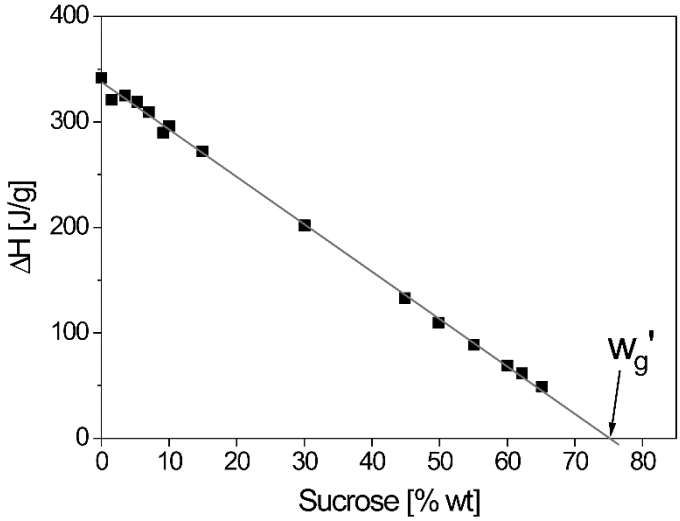

Figure 3-4: Determination of w_g' in a sucrose-water mixture using the change in melting enthalpy.

The third method directly calculates w_g' (Figure 3- 5B) from the amount of frozen water of a specific sample obtained from the DSC melting enthalpy (Figure 3-5A). The experimental ΔC_p of 2.10 ± 0.01 J/gK, T_w of 0.4 ± 0.3°C, and ΔH_w of 347 ± 2 J/g are in agreement with the literature values of 2.06 J/gK, 0°C, and 333 J/g, respectively [44, 45]. An exemplary thermogram can be found in Figure 3-6. This method results in w_g' for sucrose of 77.0% in the freeze concentrated state. The frozen water content could be determined with low standard deviation independent of the sugar concentration. But the small error in the amount of unfrozen water at low concentration samples led to high standard deviations in the calculated w_g'. Sugar concentrations of 30% and higher prompted

reliable w_g' values with a low standard deviation. Thus, 3.5% (w/w) sucrose resulted in a w_g' of 79.3 ± 10.6%, whereas 62% (w/w) sucrose showed a w_g' of 77.7% ± 0.2%. But these high solid contents are not used in lyophilisation of parenteral products.

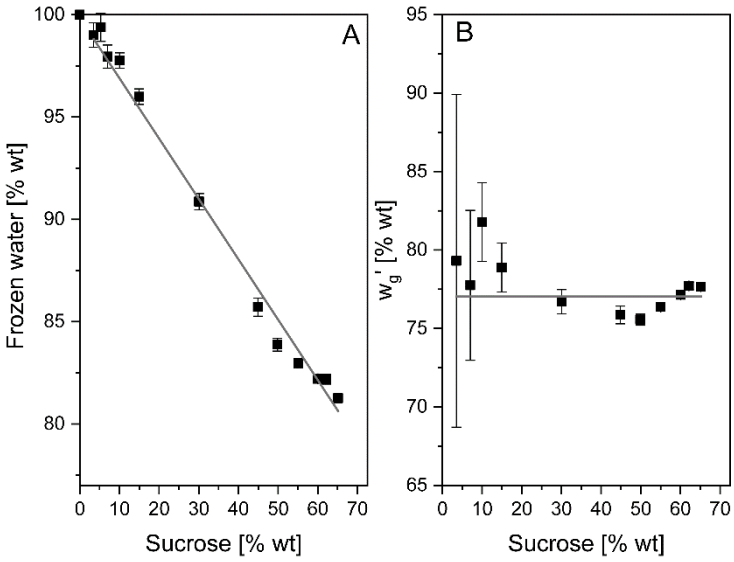

Figure 3-5: A: Frozen water content obtained from the melting enthalpy in a sucrose-water mixture and B: calculated w_g' thereof.

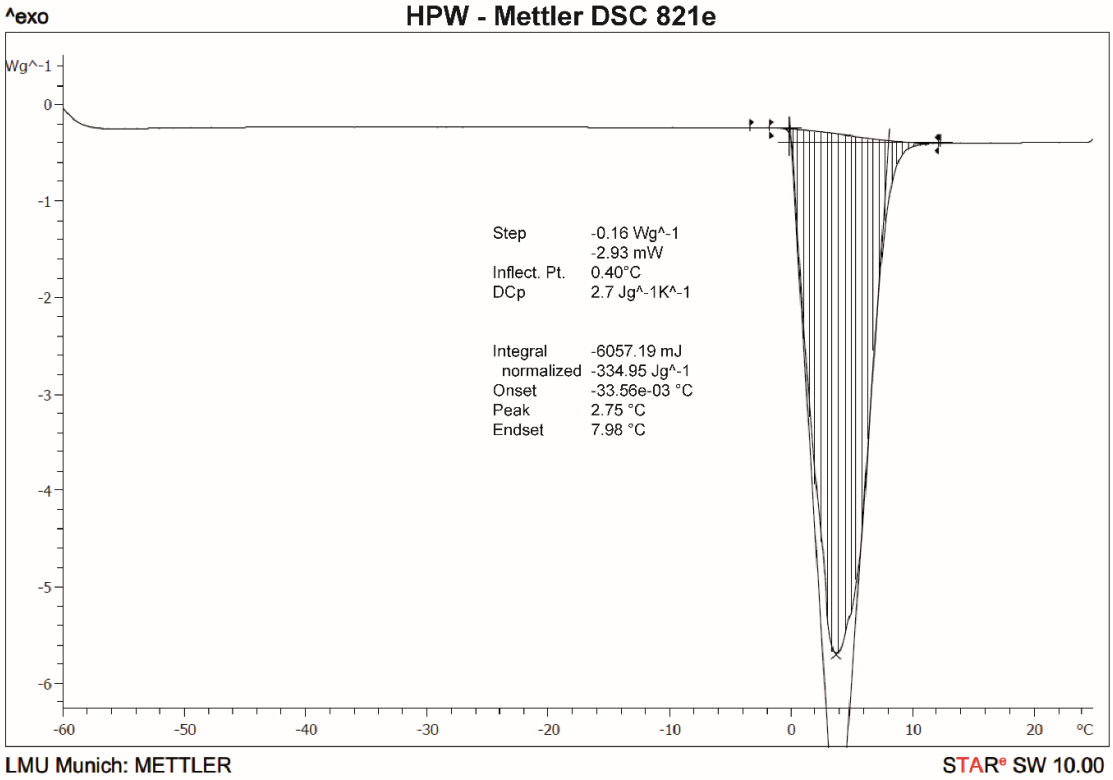

Figure 3-6: DSC thermogram of HPW obtained from Mettler DSC 821e.

All three tested methods resulted in a narrow range of w_g' values of 75.4 – 77.4%, whereas literature stated w_g' values between 64% and 83% [21, 25, 27, 29, 34]. Comparing the three methods, the first method is the most accurate since it requires only the evaluation of temperatures without integration of thermograms. However, a rather high number of samples with high solid content needs to be prepared and analysed, which is time consuming. This is mandatory to apply the Chen model to fit T_m and a new fit is required for each formulation. Considering poorly soluble excipients or rather high concentrations of proteins like antibodies, this method has its limitations. The second method could be used with less, at least five different concentrations preferably between 5% and 45%, for a simple linear fit of the melting enthalpy (same result for w_g' with $p < 0.05$ according to t-test). The third method is an even further simplification using only one single sample with a solid content of 30% or higher. With lower solid content, the error becomes inacceptable. Analysis of only one higher concentration sample showed more reliable results but still differed from the mean. DSC systems with higher sensitivity might be used with lower solid contents rendering method 3 superior. Overall, method 2 is the most applicable approach taking sample number and therefore measurement time, calculation of w_g', and reliability of the results into account. Method 2 was therefore subsequently used to analyse the w_g' of different formulations.

3.2 W_g' in protein containing samples

Subsequently method 2 was used to evaluate w_g' of sucrose and trehalose based formulations. The effect of histidine buffer and phosphate buffer at pH 5.3 and 7.4 as well NaCl was tested. In addition, the influence of the mAb concentration on the composition of the freeze concentrate was studied considering up to 200 g/L protein formulations. Furthermore, sugar free systems based on arginine hydrochloride, arginine citrate, and sodium chloride as sole excipient were tested with and without mAb.

Adding mAb to sucrose at different ratios up to Suc:mAb 7:20 did not impact w_g', with values between 74.3% and 78.0% for different mAb concentrations and 75.2 ± 0.8% for pure sucrose. mAb addition did not lead to a change in w_g' of the system (Figure 3-7). In addition, we could show that buffer did not significantly lower w_g' of Suc + His 5.3 (w_g' of 73.1 ± 1.6%), Suc + His 7.4 (73.8 ± 1.4%), Suc + Phos 5.3 (72.9 ± 1.2%), and Suc + Phos 7.4 (73.6 ± 1.5%). Addition of salt resulted in a decrease of w_g' of freeze concentrates to 72.4 ± 2.3% and 70.4 ± 2.0% for Suc + 0.4 NaCl and Suc + 0.8 NaCl as found by Her et al. [46]. NaCl alone without further excipient with mAb crystallised during the freezing and annealing process in the DSC. The eutectic concentration of NaCl-water is composed of 23.3% (w/w) NaCl [47].

Therefore, the integration was performed starting at the beginning of the eutectic temperature until the end of the melting event. W_g' of crystallised NaCl and mAb at a 0.4:1 ratio was 67.7 ± 3.4%, which was significant lower compared to the system Suc + 0.4 NaCl containing sucrose:NaCl:mAb in a ratio of 7:0.4:1 ($p < 0.05$, t-test).

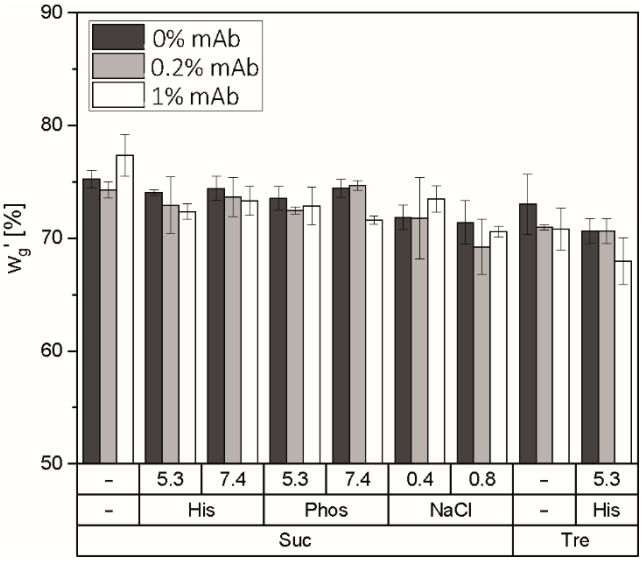

Figure 3-7: W_g' results of different sugar-based systems with varied mAb concentrations.

Trehalose as alternative disaccharide rendered a w_g' similar to sucrose with 73.0 ± 2.7%. Crystallisation of trehalose can occur in case of annealing and thereby influencing w_g' [48]. DSC thermograms did not indicate crystallisation. Again, the addition of histidine buffer pH 5.3 to the sugar did not affect w_g'.

Sugar-free formulations based on L-arginine may present an alternative for freeze-drying of protein drugs [49]. Other than sugar, arginine does not promote preferential exclusion in the solution to be freeze dried. Arginine is able to form hydrogen bonds with protein molecules and undergoes ion-dipole interactions [50], which might affect w_g'. Arginine hydrochloride can reduce protein-protein interactions, which can lead to a significant reduction in viscosity at high concentrations [51]. Such high concentrations are expected for protein containing freeze-concentrates. ArgCitr, ArgHCl, and ArgHCl with mAb (ArgHCl:mAb 5:0.2) showed w_g' values of 79.6 ± 3.1%, 72.4 ± 2.0% and 72.7 ± 2.3%, respectively. Due to the T_g' and T_g increasing influence of citric acid, w_g' of ArgCitr might be influenced [52].

Subsequently the w_g' of the mAb and its potential effect in mixtures with excipients was analysed. However, w_g' is difficult to determine since a glass transition of the mAb cannot be detected. It is

estimated to be at around -15°C [53]. According to the onset of the melting peak in the thermograms (drop in baseline) the onset temperature of T_g' could be approximated to be around -25°C ranging from 0.2% mAb up to 30% mAb (Figure 3-8). The obtained w_g' of pure mAb was 80.8 ± 3.4%.

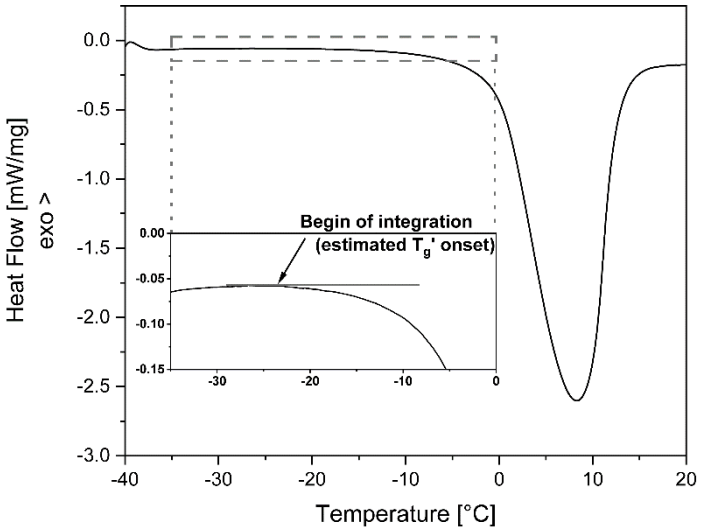

Figure 3-8: Exemplary DSC thermogram of 19% mAb in HPW. Estimation of T_g' onset is depicted in the zoom. The onset of T_g' at about -25°C was used as starting point for the integration of the melting event.

With the determined w_g' values, the composition of the maximally freeze concentrate can be calculated taking the initial solid content of the formulation into account. Other than the solid content at the concentration of w_g' only unfrozen water remains. The w_g' of mAb-sugar mixtures was independent of the mAb concentration. However, increasing the mAb concentration results in different mAb to sugar or excipient ratios in the freeze concentrate. Table 3-2 states some examples with different mAb to sucrose ratios, ranging from low to high mAb concentrations. A formulation with 20% mAb will result in around 56% (w/w) mAb in the concentrated state with only 22% (w/w) H_2O and 20% (w/w) sucrose.

Table 3-2: Exemplary freeze concentrate compositions. Ratios of solids in starting liquid upconcentrated to the total solid content w_g'. H_2O in the concentrated state is synonymous with unfrozen water.

w_g' [%]	**Starting liquid**				**Freeze concentrate**			
	Sucrose [%]	Histidine buffer [%]	mAb [%]	H_2O [%]	Sucrose [%]	Histidine buffer [%]	mAb [%]	H_2O [%]
74.3	7	--	0.2	92.8	72.2	--	2.1	25.7
72.4	7	0.23	1	92.0	61.6	2.0	8.8	27.6
78.0	7	--	20	73.0	20.2	--	57.8	22.0

Histidine buffer resulted in a marked decrease in ionic strength of 275 mM to 156 mM with increasing pH values. Phosphate buffer increased in ionic strength to 302 mM and 391 mM for a pH of 5.3 and 7.4, respectively. NaCl caused an enormous increase in ionic strength in the freeze concentrated to 1.4 M. The high ionic strength in NaCl-containing samples could possibly influence the system by decreasing T_g' and thus w_g' [14]. However, w_g' was influenced neither by different sugar concentrations, differences in ionic strength nor the mAb concentration. No systematic analysis of w_g' with different mAb containing formulations has been stated so far. The up-concentration of proteins can lead to an increase in attractive protein-protein interactions or phase separation with a loss in stabilising protein-excipient interactions. These effects in combination with high ionic strength could foster potential protein instability [4, 7].

Furthermore, the freeze concentrate was also determined to be between 72.4% and 79.6% in sugar free arginine systems. Crystallisation of NaCl as sole excipient resulted in slightly lower total solid content with around 67.6% in the up-concentrated state, which was significant lower compared to amorphous matrix forming systems.

4. Conclusion

Different DSC methods were previously reported in literature to determine the amount and the water content of the maximally freeze concentrated solution formed upon freezing solutions [24, 31, 35]. Knowledge of the composition can help to judge protein drug instability during freeze-drying and can enable to prepare the freeze concentrate and analyse its properties. We evaluated three different approaches for analysis of the freeze concentrate composition. Using the intercept of concentration dependent T_g' and T_m curves requires a large number of samples with different total solid content and samples of high total solid content of >30%. The high number of different samples results in an accurate result for the composition of the freeze concentrate. Analysis of the frozen water content and with that w_g' of only one sample is rather limited and dependent on the total solid content and the sensitivity of the DSC system. Higher solid contents are recommended for this method. A very good compromise is to determine the frozen water content of samples of 10 – 30% solid content based on the AUC of the melting event and to extrapolate. This method renders reproducible results with little sample and time consumption.

Overall, the total solid content w_g' of the maximally freeze concentrated solution of sugar based amorphous protein solutions lays between 70% and 80% total, regardless the different additional excipients. Buffers or salt tend to decrease the w_g' values, however, without statistical significance.

The mAb concentrations did not affect w_g'. Sugar free arginine formulations show similar w_g' values between 70% and 80%.

The process of freeze concentration leads to increased viscosities, potential crystallisation of solutes, phase separation, or high ionic strengths [2, 4, 7]. Thereby, increased protein concentration is accompanied by more protein-protein interactions. Eventually, the combination with pH shifts due to buffer crystallisation or high ionic strength can force protein instabilities. In following studies, freeze concentrates of different formulations will be produced and protein stability evaluated accordingly.

Acknowledgement

We would like to thank David Thomas from Vectura Group Plc., Wenqi Wu from the University of Copenhagen, and Robert Cornell from the University of Cambridge for the opportunity to use their DSC equipment and expertise. Special thanks go to Bakul Bhatnagar from Pfizer for sharing his helpful hints and tricks about DSC measurements with us.

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Chapter 4

Freeze Concentration during Freezing: How does the Maximally Freeze Concentrated Solution Influence Protein Stability?

The following chapter has been published as research article in the International Journal of Pharmaceutics and appears in this thesis with the journal's permission:

Ivonne Seifert and Wolfgang Frieß
Freeze Concentration during Freezing: How does the Maximally Freeze Concentrated Solution Influence Protein Stability?
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Abstract

During freeze drying of biologics, a highly viscous freeze concentrate (FC) is formed upon the initial freezing due to the crystallisation of ice. Protein stability in this freeze concentrated phase is not yet well understood, but can decide upon the success of the lyophilisation itself. Protein stability may be high below the T_g' as it is typically the case during primary drying but decreases above T_g', e.g. during annealing or during aggressive freeze drying above T_g' in presence of a crystalline bulking agent or, beyond freeze drying, during storage of frozen bulk. Different FCs containing monoclonal antibody, sucrose, histidine or phosphate buffer and sodium chloride were prepared via partial freeze drying and analysed for protein aggregation. No solute crystallisation is visible and the systems are vitrifying during cooling. Increasing sugar or buffer concentration showed positive effects on either melting and aggregation temperature or on protein self-interaction as indicated by A_2 values. Protein integrity in the FC was not affected by 1 month storage at temperatures above T_g'. Thus, upconcentration of solutes during freezing does not negatively impact protein stability. Exceeding T_g' during freeze drying e.g. upon annealing or, intentionally or unintentionally, during primary drying does not lead to protein aggregation.

Keywords

Antibody, protein stability, protein aggregation, protein self-interactions, freeze drying, T_g', freeze concentration

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1. Introduction

Freeze drying is the method of choice to stabilise therapeutic proteins if stability in liquid form is not adequate [1]. Thereby, the product undergoes drastic physical changes in the initial freezing step before ice sublimation starts [2-4]. During freezing, crystallisation of ice leads to an increase in solute concentration remaining in the unfrozen phase, the freeze concentrate (FC). With decreasing temperature, viscosity increases per se and even more pronounced with the increase in solute concentration. This higher viscosity may improve colloidal protein stability reducing reaction rates [4-6].

Phase separation may occur rendering a protein-rich phase, in which protein stability is potentially lowered due to a depleted ratio of stabilising excipient to protein [7-9]. As the concentration of charged solute goes up during concentration, ionic strength increases eventually leading to charge shielding and enhanced attractive protein self-interaction. This can be accompanied by a loss in protein stability [10, 11]. At the same time, the increase in protein concentration gives rise to colloidal instability [12-15]. However, in the freeze concentrated state, reaction rates for protein unfolding as well as mobility become reduced due to an increase in viscosity and proteins in high concentration behaving as polymers [2, 16, 17]. Furthermore, a pH shift of the system as a function of temperature, in extreme cases drastically enhanced by preferential crystallisation of buffer components can alter protein stability [1, 18-20]. Phosphate buffers may show a downward shift of up to 4 pH units [18, 21] and succinate buffers an increase with decreasing temperature [22]. Furthermore, proteins can undergo freezing stresses such as cold denaturation depending on pH, protein concentration, and excipients [23]. In addition to a potentially reduced protein stability in the concentrating solution, protein can enrich, unfold and aggregate at the ice-liquid interface [24, 25]. The mechanism of cold denaturation is potentially enhanced by the molecules in the quasi liquid layer between the ice and the freeze concentrated phase [26, 27]. The product cooling and the freezing rate of the system once nucleated render different rates of ice formation, ice crystal and interface area sizes affecting potential protein damage [23, 28-33]. At the end of freezing, when all ice has crystallised [29], a FC with an enormous high total solid content has formed. The maximally freeze concentrated solution of sucrose consists of around 70% to 80% (w/w) solids, termed w_g', and 20% to 30% (w/w) unfrozen water [34-38]. Thus, the sucrose concentration in the FC is above the solubility level of sucrose (0.5 g/ml [39]) at room temperature.

Aim of our study was to better understand protein behaviour in the FC and the risk of protein instability during annealing or aggressive collapse drying cycles coming with product temperatures above the glass transition temperature of the maximally freeze concentrated solution (T_g') and even storage of

frozen bulk. Direct analysis of protein stability in the FC is challenging since the FC itself is difficult to obtain. Petzold et al. described freezing of a sucrose solution to -20°C and separation of the FC from the ice formed using a suction unit similar to a filter flask at ambient temperature [40]. Similarly, a protein solution frozen to -10°C may be centrifuged with the FC accumulating at the bottom [41]. In need for a simple preparation method without separating the FC from the ice interface, we developed a method based on partial freeze drying. With the FC in hand, we evaluated the influence of the high solutes and protein concentration on protein stability. Protein self-interaction was analysed for a mAb with increased sucrose, buffer, and salt levels. As the analysis in the FC was not always possible, comparable concentrated solutions were used for interaction studies. Ultimately, the protein aggregation as function of time and temperature reflecting freeze drying with sucrose as stabiliser with histidine and phosphate buffer pH 5.3 and pH 7.4 was analysed, followed by an evaluation of the effect of NaCl expressing potentially present additional ionic species in a lyophilised formulation [42].

2. Materials and Methods

2.1 Materials

Sucrose, L-histidine base, L-histidine hydrochloride monohydrate, sodium phosphate dibasic dihydrate, and sodium phosphate monobasic dihydrate were purchased from Merck KGaA, Darmstadt, Germany, sodium chloride from VWR International, Ismaning, Germany, and D-(+)-trehalose dihydrate from Pfanstiehl Technologies, Waukegan, IL, USA.

A rather hydrophobic monoclonal IgG_1 antibody without specific pharmaceutically relevant target (94 mg/ml, MW ca. 148 kDa, ϵ = 1.37 mL/mg·cm, referred to as mAb) in 15 mM histidine buffer pH 5.3 was used as model protein. mAb used for stability analysis was dialysed against highly purified water (HPW) in a 33 mm dialysis tubing cellulose membrane (14 kDa cut-off, Merck KGaA). 20 mL mAb were dialysed against 5 L HPW with a 3-times media exchange. Concentration was determined via UV absorption (Nanodrop 2000, ThermoFisher Scientific, Waltham, MA, USA). Formulations were filtrated with 0.2 µm polyethersulfone (PES) membrane syringe filters (VWR International) prior to use.

2.2 Methods

2.2.1 Freeze-Thaw (FT)

Freeze-thaw experiments were performed using an Epsilon 2-6D freeze drier (Martin Christ, Osterode am Harz, Germany). Samples were cooled from 20°C to -50°C at a ramp of 1°C/min followed by an

isothermal hold for 3 h, thawed back to 20°C at a ramp of 1°C/min followed by an isothermal hold of 3 h. This cycle was repeated three times. Thermocouples confirmed complete ice crystallisation and thawing in selected vials by reaching -50°C and 20°C, respectively.

2.2.2 Preparation of freeze concentrates

For the preparation of freeze concentrates (Figure 4-1) solutions with an already approx. 4x increased starting total solid content based on 30% (w/w) sucrose were filled in DIN 10R Vials (Fiolax®, Schott AG, Mainz, Germany). The vials were semi-stoppered with lyophilisation stoppers (igloo stoppers with B2-TR coating, West Pharmaceutical Services, Eschweiler, Germany). The formulations were partially freeze dried in an Epsilon 2-6D freeze drier (Martin Christ, Osterode am Harz, Germany). After freezing to -50°C at 1°C/min and 1 h hold time, freeze drying was performed at -5°C at 0.1 mbar until comparative pressure measurement with Pirani and capacitance sensor aligned. The weight of the vial was recorded prior to and after freeze drying. Water was added to match the solid and water content of the FC as determined previously (Table 4-2) [34] and the vial equilibrated 2 to 24 hours at 2 – 8°C. FCs tested in a 1-month stability study at 25°C, -10°C, and -80°C are listed in Table 4-1.

Table 4-1: FCs tested in a 1 month stability study. Numbers reflect the ratios of different excipients including sugar and buffer to mAb.

	Base formulation for FC reflecting the solute ratios			
Formulation	**Sucrose**	**Buffer**	**NaCl**	**mAb**
Suc	7	-	-	0, 0.2, 1
Suc + His 5.3	7	0.23	-	0, 0.2, 1
Suc + His 7.4	7	0.23	-	0, 0.2, 1
Suc + Phos 5.3	7	0.15	-	0, 0.2
Suc + Phos 7.4	7	0.15	-	0, 0.2
Suc + 0.4 NaCl	7	-	0.4	0, 0.2, 1
Suc + 0.8 NaCl	7	-	0.8	0, 0.2, 1

Table 4-2: Composition of FCs with different mAb concentrations in % w/w according to [32].

Formulation	Suc [%]	His [%]	Phos [%]	NaCl [%]	mAb [%]	H_2O [%]
Suc	75.2	-	-	-	-	24.8
	72.2				2.1	25.7
	67.6				9.7	22.7
Suc + His 5.3	71.7	2.4	-	-	-	25.9
	68.7	2.3			2.0	27.1
	61.5	2.0			8.8	27.7
Suc + His 7.4	72.0	2.4	-	-	-	25.6
	69.4	2.3			2.0	26.3
	62.3	2.0			8.9	26.7
Suc + Phos 5.3	72.0	-	1.5	-	-	26.5
	69.0		1.5		2.0	27.5
Suc + Phos 7.4	72.8	-	1.6	-	-	25.6
	71.1		1.5		2.0	25.3
Suc + 0.4 NaCl	67.9	-	-	3.9	-	28.2
	66.1			3.8	1.9	28.2
	61.3			3.5	8.8	26.5
Suc + 0.8 NaCl	64.1	-	-	7.3	-	28.6
	60.6			6.9	1.7	30.8
	56.2			6.4	8.0	29.4

2.2.3 Rheology

Approximately 0.3 ml samples were loaded between two parallel plates (upper plate 25 mm diameter) with a gap of 0.5 mm in a rheometer MCR 100 (Anton Paar, Graz, Austria). During a temperature range from 20°C to -15°C with -2°C/min viscosity was measured at a shear rate of 300 1/s. The diffusion coefficient D of the mAb was calculated based on the Stokes-Einstein equation [43].

2.2.4 Dynamic light scattering (DLS)

For DLS analysis, samples with 2 – 20 mg/ml mAb were centrifuged at 10,000 xg for 10 min and 20 µl filled in a 364 well plate (Corning, Corning, NY, USA). The plate was centrifuged at 546 xg for 2 min followed by sealing each well with 5 µl silicon oil and a second centrifugation. The samples were

measured on a DynaPro plate reader III (Wyatt Technology, Santa Barbara, CA, USA) using 10 acquisitions of 5 s at 25°C. The autocorrelation function, the mutual diffusion coefficient, and the polydispersity index were determined with the Dynamics V7.8 software. The interaction parameter k_D was calculated from the mutual diffusion coefficient and transferred to A^*_2 based on the TIM-equation [11]. All DLS measurements were performed in 6 replicates per sample.

2.2.5 Composition-gradient multi-angle light scattering (CG-MALS)

10 mg/ml mAb in 15 mM histidine buffer pH 5.3 and 0% to 5% (w/w) sucrose samples were used for CG-MALS measurement. Before use, all samples were filtrated with 0.2 µm PES filters. Light scattering and protein concentration were analysed with an automated CG-MALS instrument equipped with a dual syringe-pump Calypso-II sample and preparation unit, a Dawn Heleos-II multi-angle laser light scattering detector, and an Optilab® T-rEX dRI detector (Wyatt Technologies). Calypso 2.1.5 software was used to obtain Zimm Plots and A_2 values [44].

2.2.6 nanoDSF®

NanoDSF was used to study thermal unfolding of the mAb in different formulations. The samples were filled in standard glass capillaries and sealed afterwards. A Prometheus® NT.48 (NanoTemper Technologies, Munich, Germany) was used starting from 25°C up to 95°C or 110°C at a ramp of 1°C/min. Intrinsic protein fluorescence intensity at 330 nm and 350 nm was measured after excitation at 280 nm (± 10 nm). Furthermore, back-reflection intensity was measured to detect protein aggregation and precipitation. The fluorescence intensity ratio (FI350/FI330) was used to determine protein thermal unfolding, calculated by PR.ThermControl V2.1 software (NanoTemper).

2.2.7 Light obscuration

Subvisible particles were characterised by light obscuration. The freeze concentrates were diluted with HPW to 7% (w/w) sucrose and analysed using a PAMAS SVSS-C35 particle counter with an HCB-LD-25/25 sensor (Partikelmess- und Analysensysteme GmbH, Ruthesheim, Germany). The system was rinsed with HPW followed by a sample pre-rinse of 0.2 ml. Samples were analysed 4 times with 0.2 ml each [45].

2.2.8 Size exclusion chromatography (SEC)

SEC was performed with an Agilent 1100 series HPLC system equipped with a UV/Vis detector measuring at 280 nm (Agilent Technologies, Santa Clara, CA, USA). A Waters AQUITY UPLC Protein BEH 200Å column (1.7 µm; 4.6 x 150 mm; Waters GmbH, Eschborn, Germany) was used with a 50 mM sodium phosphate and 400 mM sodium perchlorate buffer at pH 6.0 at 0.4 ml/min. All samples were diluted to 2 mg/ml mAb and centrifuged at 7,000 rpm for 10 min prior to use. The area under the peaks as well as relative areas of monomer, fragments, and aggregates were determined after blank subtraction using ChemStation software (Agilent Technologies).

3. Results and Discussion

3.1 FC properties

For preparation of FCs partial freeze drying was performed, interrupting the process in the course of primary drying to reach a water level of slightly less than what was determined by DSC previously [34] followed by adjustment to the final water content. Regardless of the composition, all FCs were transparent with some air bubbles entrapped, highly viscous and solidified solute, ice crystals, or phase separation were not noticeable (Figure 4-1). Due to the collapse upon partial freeze drying and further viscosity increase, mAb containing FCs had a more foam-like appearance. No ice or solute crystallisation but only vitrification was observed when cooling the FCs to -80°C. Furthermore, the visual appearance remained unchanged upon storage.

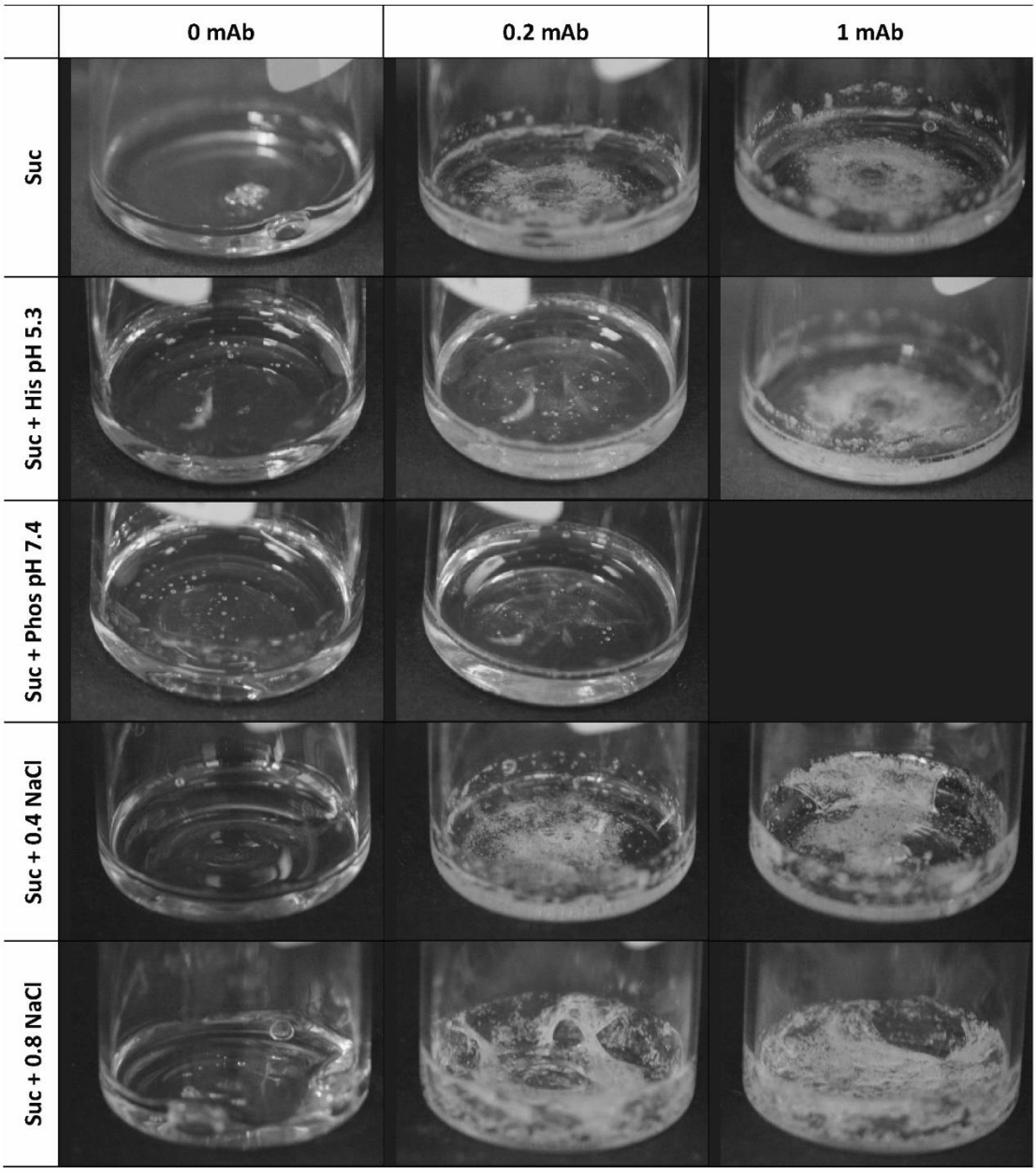

Figure 4-1: Visual appearance of exemplary FCs.

The sucrose FCs showed a viscosity of 2.5 Pa·s at 20°C (Figure 4-2). In comparison, the viscosity of a 7% (w/w) and 20% (w/w) sucrose solution is only around 1.5 mPa·s and 2 mPa·s at 20°C, respectively [46]. Thus, already at room temperature, diffusion and self-interaction of protein molecules will be markedly reduced. At -15°C the viscosity is approx. 100 times higher with approx. 400 Pa·s. Due to limitations of the instrument we could not determine the viscosity of the FCs below -15°C. But based on the Angell's Plot [47], Suc FC viscosity can be extrapolated and we obtained values of 703, 1355, and 2612 Pa·s at -20°C, -25°C, and -30°C, respectively. Thus, at product temperatures used during

freeze drying for annealing, e.g. -20°C, the viscosity in a FC is around $5 \cdot 10^5$ times higher compared to the initial 7% sucrose solution at room temperature which can drastically reduce colloidal interaction and unfolding times [48].

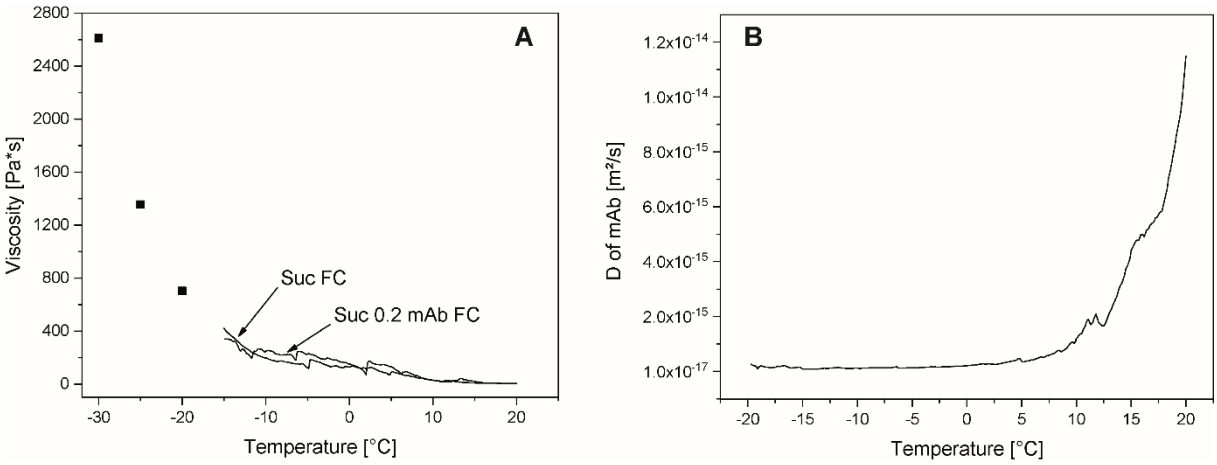

Figure 4-2: (A) Viscosity of pure sucrose and sucrose with 0.2 mAb FC (black squares: theoretical values of Suc FC obtained based on the Angell's plot [45] and (B) Diffusion coefficient of mAb in sucrose with 0.2 mAb FC as a function of temperature.

We attempted to measure the self-interaction propensity at high sucrose concentrations. Methods such as DLS and small-angle X-ray scattering (SAXS) did not work for FCs as the high sucrose concentrations interfered with the protein signal in case of DLS and missing contrasts did lead to inconclusive results for SAXS. Therefore, CG-MALS was performed for A_2 analysis in presence of up to 5% sucrose and DLS for comparative analysis of A_2^* in sugar free solutions.

A_2^* of mAb in His pH 5.3 resulted in a negative value indicating net attractive self-interaction of the mAb (Table 4-3) [49]. Changing the buffer system to Phos pH 7.4 did not substantially modify this behaviour. Furthermore, adding NaCl and increasing the ionic strength of these formulations did not affect with the self-interactions by changing A_2^*. At the pH values relatively close to the pI of the mAb of between 6 and 7 for different charge species attraction was slightly more dominant with little impact of pH and ionic strength [10]. An increased buffer and NaCl concentrations by a factor of 10, reflecting the buffer and salt concentration of the FC, resulted in less negative A_2^* values for all formulations and thus a shift of the self-interaction towards less attraction.

Table 4-3: A_2^* in solutions obtained by DLS from a 2 – 20 mg/ml mAb concentration series.

Formulation	A_2^* [mol·ml/g²]	Formulation	A_2^* [mol·ml/g²]
15 mM His pH 5.3	$-9.37 \cdot 10^{-5}$	150 mM mM His pH 5.3	$-2.05 \cdot 10^{-5}$
15 mM His pH 5.3, 20 mM NaCl	$-9.03 \cdot 10^{-5}$	150 mM His pH 5.3, 200 mM NaCl	$-9.71 \cdot 10^{-6}$
15 mM Phos pH 7.4	$-6.45 \cdot 10^{-5}$	150 mM Phos pH 7.4	$-2.21 \cdot 10^{-6}$
15 mM Phos pH 7.4, 20 mM NaCl	$-3.36 \cdot 10^{-5}$	150 mM Phos pH 7.4, 200 mM NaCl	$-2.94 \cdot 10^{-6}$

Sucrose containing formulations resulted in multimodal size distributions in DLS, which do not allow determination of A_2^*. Therefore, A_2 was determined by CG-MALS for a 10 mg/ml mAb solution in 15 mM His pH 5.3 (Figure 4-3). The A_2 value obtained by CG-MALS of the mAb in His pH 5.3 without sucrose was comparable with the one determined by DLS. With the addition of sucrose, A_2 increased. At 2% sucrose positive values resulted, indicating repulsive interaction and qualifying sucrose as protein stabiliser [50]. Higher sucrose concentrations are expected to result in even higher repulsive forces making the FC favourable for more repulsive protein self-interaction.

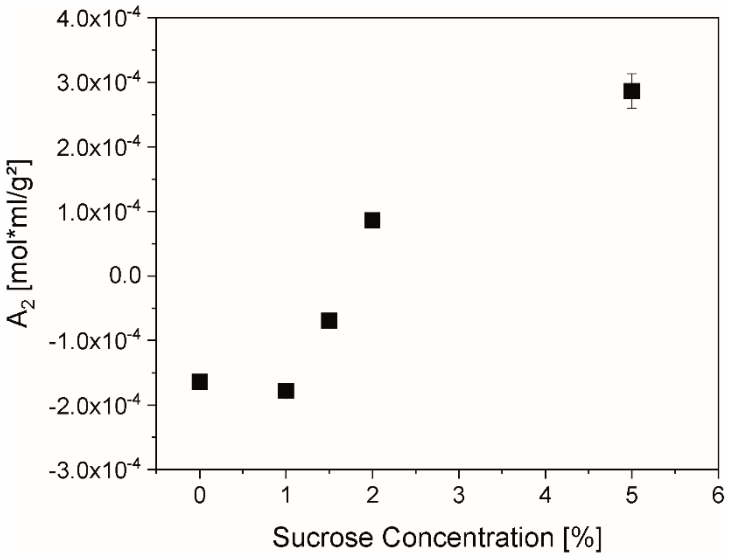

Figure 4-3: A_2 of 10 mg/ml mAb in 15 mM His pH 5.3 at 0 – 5% sucrose obtained by CG-MALS.

To identify the influence of the increased solute content in the FC on the conformational stability, the first melting temperature T_m of the mAb and the onset temperature of aggregation T_{agg} were analysed by nanoDSF®. The trend at high temperatures (hot denaturation) is comparable with low temperatures (cold denaturation) for various proteins including mAbs [51, 52]. No difference in T_m was found

between the different excipients and mAb concentrations in the initial solutions based on 7% sucrose (Table 4-4). All T_m values were comparable to the pure mAb in 15 mM His buffer pH 5.3 without sucrose. In the NaCl containing formulations and the pH 7.4 formulations except for Phos 7.4 with low mAb concentration, aggregation started simultaneously with unfolding. Thus, with the increase in hydrophobicity upon unfolding the mAb self-interaction becomes highly attractive. Aggregation did not set in with unfolding and was not noticed up to 95°C for pure mAb, Suc, at pH 5.3, and in Suc + Phos 7.4 (0.2% mAb) indicating that despite unfolding repulsive interactions are still dominant under these conditions.

Table 4-4: T_m and T_{agg} of mAb in liquid and freeze concentrated formulations. *not the FC tested, but a 10-fold concentrated mAb (2%), comparable to the concentration in the FC. Mean of n = 2.

	0.2% mAb				**1% mAb**			
	Initial solution		**FC**		**Initial solution**		**FC**	
	T_m [°C]	T_{agg} [°C]	T_m [°C]	T_{agg} [°C]	T_m [°C]	T_{agg} [°C]	T_m [°C]	T_{agg} [°C]
His 5.3	69.0	>95	68.9*	>95*	--	--	--	--
Suc	69.2	>95	87.6	>110	68.8	>95	86.2	84.6
Suc + His 5.3	68.0	>95	83.1	80.8	68.4	>95	84.2	82.6
Suc + His 7.4	68.0	67.1	86.7	84.2	69.1	72.2	86.2	89.0
Suc + Phos 7.4	66.5	>95	85.8	84.0	68.6	66.7	85.7	>110
Suc + 0.4 NaCl	69.2	68.8	81.7	80.2	68.3	66.5	80.1	78.5
Suc + 0.8 NaCl	69.1	68.8	78.3	76.8	68.3	66.7	77.7	76.8

The FCs showed 9°C to 18°C higher T_m and T_{agg} values compared to the initial solutions [53]. For FCs with additional NaCl this difference was less than for the NaCl-free FCs indicating that charge shielding by NaCl, i.e. higher ionic strength, led to destabilisation of the protein structure [10]. The mAb aggregation behaviour in the FC was similar to that in the initial solution. In the FCs, aggregation of Suc 0.2% mAb and Suc + Phos 7.4 1% mAb was not completed up to 110°C. The more repulsive interaction of the mAb we saw by CG-MALS in presence of sucrose do not have a significant impact when it comes to aggregation of the more hydrophobic unfolded mAb.

Thus, with respect to the physico-chemical properties of the FCs studied, viscosity is enormously increased at lower temperatures compared to the initial solution leading to slow reaction rates. Thereby, protein interactions are decreased. The presence of a high sucrose concentration in the FCs leads to a substantial increase in T_m and T_{agg} which indicates also improved stability against cold

denaturation. NaCl causes more attractive mAb self-interaction and higher susceptibility of unfolding and aggregation, but this negative impact of the high solute content in the FC is outweighed by the positive impact of sucrose.

3.2 Stability of mAb in FCs

Subsequently to the characterisation of the FC properties, the aggregation of the mAb in the FC over one 1 month was to be analysed in order to learn about protein physical stability itself in the FC liquid without ice interface. Thereby, different buffer conditions and the increase in ionic strength through NaCl addition were evaluated critically. Initially, the formulations forming the basis of the FCs were stressed by freezing and thawing to learn about the short term stability of the mAb in the FC and about the impact of the ice interface. Neither visible nor subvisible particles were formed upon three freezing and thawing cycles (Figure 4-4).

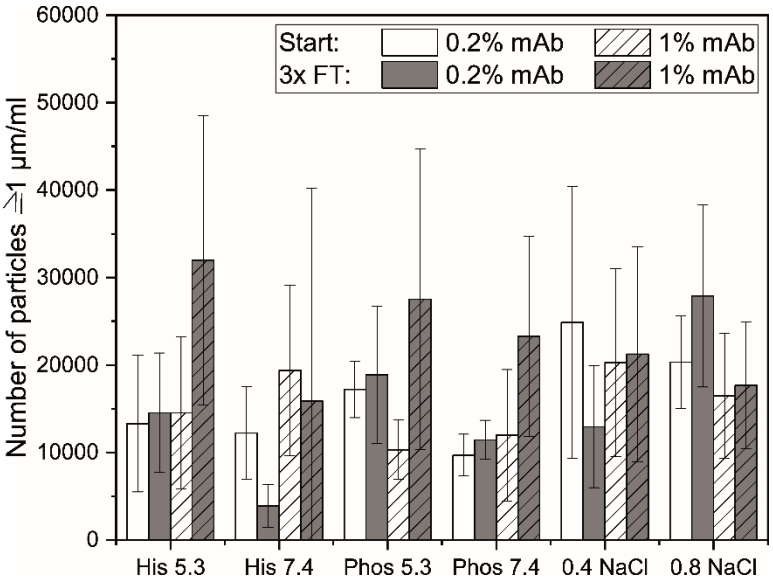

Figure 4-4: Number of subvisible particles ≥ 1 µm/ml of the initial solutions with 7% sucrose containing 0.2% or 1% mAb before and after 3 freeze-thaw cycles.

The corresponding FCs were stored for 1 month at 25°C to trigger protein self-interaction, at -10°C above T_g' reflecting collapse and annealing conditions during freeze-drying and at -80°C as reference with inhibited mobility below T_g' and evaluated for mAb aggregation. T_g' of sucrose is at approximately -32°C and 0.2% NaCl in the solution already reduce it by almost 4°C [35, 54, 55]. Overall, the subvisible particle concentrations in all FCs was low and no increase was in observed regardless of storage

temperature, mAb concentration or buffer system (Figure 4-5). The HMWS levels were at approx. 1% to 1.5% after preparation, slightly higher for Suc and Suc + His 5.3 1% mAb. Upon storage, the HMWS in the 0.2% mAb FCs did not change at any storage temperature. His 7.4 and both NaCl 1% mAb FCs showed an increase in HMWS levels up to 2.5 – 2.7% at all storage temperatures. The fragment levels were below 0.5% in all 0.2% FCs and remained consistent over storage. In the 1% mAb FCs the LMWS level was increased already at t_0 for the pure Suc FC but did not rise further upon storage. Again, His 7.4 and both NaCl 1% mAb FCs showed signs of additional aggregation to the same extent at all three storage temperatures. Thus, we could not see signs of physical instability of the mAb in the FCs based on 0.2% initial solution concentration. The FCs based on 1% initial solution concentration and containing NaCl showed slight changes in both HMWS and LMWS in SEC, as well as the His 7.4 system, independent of storage temperature. The effect in the NaCl may be related to the increased ionic strength promoting attractive self-interaction.

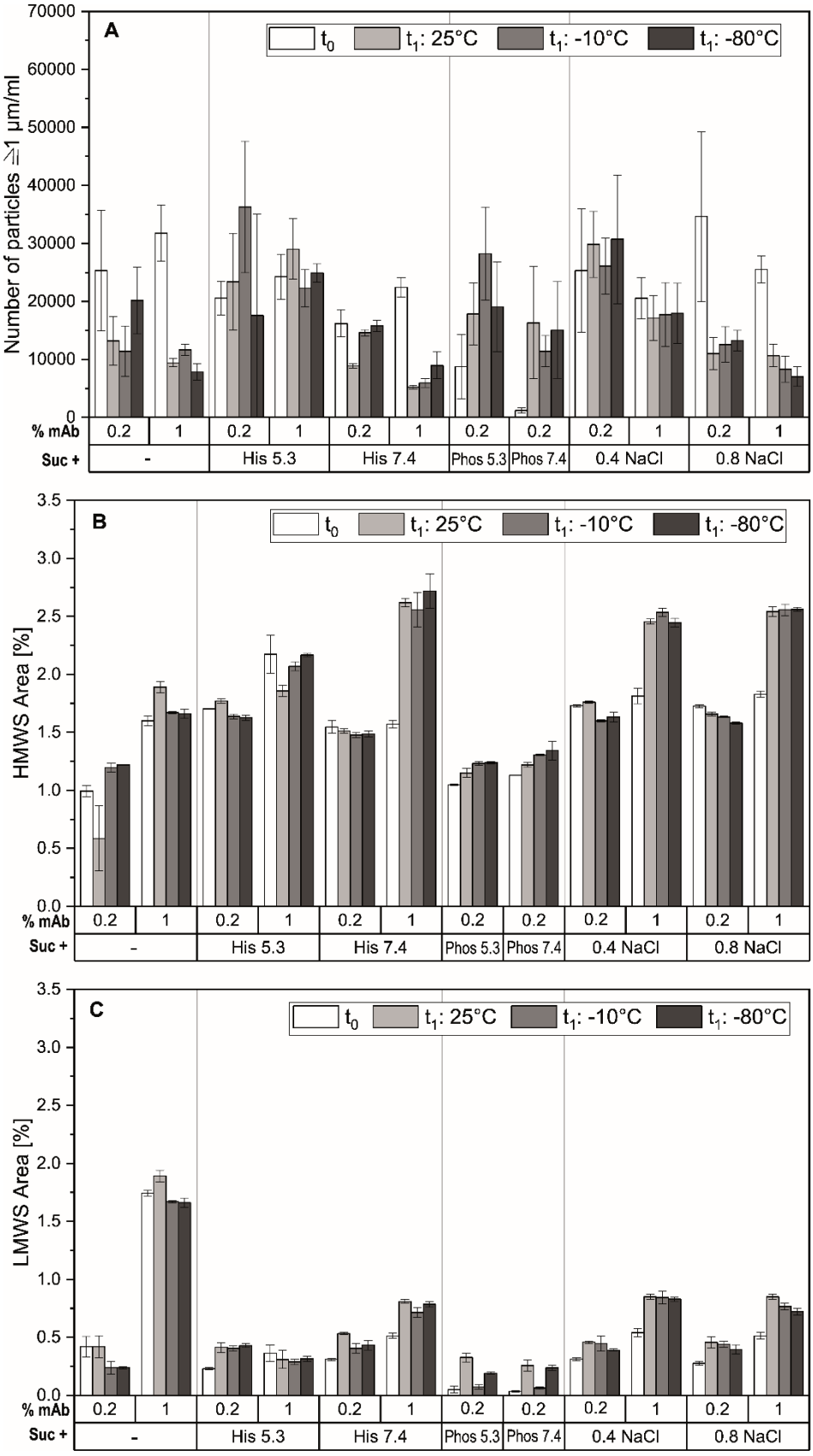

Figure 4-5: (A) Subvisible particle, (B) HMWS and (C) LMWS levels of FCs at t_0 and after storage at 25°C, -10°C, and -80°C for 1 month (t_1).

4. Conclusion

The aim of this study was to understand and evaluate protein stability in a maximally freeze concentrated solution and the influence of sucrose, buffer, and salt. While protein stability is well at temperatures below T_g' during freeze drying, it could be significantly different above T_g' during annealing or aggressive freeze drying. Furthermore, storage of frozen bulk at temperatures higher T_g' could lack in protein stability.

All FCs were visibly clear without phase separation or solute solidification at any time. An increase in sucrose concentration, which arises during freeze concentration, led to a drastic increase in viscosity reducing diffusivity and potentially reducing unfolding. We could not directly analyse the mAb self-interaction in the FC due to analytical challenges. A_2 in our case was less affected by pH and ionic strength but increasing the sucrose concentration led to stronger repulsion of the molecules. T_m was strongly increased in the freeze-concentrate due to the high sugar concentration. This should mirror in improved protein stability against cold denaturation. T_{agg} was increased according to T_m and both in the initial formulation and the FC aggregation occurred immediately with unfolding or was completely suppressed. Thus overall, the high sucrose concentration in the FC substantially improves mAb stability with respect to self-interaction, aggregation and denaturation. Over 1 month at 25°C, -10°C, and -80°C we did not observe visible and subvisible particle formation, and in SEC only in samples with higher mAb concentrations a slight HMWS formation was identified.

Thus, with respect to a lyophilisation process, we conclude no critical impact on mAb quality in the FC when the product temperature exceeds T_g' for a short time during annealing or primary drying. The amorphous sugar is an important stabilising factor in this context. These findings are additionally important for storage on bulk drug substance. In further studies, different proteins should be analysed regarding their stability in the FC, especially at high concentration.

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Chapter 5

The Influence of Arginine and Counter-Ions: Antibody Stability during Freeze Drying

The following chapter has been published as research article in the Journal of Pharmaceutical Sciences and appears in this thesis with the journal's permission:

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Abstract

Amino acids, for example L-arginine, are used in lyophilisation as crystalline bulking, buffering, viscosity reducing or stabilising excipients. In this study, arginine was formulated with different counter ions (hydrochloride, citrate, lactobionate, phosphate, and succinate). A monoclonal antibody was investigated in sugar-free arginine formulations and mixtures with sucrose regarding cake appearance and protein aggregation and fragmentation. Arginine hydrochloride formulations collapsed during lyophilisation due to its low T_g' and partially crystallised during storage, but provided the best protein stability at low antibody concentration, followed by arginine succinate. Arginine citrate/ phosphate/ lactobionate formulations resulted in amorphous elegant cakes, but inferior protein stability. Addition of sucrose improved cake appearance and protein stability. Arginine phosphate with sucrose resulted in similar protein stability as the sucrose reference. Mixtures of sucrose with arginine hydrochloride/ lactobionate/ succinate provided better stability than sucrose alone. While 50 mg/ml antibody improved the cake appearance, only arginine lactobionate provided sufficient protein stability next to sucrose. Overall, sugar-free arginine hydrochloride and lactobionate lyophilisates stabilised the antibody comparably or better than sucrose depending on antibody concentration. The best protein stability was found for mixtures of arginine hydrochloride/ lactobionate/ succinate with sucrose.

Keywords

Freeze-drying, Arginine, Sucrose, Protein aggregation, Antibody(s), Lyophilization

Table of Contents

1. Introduction

In order to prolong the shelf life of biopharmaceutics, freeze drying can be used [1]. Sucrose is the most frequently utilised excipient due its cryo- and lyoprotecting effect. Its moderate T_g' of -32°C and relatively high T_g of 75°C make sucrose favourable with respect to both process and storage stability [2]. Furthermore, isotonic sucrose solutions result in amorphous and elegant cakes [3]. Sucrose is a non-reducing sugar, but the glycosidic bond between the two building blocks, glucose and fructose, may be broken and the reducing monosaccharides can lead to protein glycation at accelerated or long term storage [4].

Besides saccharides like sucrose or trehalose, other bulk stabilising excipients are utilised with amino acids comprising an interesting group already used in commercially available products [5-8]. A few sugar free lyophilisation formulations based on amino acids for biologicals are on the market, e.g. ATryn® (antithrombin alfa), with glycine and sodium citrate, Omnitrope® (somatropin), with glycine, and Metalyse® (tenecteplase), with arginine and phosphoric acid as main excipients [9]. Various lyophilisates comprise both amino acid and sugar, the amino acid in most cases acting as buffer [9].

The amino acids differ in their physical and chemical behaviour after freeze drying. L-arginine, L-histidine, L-lysine, and L-citrulline form amorphous glasses, whereas glycine, L-phenylalanine, L-leucine, and L-isoleucine crystallise [6, 7, 10]. Crystallising amino acids are able to act as scaffolds to achieve elegant cake appearance [6, 11]. Nevertheless, proteins lack in stability when freeze dried in crystalline excipients only [12-14]. Glass forming amino acids can stabilise proteins by the same mechanisms as sugars, i.e. preferential exclusion, water replacement or void filling [5, 15-17].

Arginine has received specific attention as it can reduce protein-protein interaction and aggregation in liquid formulations and reconstituted lyophilisates [18, 19]. Its stabilising effect derives from a weak binding affinity to the protein surface via hydrogen bond formation and ion-dipole interactions [20-22]. Arginine containing formulations have shown their protein stabilising efficacy in freeze dried products depending on the protein, further excipients and concentrations [23]. Arginine in combination with further amino acids was able to prevent changes in the secondary structure of anti-CD11a and anti-IgE antibodies during lyophilisation [24]. A mixture of the three amino acids arginine, glutamic acid, and isoleucine was suitable to stabilise b-domain-deleted recombinant FVIII (GreenGene™ F) during production and storage to overcome the need for albumin as stabiliser [25]. In a potassium phosphate buffered solution pH 7.0, the addition of at least 2% (w/v) arginine preserved the stability of bovine liver catalase during lyophilisation [26].

The basic pH of 10.4 of an arginine base solution is not suitable for protein formulation and an acid or counter ion is essential for pH adjustment [10]. Furthermore, arginine base shows a very low T_g' of -42.7°C [27]. Different multivalent acids such as phosphoric acid, citric acid, or tartaric acid lead to an increase of T_g' [10]. The counter ion also affects protein stability. After 6 months at 40°C antibody formulations containing sucrose and arginine citrate, phosphate or succinate showed more aggregates than those with sucrose and arginine hydrochloride [28, 29]. Schersch et al. showed a protein stabilizing effect of arginine phosphate for lyophilisates which collapsed during freeze-drying [30].

Although, the stabilising potential of amino acids and especially of arginine containing formulations has been shown, a detailed investigation of the stabilising potential of arginine in combination with different counter ions and in the absence of additional excipients is still lacking [28, 29]. Therefore, we evaluated sugar-free based solely on arginine in combination with hydrochloride, citrate, lactobionate, phosphate, and succinate as counter ions and compared them to sucrose-based formulations. The formulations were studied with respect to their freeze drying properties, cake characteristics, and stabilisation of a monoclonal antibody (mAb) at 2 mg/ml in the pH range between 5.0 and pH 7.0. The arginine formulations were also combined with sucrose or high protein concentration of 50 mg/ml to evaluate the potential to improve the characteristics of the lyophilised product as well as mAb stability.

2. Materials and Methods

2.1 Materials

Stock solutions of sucrose (Merck KGaA, Darmstadt, Germany), L-arginine base (Sigma-Aldrich Chemie GmbH, Steinheim Germany), citric acid anhydrous (VWR International, Ismaning, Germany), succinic acid (Merck KGaA), and lactobionic acid (Acros Organics, Geel, Belgium) were prepared with highly purified water (HPW) or in 15 mM L-histidine buffer (Sigma-Aldrich Chemie GmbH) pH 5.0, 6.0, and 7.0. In addition, 1 M hydrochloric acid and conc. phosphoric acid (both VWR International) were used.

The pH of arginine base stock solution was titrated to pH 7.0, 6.0 or 5.0 with citric acid (ArgCitr), hydrochloric acid (ArgHCl), lactobionic acid (ArgLacto), phosphoric acid (ArgPhos), and succinic acid (ArgSucc). For sucrose formulations (Suc), sucrose was dissolved in the corresponding histidine buffer. Arginine formulations containing sucrose were prepared by adding sucrose to the titrated arginine formulations at pH 6.0. The following acronyms were used to describe these formulations: SucArgCitr, SucArgHCl, SucArgLacto, SucArgPhos, SucArgSucc. Suc based formulations contained 7% (w/w) sucrose in 15 mM histidine buffer, Arg based formulations 4% (w/w) arginine and mixed formulations 3.5% (w/w) sucrose and 2% (w/w) arginine.

A monoclonal IgG1 antibody (MW ca. 148 kDa, ϵ = 1.37 mL mg-1 cm-1, referred to as mAb) in 15 mM histidine buffer pH 5.3 was used as model protein. Formulations contained 2 mg/ml mAb. Formulations with high mAb concentration contained 50 mg/ml mAb and were named ArgHCl-HC, ArgCitr-HC, ArgLacto-HC, ArgPhos-HC, ArgSucc-HC, Suc-HC. For HC formulations the mAb was dialysed against HPW with a 10-fold buffer exchange in Vivaspin® 20 Ultrafiltration Uni (30 kDa, Sartorius, Goettingen, Germany) and up-concentrated.

The formulations were filtrated with 0.2 µm polyethersulfone membrane syringe filters (VWR International) prior to filling.

2.2 Methods

2.2.1 Freeze-thaw

Freeze-thaw experiments were performed using a FTS LyoStar™ 3 freeze dryer (SP Scientific, Stone Ridge, NY, USA). DIN 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with highly purified water and dried at 60°C for 8 h. The vials were filled with 1.5 mL of sample solution and stoppered with lyophilisation stoppers (B2-TR coating, West Pharmaceuticals Services Deutschland GmbH & Co. KG, Eschweiler, Germany). Samples were cooled from 20°C to -50°C at a ramp of 1°C/min followed by an isothermal hold for 3 h, thawed back to 20°C at a ramp of 1°C/min followed by an isothermal hold of 3 h. This cycle was repeated three times. Thermocouples in selected vials confirmed complete ice crystallisation and thawing and that -50°C and 20 °C resp. were reached in the samples.

2.2.2 Lyophilisation

Lyophilisation stoppers (B2-TR coating, West) and DIN 2R Vials (Fiolax®, Schott) were cleaned with highly purified water and dried at 60°C for 8 h. The vials were filled with 1.5 mL and semi-stoppered subsequently. The two outer rows of vials in each batch were not used for analysis. The product temperature in different vials at different position over the whole shelf area was record with thermocouples. Formulations were freeze-dried according to the protocols shown in Table 5-1 using a FTS LyoStar™ 3 freeze dryer (SP Scientific). End of primary drying was controlled by comparative pressure measurement between Pirani and MKS sensor. The vials were stoppered after secondary drying under nitrogen atmosphere at 800 mbar and crimped with flip-off seals.

For accelerated stability studies, sucrose reference and sugar free arginine samples pH 5 to 7 were stored at 50°C for 6 months, mixtures with sucrose pH 6 and high concentration pH 6 samples were stored at 40°C for 6 months.

Table 5-1: Freeze-drying cycles used.

Step	Ramp [°C/min]	Shelf temperature [°C]	Pressure [µbar]	Hold time [h]
Cycle 1: Sucrose, ArgCitr, ArgLacto, ArgPhos (pH 5 – 7) ArgCitr-HC, ArgHCl-HC, ArgLacto-HC, ArgPhos-HC, ArgSucc-HC, Suc-HC (pH 6)				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	54.5 / 64.7 (HC)
Secondary Drying	0.4	+40	60	8.3
Cycle 2: ArgHCl, ArgSucc (pH 5 – 7)				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	40	49.5
Secondary Drying	0.4	+50	40	5
Cycle 3: SucArgCitr, SucArgHCl, SucArgLacto, SucArgPhos, SucArgSucc (pH 6)				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	46.5
Secondary Drying	0.4	+30	60	8.3

2.2.3 Cake appearance

Images of the lyophilised products were taken with a Nikon D5300 camera (Nikon GmbH, Düsseldorf, Germany) in front of a black background.

2.2.4 Differential scanning calorimetry (DSC)

For the analysis of the glass transition temperature of the maximally freeze-concentrated solution T_g', 15 µl sample were filled in aluminium 40 µl crucibles (Mettler Toledo, Gießen, Germany), hermetically sealed and analysed with a DSC 821^e (Mettler Toledo). The samples were cooled to -60°C at 10 K/min, held at -60°C for 5 min and reheated to 20°C at 2 K/min. The T_g' was determined as the midpoint of the phase transition using the STAR^e software for data analysis.

The glass transition temperature (T_g) of the lyophilisates was determined with a DSC 821^e (Mettler Toledo) or a Polyma 314 (Netzsch, Selb, Germany). 5 to 10 mg of crushed cake were filled under controlled humidity conditions (less than 10% rel. humidity) into aluminium 40 µl crucibles (Mettler Toledo) or concavous 40 µl crucibles (Netzsch) and sealed hermetically. Samples containing sucrose or

ArgHCl were heated to 80°C at 10°/min, cooled to 20°C at 10°/min and reheated to 120°C at 10°/min. Other samples were heated to 120°C at 10°/min, cooled to 20°C at 10°/min and reheated to 150°C at 10°/min. T_g was determined as the midpoint of the phase transition during the second heating using the STAR^e software (Mettler Toledo) or Proteus 7.1 software (Netzsch) for data analysis.

2.2.5 Karl-Fischer titration

The residual moisture (RM) was analysed by coulometric Karl-Fischer titration using the AQUA 40.00 titrator with a headspace module (Analytik Jena AG, Halle, Germany). For the measurement about 20 mg of the lyophilised sample was prepared in a dry atmosphere and heated in the headspace module up to 100°C. The evaporated water was transferred into the titration solution and the remaining moisture determined.

2.2.6 X-Ray powder diffraction (XRPD)

The crystallinity of the lyophilised samples was analysed with the X-Ray Diffractometer XRD 3000 TT (Rich. Seifert & Co. GmbH & Co. KG, Ahrensberg, Germany), equipped with Cu Kα (40 kV, 30 mA, wavelength 154.17 pm). The powders were analysed from 5 – 45° 2°θ with steps of 0.05° 2°θ and 2 seconds per step.

2.2.7 Reconstitution time

Reconstitution time was determined by dissolving the lyophilisates with the required volume of HPW. The required volume was calculated based on the solid content of each formulation. The time span from adding the HPW until complete dissolution was determined visually and considered as reconstitution time. During reconstitution, the vials were gently rolled by hand.

2.2.8 Light obscuration

Light obscuration was used to characterise subvisible particles in the range of 1 to 200 µm. For this purpose, a PAMAS SVSS-C35 particle counter with an HCB-LD-25/25 sensor (Partikelmess- und Analysensysteme GmbH, Ruthesheim, Germany) was used. Samples were analysed 4 times with 0.2 ml each after rinsing the system with 0.2 µm filtrated HPW and a sample pre-rinse with 0.2 ml according to USP 788 [31]. At least 3 samples were measured to evaluate the subvisible particle count per ml of particles ≥1 µm.

2.2.9 Turbidity

To further assess protein aggregation the turbidity was measured by static light scattering at 90° (λ = 860 nm) using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany). The turbidity was recorded in formazine nephelometric units (FNU). 1.5 ml of each sample was analysed in triplicates.

2.2.10 High performance size exclusion chromatography (HP-SEC)

Size exclusion chromatography was performed with an Agilent 1100 series HPLC system with UV detection at 280 nm (Agilent Technologies, Santa Clara, CA, USA). A Waters AQUITY UPLC Protein BEH 200Å column (1.7 µm; 4.6 x 150 mm; Waters GmbH, Eschborn, Germany) and 50 mM sodium phosphate with 400 mM sodium perchlorate buffer at pH 6.0 were used as stationary and mobile phase, respectively. The flow rate was set to 0.4 mL/min. Prior to analysis, samples were centrifuged (10 min, 7000 rpm, Force7, Denver Instruments, Bohemia, NY, USA). The area of the peaks as well as relative areas of monomer, aggregates and fragments were determined after blank subtraction using the ChemStation software (Agilent Technologies).

3. Results and Discussion

3.1 Evaluation of arginine formulations during freeze-thaw cycles

Already the freezing step during freeze drying can result in a loss in product quality and was therefore addressed separately as a first step by characterising the influence of freezing and thawing on mAb stability in the presence of arginine and different counter ions [32].

Freezing and thawing of sucrose reference formulations (pH 6.0) resulted in formation of approx. 50,000 particles ≥ 1 µm/ml (Figure 5-1). The addition of 0.05% polysorbate 80 to sucrose formulations reduced particle formation to less than 10,000 particles ≥ 1 µm/ml. After freezing and thawing, ArgPhos pH 5 resulted in the most pronounced particle formation with 1.2 million, followed by ArgCitr pH 5 and SucArgCitr with around 100,000, and ArgPhos pH 6 and pH 7 as well as SucArgSucc with around 80,000 particles ≥ 1 µm/ml. The other samples were comparable or better than the sucrose reference. Overall, there was a trend towards less particles with higher pH.

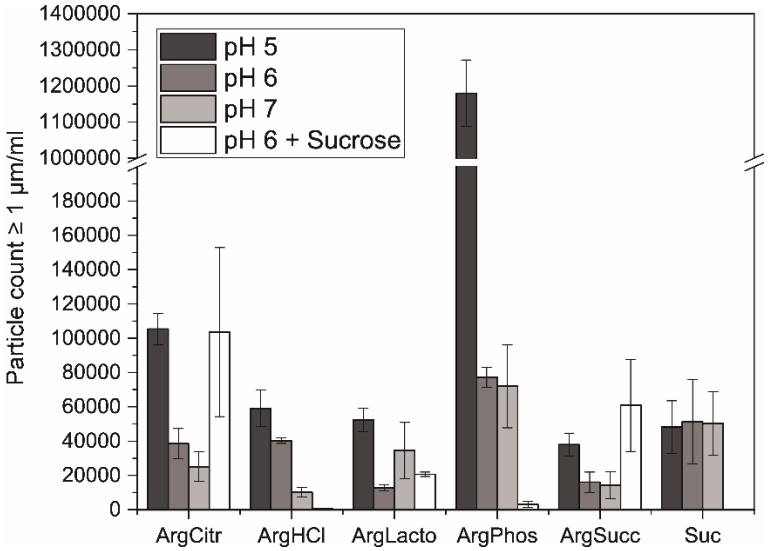

Figure 5-1. Subvisible particle count after 3 freeze-thaw cycles.

3.2 Protein stability after freeze drying

Subsequently the stabilising effect of arginine both in pure amino acid based as well as in arginine and sucrose containing mAb formulations with different pH and counter-ions was evaluated. The formulations were studied with respect to their lyophilisation properties and cake characteristics by characterising T_g' of the frozen liquid as well as T_g, residual moisture, reconstitution times, and cake appearance. The colloidal stability of the mAb was assessed by characterising the formation of soluble aggregates as well as subvisible particles and turbidity. Chemical degradation of a mAb was assessed on the basis of fragmentation.

3.2.1 T_g' of arginine based formulations

Pure arginine base has a rather low T_g' of -42.7°C [27]. The T_g' of arginine salts strongly depends on the counterion and the salt form and does not only reflect the difference in pH value resulting from a different amount of acid added. Multivalent acids increase the T_g' of arginine until a 1:1 mixture is reached, whereas monovalent acids tend to decrease T_g'. For example, citric acid brings the T_g' up to around -25°C, whereas acetic acid shows a continuous decrease in T_g' to values below -50°C [10, 23, 33]. Too low T_g' values can cause issues during conventional freeze drying causing poor cake appearance due to collapse [34].

As shown in Table 5-2, ArgHCl and ArgSucc exhibited T_g' values of approx. -46.5°C and -38.0 °C, respectively, which was not affected by pH and was below the T_g' of sucrose of approx. -32.5 °C. The

other sugar free arginine formulations ArgCitr and ArgLacto showed substantially higher T_g' values. ArgPhos exhibited a pH dependence of T_g' with -30.6°C at pH 5 and -27.3°C at pH 7. As the strong pH dependent shift of T_g' was only seen for phosphoric acid but not for the other trivalent acid, citric acid, a correlation with nearing one of the pK_a values of phosphoric acid cannot be concluded. Furthermore, a dependency of T_g' with respect to different acids in combination with arginine or with increasing phosphoric acid concentration were reported without a correlation to the pK_a [10, 33]. Addition of sucrose led to a shift towards the T_g' of pure sucrose. The T_g' for SucArgCitr, SucArgLacto, and SucArgPhos became decreased by approx. 3°C and we observed an increase for ArgHCl and ArgSucc by 8 and 5 °C respectively. This shift can be explained by the Gordon-Taylor equation, which states a mixed T_g' consisting of the mass fraction and the T_g' values of the individual components [35]. Nevertheless, the bell-shaped transition temperature profiles of arginine with organic acids is not explainable by Gordon-Taylor [10]. Interestingly, T_g' of ArgLacto was higher if compared to the pure arginine T_g', although lactobionic acid belongs to the monovalent acids [33]. T_g' of a pure lactobionic acid solution was -30.2 ± 0.3°C leading to a mixed T_g' explainable by the Gordon-Taylor equation [10]. Increasing the mAb concentration from 2 mg/ml to 50 mg/ml mAb led to an increase of T_g' [34, 36]. Suc-HC exhibited an increase of about 2°C, whereas the T_g' values for ArgHCl-HC and ArgSucc-HC was increased by almost 15 °C.

Table 5-2: T_g' values of sucrose and arginine formulations with mAb.

Formulation	**T_g' [°C]**				
	pH 5	**pH 6**	**pH 7**	**with sucrose (pH 6)**	**high conc (pH 6)**
Suc	-32.5 ± 0.3	-33.5 ± 0.3	-31.3 ± 0.6	--	-31.6 ± 0.4
ArgCitr	-26.4 ± 0.1	-27.1 ± 0.1	-27.2 ± 0.03	-30.4 ± 0.03	-23.1 ± 0.1
ArgHCl	-46.4 ± 0.1	-46.3 ± 0.2	-46.4 ± 0.1	-38.2 ± 0.1	-32.5 ± 0.3
ArgLacto	-26.2 ± 0.3	-25.6 ± 0.05	-25.2 ± 0.4	-28.8 ± 0.2	-21.4 ± 0.1
ArgPhos	-30.6 ± 0.01	-28.7 ± 0.3	-27.3 ± 0.1	-32.1 ± 0.2	-23.8 ± 0.03
ArgSucc	-37.7 ± 0.1	-38.0 ± 0.1	-37.9 ± 0.1	-33.8 ± 0.3	-23.6 ± 0.02

Thus, most of the arginine formulations can be handled by a standard conventional lyophilisation process and are expected to result in pharmaceutically acceptable cakes. ArgHCl and ArgSucc must be seen critical due to their low T_g' values. T_g' could be increased by adding sucrose or using high mAb concentrations.

3.2.2 Powder characteristics of the lyophilisates

Subsequently, sucrose and arginine formulations pH 5 to 7 were freeze dried according to cycle 1. ArgCitr, ArgLacto, and ArgPhos resulted in pharmaceutically elegant cakes (Figure 5-3). The pure sucrose lyophilisates showed some defects. The pH value did not affect the macroscopic appearance. ArgHCl and ArgSucc were substantially collapsed and shrunken since the product temperature of -32°C during primary drying in cycle 1 was significantly above T_g' (Figure 5-2). Less defects could be observed when lyophilised at a lower pressure according to cycle 2 resulting in a product temperature of -34°C during primary drying (Figure 5-3). Secondary drying temperature was decreased to 30°C for freeze drying the mixed arginine sucrose formulations according to cycle 3 (Figure 5-3). The addition of sucrose did not influence cake appearance of ArgCitr, ArgLacto, and ArgPhos based lyophilisates and improved the appearance of ArgHCl and ArgSucc based cakes. 50 mg/ml mAb formulations freeze dried according to cycle 1 resulting in a product temperature of -36°C during primary drying were all elegant products due to the higher T_g' values and the increased total solid contents. Cake appearance of all the formulations did not change over 6 months storage at 40°C or 50°C.

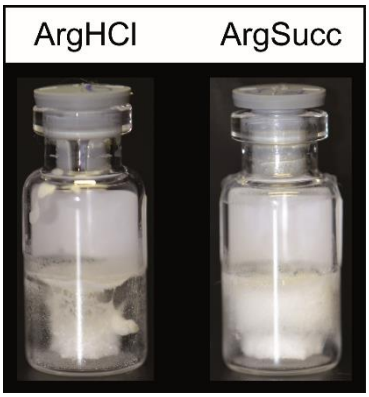

Figure 5-2. Macroscopic appearance of ArgHCl and ArgSucc at pH 6 lyophilized according to cycle 1.

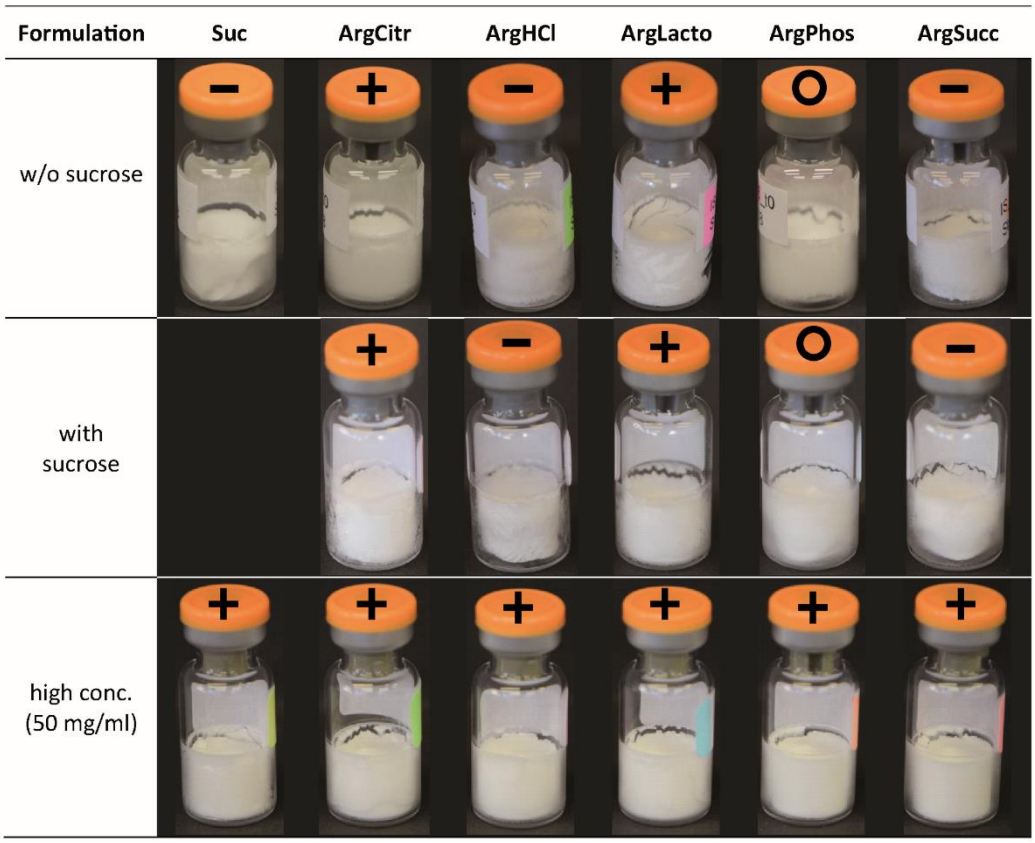

Figure 5-3. Macroscopic appearance of sucrose and arginine lyophilisates pH 5.0, 6.0, and 7.0 that differ in the counter ion, the addition of sucrose, and mAb content. Freeze drying cycle 1 was used for Suc, ArgCitr, ArgLacto, and ArgPhos without sucrose, as well as all 50 mg/ml mAb lyophilisates; cycle 2 was used for ArgHCl and ArgSucc without sucrose; cycle 3 was used for all arginine-sucrose mixtures. The symbol + represents good cake appearance, o minor cake shrinkage/minor structural defects, and – major cake shrinkage/structural defects.

Overall, ArgHCl and ArgSucc required low product temperatures during primary drying to gain elegant cakes. A mixture with sucrose or the addition of a high mAb concentration can improve slow primary drying times due to their low T_g' values and resulted in acceptable cakes. Other arginine formulations showed elegant cakes after freeze drying in a standard procedure.

The sucrose reference showed a T_g value of 65°C and a RM of 0.5% after lyophilisation [37]. T_g did not significantly change upon storage at 50°C for up to 6 months while RM increased slightly to 0.9% (Figure 5-4). Overall, the Arg based lyophilisates showed very high T_g and low RM values after production. Similar to T_g', the T_g of freeze dried arginine with multivalent counter ions increased with increasing pH [33], whereas ArgHCl and ArgLacto lyophilisates were not affected by pH. ArgCitr showed the highest T_g values of 90°C to 120°C (RM of 0.3%), followed by ArgPhos and ArgSucc. ArgHCl and ArgLacto lyophilisates had similar T_g values of 80°C independent of pH. After 6 months storage at 50°C, arginine based products increased in RM to between 1% and 2% and correspondingly the T_g values

decreased by up to 15°C [19, 38]. Even after storage, T_g was above storage temperature indicating potential protein stability through immobilisation in the amorphous matrix [39, 40].

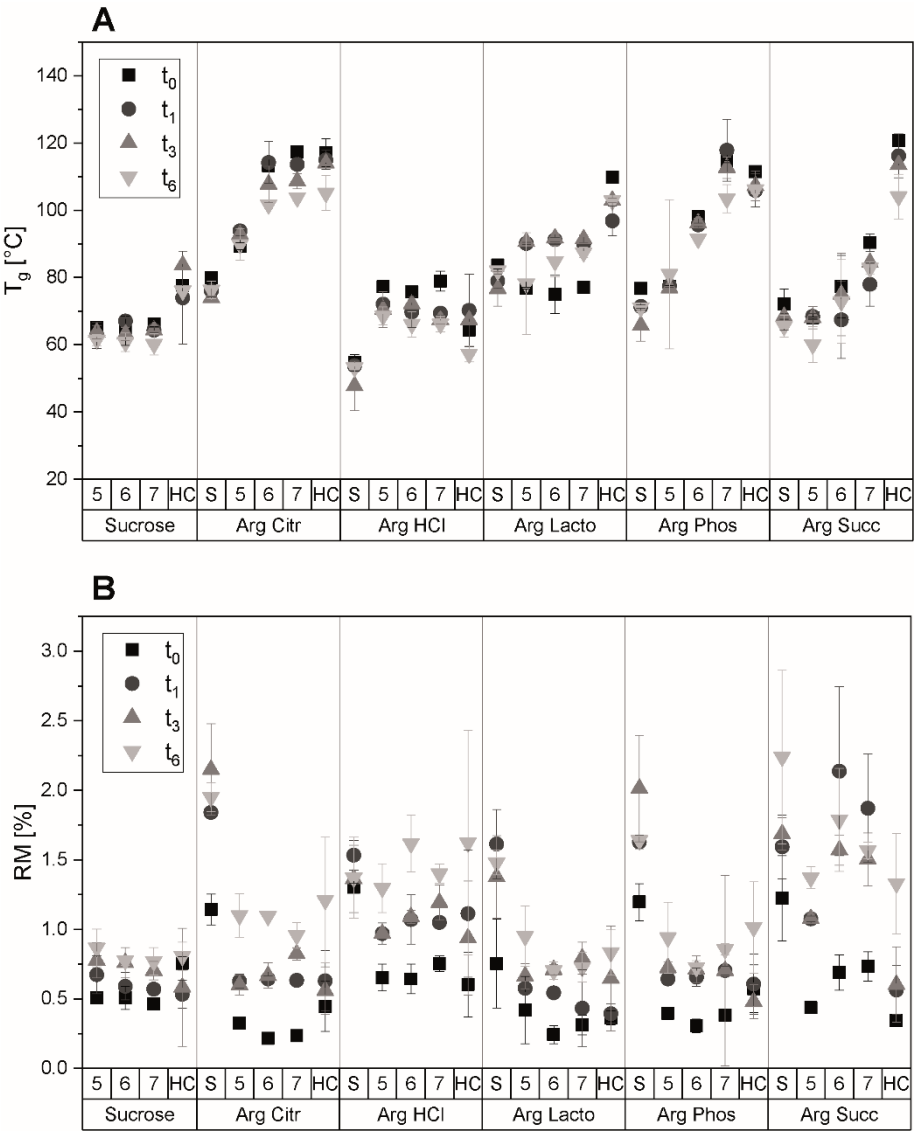

Figure 5-4. T_g (A) and RM (B) of sucrose and arginine based 2 mg/ml mAb lyophilisates at pH 5, 6 and 7 (5, 6, 7) stored at 50°C, as a mix with sucrose at pH 6 (S) and at pH 6 with 50 mg/ml mAb (HC) after lyophilisation (t_0) and upon storage at 40°C for 1 (t_1), 3 (t_3) and 6 months (t_6).

Mixtures of sucrose and arginine salts were freeze dried according to cycle 3 with a lower secondary drying temperature resulting in higher RM levels after production except for ArgLacto. The higher RM levels and the addition of sucrose led to a decrease in T_g to 55°C for SucArgHCl and between 72°C and slightly above 80°C for SucArgCitr, SucArgPhos, SucArgSucc and SucArgLacto. During 6 months storage at 40°C, the T_g slightly decreased by approx. 5°C.

Products with 50 mg/ml mAb resulted in similar RM and the higher protein concentrations led to even further increased T_g values [41]. An increase by 10°C up to 20°C was found for ArgPhos-HC, ArgLacto-HC, and Suc-HC, a drastic increase by 40°C for ArgSucc-HC. Hardly any change was found for ArgCitr-HC, which already exhibited an extremely high T_g, and ArgHCl-HC, which was unexpected as an increase was identified in literature [28].

Sugar-free Arg based formulations exhibited extremely high T_g values compared to the sucrose reference formulation pointing towards high protein stability in the immobilised amorphous phase [39, 40]. In combination with sucrose, T_g values were decreased slightly according to a mixed T_g based on the Gordon-Taylor equation [10] but the values are still high overall.

All samples showed a T_g in DSC analysis and were fully amorphous according to XRPD except for ArgHCl stored for 3 or 6 months, independent of pH (Figure 5-5). The addition of sucrose as well as a high mAb concentration of 50 mg/ml suppressed the crystallisation of ArgHCl upon storage.

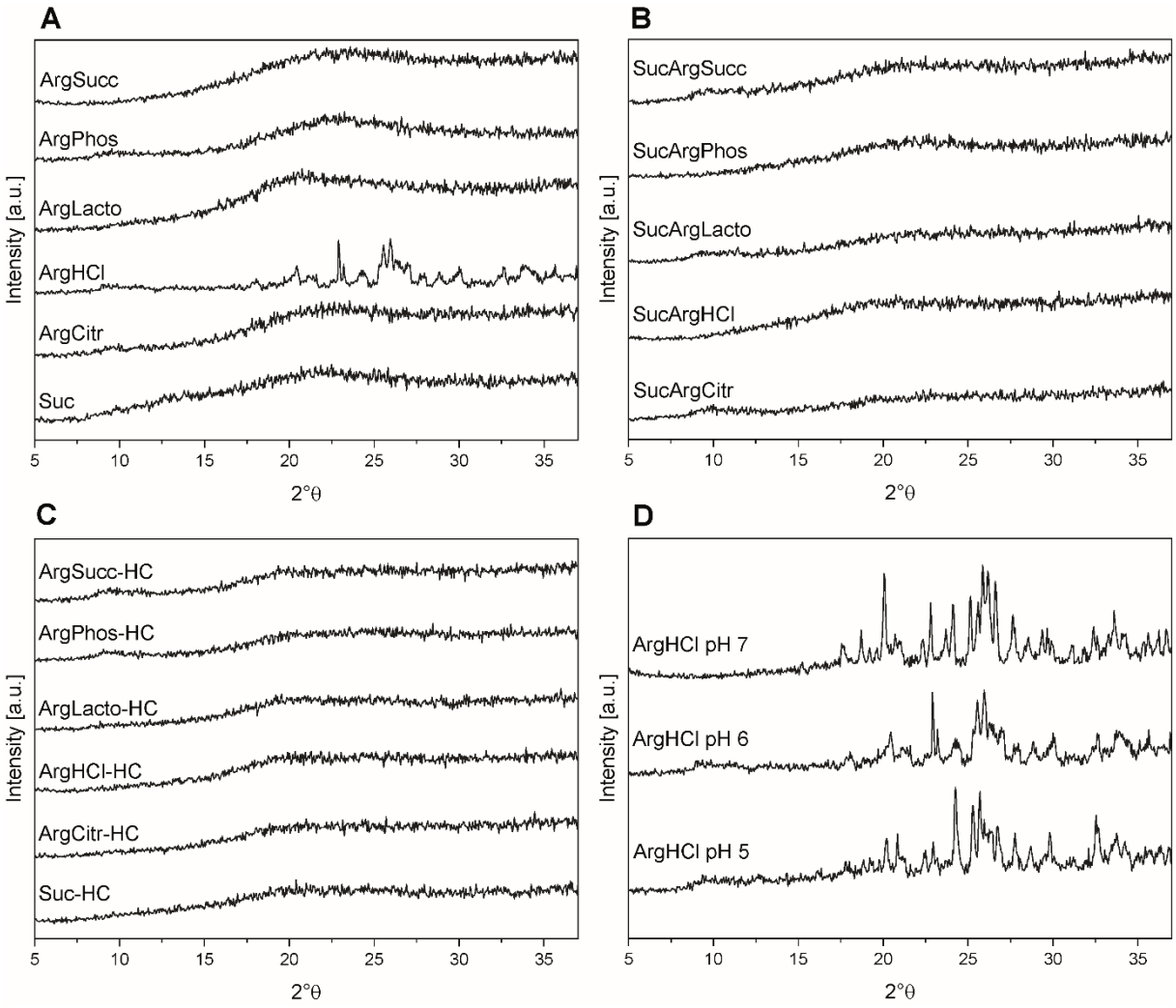

Figure 5-5. XRPD of (A) 2 mg/ml mAb pH 6 samples after 6 months storage at 50°C, (B) mixtures of arginine and sucrose pH 6 samples after 6 months storage at 40°C, (C) 50 mg/ml mAb pH 6 samples after 6 months storage at 40°C, and (D) ArgHCl pH 5, 6, and 7 after 3 months storage at 50°C.

Reconstitution of all 2 mg/ml mAb samples was completed within 30 s at t_0 and after storage. The 50 mg/ml mAb samples based on ArgHCl showed the same fast reconstitution. For all other formulations, reconstitution took longer with up to 60 s for ArgCitr-HC, ArgLacto-HC, and ArgSucc-HC and 3 min for ArgPhos-HC, which increased further upon storage. Thus, arginine based lyophilisates dissolve which makes especially ArgHCl a promising alternative candidate for highly concentrated mAb sugar based lyophilisates with delayed dissolution times [6, 42].

3.2.3 mAb stability during lyophilisation and upon storage

The mAb process and storage stability was analysed with respect to aggregation by SEC, turbidity and subvisible particle analysis by light obscuration in order to assess colloidal stability of the mAb. Furthermore, information on fragmentation was collected via the SEC measurements in order to evaluate chemical stability of the formulations.

The sucrose reference formulations pH 5, 6, and 7 resulted in low particle counts after lyophilisation with less than 30,000 particles ≥ 1 µm/ml and a turbidity of < 5 FNU (Table 5-3). Less than 2% HMWS were found for all pH values (Figure 5-6). After 6 months storage at 50°C, particle count and turbidity increased in a pH dependent manner. While pH 5 revealed no changes, pH 7 resulted in around 90,000 particles and 11.7 FNU turbidity. Additionally, large and rapidly sedimenting visible particles had formed. The HMWS level did not increase but the relative monomer recovery compared to t_0 decreased to 85 – 90% due to larger particle formation [43].

Table 5-3: Subvisible particle count and turbidity data of sucrose and arginine based 2 mg/ml mAb lyophilisates at pH 5, 6, and 7 (5, 6, 7) stored at 50°C, as a mix of sucrose at pH 6 (S) stored at 40°C, and at pH 6 with 50 mg/ml mAb (HC) after lyophilisation (t_0) and after 6 months storage (t_6) at 40°C. Turbidity data marked with * were analysed after 3 months storage.

		Subvisible particle count ≥ 1 µm/ml		**Turbidity [FNU]**	
		t_0	**t_6**	**t_0**	**t_6**
Sucrose	**5**	23,425 ± 947	8,003 ± 1,257	3.3 ± 0.4	3.1 ± 0.1
	6	24,623 ± 2,334	53,550 ± 7,280	3.3 ± 0.8	7.7 ± 0.3
	7	28,520 ± 2,926	90,296 ± 17,487	4.6 ± 1.5	11.7 ± 1.0
	HC	15,516 ± 2,372	62,851 ± 16,681	13.3 ± 1.1	14.8 ± 0.4*
ArgCitr	**S**	31,045 ± 9,252	42,826 ± 9,904	2.7 ± 0.5	6.4 ± 0.8
	5	60,562 ± 18,570	118,557 ± 22,679	4.9 ± 3.6	12.9 ± 0.7
	6	15,705 ± 3,704	144,738 ± 25,431	2.8 ± 0.7	11.5 ± 1.7
	7	14,450 ± 1,439	144,651 ± 24,362	4.6 + 2.2	11.9 ± 0.4
	HC	15,951 ± 5,481	71,050 ± 21,407	11.3 ± 0.4	13.2 ± 0.9*
ArgHCl	**S**	10,559 ± 1,281	12,726 ± 3,208	2.6 ± 0.4	4.8 ± 1.0
	5	4,638 ± 716	23,947 ± 7,182	3.4 ± 1.1	4.2 ± 0.5
	6	1,311 ± 475	41,502 ± 4,358	2.4 ± 0.1	5.6 ± 0.3
	7	1,685 ± 229	74,419 ± 10,053	3.4 ± 1.1	7.1 ± 0.02
	HC	7,590 ± 4,864	15,447 ± 5,027	4.2 ± 0.2	5.2 ± 0.3*
ArgLacto	**S**	23,017 ± 5,325	54,532 ± 10,957	3.1 ± 0.3	7.0 ± 0.2
	5	58,353 ± 26,010	101,698 ± 32,743	5.7 ± 1.1	14.5 ± 1.2
	6	56,680 ± 13,404	113,148 ± 36,471	6.7 ± 1.3	16.9 ± 0.7
	7	42,961 ± 12,292	122,398 ± 40,258	3.9 ± 0.7	18.3 ± 0.7
	HC	12,870 ± 5,141	42,001 ± 14,172	8.8 ± 0.5	9.3 ± 0.1*
ArgPhos	**S**	13,315 ± 3,783	29,408 ± 4,107	2.6 ± 0.4	5.9 ± 0.1
	5	53,938 ± 12,009	144,320 ± 16,079	6.0 ± 1.2	17.0 ± 1.1
	6	30,348 ± 5,273	135,814 ± 20,690	3.9 ± 0.5	13.1 ± 1.1
	7	46,976 ± 5,529	116,887 ± 17,356	5.6 ± 0.7	11.7 ± 0.2
	HC	10,911 ± 5,049	76,135 ± 22,258	10.2 ± 0.2	12.4 ± 0.7*
ArgSucc	**S**	14,294 ± 2,929	33,445 ± 4,962	2.5 ± 0.3	7.9 ± 1.1
	5	16,852 ± 3,368	48,648 ± 9,856	4.2 ± 0.2	5.8 ± 0.4
	6	9,692 ± 1,903	112,868 ± 18,386	2.8 ± 0.5	8.1 ± 3.6
	7	9,238 ± 2,327	116,937 ± 18,206	2.8 ± 0.6	16.3 ± 1.0
	HC	15,966 ± 2,923	73,258 ± 23,688	12.2 ± 0.3	13.9 ± 0.9*

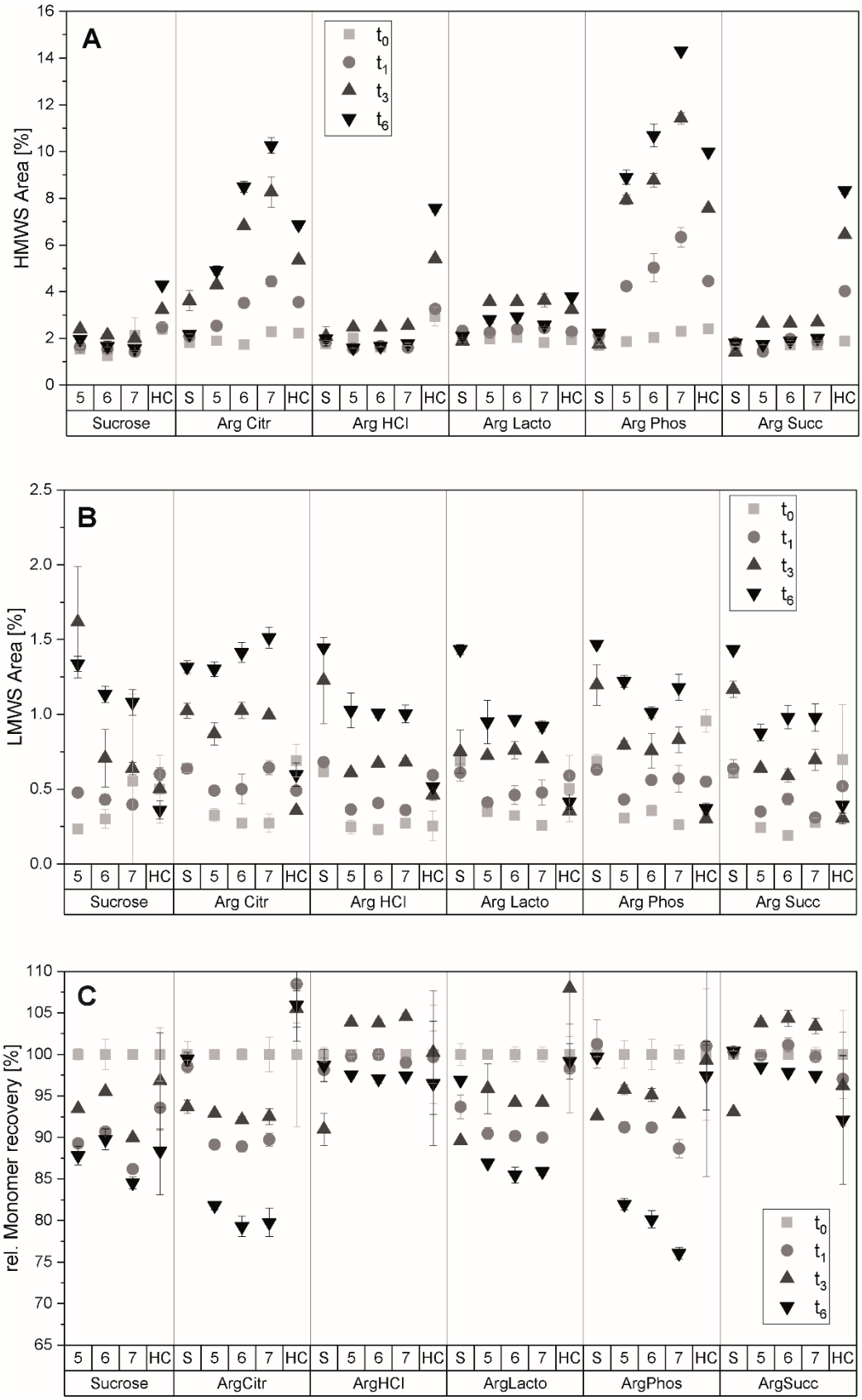

Figure 5-6. (A) Higher molecular weight species (HMWS), (B) lower molecular weight species (LMWS), and (C) relative monomer recovery of sucrose and arginine based 2 mg/ml mAb lyophilisates at pH 5, 6 and 7 (5, 6, 7), as a mix with sucrose at pH 6 (S) and at pH 6 with 50 mg/ml mAb (HC) after lyophilisation (t_0) and upon storage for 1 (t_1), 3 (t_3) and 6 months (t_6).

The sugar-free arginine based 2 mg/ml mAb products differed depending on counter ion and pH. Whereas the number of particles ≥ 1 µm/ml was similar or slightly higher for ArgCitr, ArgLacto and ArgPhos, the mAb showed substantially better process stability in ArgHCl and ArgSucc with less than 5,000 and < 17,000 particles, respectively. Turbidity and HMWS content were similar for all formulations and comparable to sucrose.

After 6 months storage at 50°C, the particle count of ArgCitr, ArgLacto, and ArgPhos was higher than 100,000 and turbidity reached values above 10 FNU. These formulations showed a slight pH dependency. ArgCitr and ArgLacto showed an increase in particle count with increasing pH, moving closer to the IEP of the mAb of around 8.0. This tendency is less pronounced for ArgPhos, which correspond to the freeze/thaw experiments where higher particle formation only occurred at pH 5. Overall similar trends were observed in HMWS content and relative monomer recovery. The HMWS content increased up to 4% in ArgLacto and up to 10% and 14% at pH 7 for ArgCitr and ArgPhos respectively. The relative monomer recovery decreased to 85% in ArgLacto, 80% in ArgCitr, and 75 – 82% in ArgPhos. The mAb stability after 6 months at 40°C in ArgSucc lyophilisates was superior at pH 5 with less than 50,000 particles ≥ 1 µm/ml, turbidity below 10 FNU, approx. 3% HMWS and more than 97% monomer recovery. At pH pH 6 and 7 the particle numbers were higher. ArgHCl formulations exhibited the best stability with the lowest particle numbers, turbidity values below 10 FNU, no increase in HMWS level and no monomer loss in SEC.

In general, mixtures of sucrose and arginine at pH 6 showed a better process stability. Only for SucArgHCl a slightly higher particle count compared to pure ArgHCl product was seen. After 6 months storage at 40°C, all formulations resulted in a less pronounced increase in particle count compared to the sugar free formulations. Most particles were found for SucArgLacto with around 55,000 particles, which was comparable to pure sucrose. The turbidity was similar or lower compared to both pure sucrose and the sugar-free products. The HMWS levels increased and the relative monomer recovery decreased in none of the formulations. Only a slight increase in HMWS formation from 2.9% to max. 4.1% upon storage at 40°C was observed in literature for sucrose mixtures with 50 mg/ml mAb [29].

Increasing the mAb concentration to 50 mg/ml resulted in similar of slightly lower particle levels. The turbidity values were significant higher with around 10 FNU compared to 2 mg/ml products, except for ArgHCl-HC which was low with 4.2 FNU. Aggregate levels of around 3% HMWS were found. After 6 months storage at 40°C, particle counts increased less compared to the corresponding 2 mg/ml samples. Again, ArgCitr-HC, ArgPhos-HC, and ArgSucc-HC were comparable to the sucrose reference Suc-HC reference and ArgLacto-HC and ArgHCl-HC were superior. The turbidity increased slightly in all formulations and the HMWS levels in Suc-HC and ArgLacto-HC went up to 4% and to 7 – 10% in all

other arginine formulations including ArgHCl-HC. A decrease in relative monomer recovery was not observed. Thus, at high mAb concentration ArgHCl and ArgLacto were beneficial with ArgHCl forming the least amount of larger aggregates and ArgLacto the least soluble aggregates.

All formulations showed comparable stability against cleavage. Overall, the mAb did not undergo severe fragmentation in any formulation upon freeze drying and storage (Figure 5-6B).

Thus, despite providing the lowest T_g of all tested counter ions, the monovalent hydrochloric acid was the most suitable counter ion for arginine to stabilise the mAb during freeze-drying and storage, independent of the pH value and addition of sucrose. Overall it provided better protein stability compared to the sucrose reference formulations. This is in good agreement with previously published data similarly concluding best protein stability for ArgHCl lyophilisates [29]. Multivalent counter ions resulted in reduced protein stability compared to ArgHCl, with the counter ion succinic acid being an exception at pH 5. The amorphous matrices of arginine with multivalent counter ions are different to ArgHCl matrices, indicated by high T_g values of up to 120°C. Accordingly, they seem to act differently in terms of stabilising a mAb. The use of arginine lyophilisates in marketed products further supports the potential of sugar-free arginine formulations [9].

The addition of sucrose to arginine formulations resulted in good or better results for particle formation and protein stability compared to the sucrose reference, again similar to previous reports [28, 29]. A high mAb concentration of 50 mg/ml resulted in overall low particle counts and ArgLacto-HC turned out to be an interesting formulation candidate next to ArgHCl-HC.

4. Conclusion

The aim of this study was to test sugar-free arginine based formulations as alternatives to sucrose or sucrose-containing arginine formulations for lyophilisation of biopharmaceuticals. By using a sugar-free formulation approach, potential glycation issues, which may occur in sucrose based formulations, can be circumvented in an elegant way [2, 4]. We studied the freeze-drying performance of sugar-free arginine formulations with different counterions at different pH with 2 and 50 mg/ml of an exemplary mAb. The freeze-thaw stability of the mAb as well as the physicochemical characteristics and the mAb stability during freeze-drying and upon subsequent storage up to 6 months at 40°C or 50°C were studied. The sucrose references pH 5 to pH 7 showed good cake appearance and remained amorphous over storage. They were characterised by a T_g of 65°C [37]. The lyophilisates exhibited low particle counts and did not show an increase in HMWS but substantial subvisible and visible particle formation, which led to a loss in monomer recovery [43].

Stability of the antibody upon freezing and thawing in ArgHCl, ArgLacto or ArgSucc formations were found to be comparable or better than in a sucrose formulation. Potential protein instabilities during the freezing step of the applied freeze-drying process for arginine formulations could be excluded for these formulations.

T_g' was increased compared to the sucrose formulation by using arginine in combination with citric, lactobionic, and phosphoric acid potentially enabling a more robust and faster lyophilisation process. The T_g' values of ArgHCl and ArgSucc at 2 mg/ml mAb were very low with -46°C and -38°C, respectively, which poses challenges in the opposite way. This challenge can be overcome by adding sucrose or using high mAb concentration leading to an increase in T_g'. By running an adapted freeze drying cycle for ArgHCl and ArgSucc, all formulations resulted in good cake appearance which did not change over storage. The T_g values of ArgHCl and sucrose lyophilisates were similar with approx. 65°C whereas the other arginine formulations showed even higher T_g values of up to 120°C. All formulations exhibited T_g values well above the intended storage temperature providing a good forecast for protein stabilisation through protein immobilisation in the amorphous matrix [39, 40]. ArgHCl partially crystallised after 3 months at 50°C but crystallisation was suppressed in the sucrose mixtures or at 50 mg/ml mAb at 40°C storage temperature. The Arg based lyophilisates reconstituted fast. At 50 mg/ml mAb, reconstitution times were prolonged except for ArgHCl-HC taking only 30 sec for complete dissolution, which could be highly valuable asset for high concentration formulation or large fill volumes [42]. Both process and storage stability of the mAb was superior in ArgHCl and ArgSucc if compared to sucrose with respect to aggregation. Mixtures of arginine and sucrose improved both process and storage stability of the mAb compared to a sucrose formulation without arginine. At high mAb concentration ArgHCl-HC and ArgLacto-HC provided good process and storage stability with respect to aggregation and fragmentation.

In summary, this study presented sugar-free Arg based formulations as potential alternative to sucrose or sucrose-containing arginine lyophilisates for biopharmaceuticals, which were already reported in literature [28, 29]. While multivalent acids as counter ions enhance T_g' and T_g to values above corresponding sucrose formulations and provide good process characteristics, they showed a lack in protein stabilisation. Arginine formulations in combination with the counter ions succinate and lactobionate were comparable in both cake appearance and protein stabilisation efficiency to the sucrose reference formulation. The monovalent hydrochloride rendered outstanding protein stability. The low T_g' values of ArgSucc and ArgHCl with low mAb concentration may pose challenges in freeze drying and corrupt the cake appearance but this can be overcome by addition of sucrose and potentially other crystalline bulking agents. Further studies should address these drawbacks while

maintaining protein stability. Overall, arginine based lyophilisates do have both advantages and disadvantages but should be used more frequently as alternatives to sugar based formulations.

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Chapter 6

The Effect of Residual Moisture on a Monoclonal Antibody Stability in L-arginine Based Lyophilisates

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The Effect of Residual Moisture on a Monoclonal Antibody Stability in L-arginine Based Lyophilisates
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Abstract

Amino acids are not only used as buffering agents in lyophilisation, but also exhibit cryo- and lyoprotecting characteristics. L-Arginine based lyophilisates were tested regarding their ability to stabilise a monoclonal antibody (mAb) at different residual moisture (RM) levels. Arginine base was formulated with citric, hydrochloric, lactobionic, phosphoric, and succinic acid for pH adjustment. Lyophilisates with less than 0.5% and approx. 2.5% RM were stored for up to 6 months at 40°C. The mAb aggregation in arginine in combination with hydrochloric acid and succinic acid was similar or even less compared to a sucrose reference formulation. Arginine in combination with citric acid, lactobionic acid, and phosphoric acid resulted in lower protein stability. Overall, arginine formulations with high RM levels resulted in better protein stabilisation despite decreased glass transition temperatures (T_g). Whereas we detected mAb glycation in the sucrose based formulations, this chemical reaction did not occur in arginine based formulations. Arginine hydrochloride and succinate, especially at high RM levels, could be promising alternatives to sucrose for stabilisation of mAb in lyophilisates.

Keywords

Lyophilisation, mAb, Aggregation, Residual moisture, Arginine, Sugar-free, Glycation

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1. Introduction

Lyophilisation of is frequently used to improve the long-term storage stability of protein pharmaceuticals. Protein stability is typically attributed to the reduced mobility in the glassy amorphous matrix [1]. Stabilisation has also been ascribed to replacement of water molecules by sugar molecules, which can form hydrogen bonds with the protein [2, 3]. This raises the question whether all water molecules stabilising the native state of the protein can be replaced or how many water molecules should remain to grant optimal protein stability. It is commonly stated that products should be freeze dried below 1% residual moisture (RM) [1, 4-6]. An enhanced loss of protein activity may occur with overdried samples and chemical and physical instability may be more pronounced at higher RM of 3 – 10% [7-9]. Overall, individual formulations have to be freeze dried to their optimal RM area also keeping in mind that product specifications have to be set based on a rational.

Only a few sugar free lyophilisates for biopharmaceuticals based on amino acids are available on the market, e.g. Metalyse® (tenecteplase) with arginine and phosphoric acid as main excipients [10]. Arginine has the ability to reduce protein aggregation in liquid and after reconstitution, which makes arginine salts of special interest for formulation development [11-13]. The protein stabilising effect of arginine containing lyophilisates is dependent on the protein, other excipients, and the concentrations used [14, 15]. Since the pure base has a pH of 10.4 counter ions are necessary for pH adjustment [16, 17]. Furthermore, the glass forming ability depends on the counter ion [16, 18, 19]. Exceptionally high glass transition temperatures (T_g) have been described for arginine with multivalent anions like phosphate and citrate, whereas markedly lower T_g values result for monovalent anions like hydrochloride [16, 20, 21]. The low T_g of the arginine and hydrochloride combination is related to a low T_g' of -42.5°C which may lead to macro- or microcollapse, eventually resulting in higher RM levels [20].

So far, the effect of RM on the protein stability in sugar-free arginine based lyophilisates has not been addressed in research. A slight increase in RM during storage in sucrose formulations can cause a strong decrease in T_g [7]. An increase in RM in arginine formulations with an extremely high T_g might result in sufficiently high T_g values, also over storage, to maintain protein stability.

We analysed mAb aggregation in arginine salt (citrate, hydrochloride, lactobionate, phosphate, succinate) lyophilisates at two different RM levels. Formulations were tested at less than 0.5% RM to simulate overdrying and approx. 2.5% RM as high moisture level. Over 6 months storage at 40°C less mAb aggregation occurred at high compared to low RM levels of all arginine formulations. The protein stability in arginine succinate was similar to that in the sucrose reference formulation. High RM arginine hydrochloride cakes collapsed due to their lower T_g over storage, but they did not show

particle or HMWS formation providing superior stability over sucrose. The sucrose reference formulation provided markedly less protein stabilization at high RM and additionally glycation occurred. Glycation was further assessed in sucrose containing formulations (sucrose and mannitol:sucrose 4:1) over 9 months at 60°C and compared to arginine citrate and hydrochloride lyophilisates.

2. Materials and Methods

2.1 Materials

Sucrose and succinic acid (Merck KGaA, Darmstadt, Germany), L-arginine base, L-histidine base, L-histidine hydrochloride monohydrate, and D-Mannitol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), citric acid anhydrous, di-sodiumhydrogen phosphate dihydrate, and sodiumdihydrogen phosphate dihydrate (VWR International, Ismaning, Germany), and lactobionic acid (Acros Organics, Geel, Belgium), 1 M hydrochloric acid (VWR International), and conc. phosphoric acid (VWR International) were used to prepare stock solutions in highly purified water (HPW, Arium Pro, Sartorius, Göttingen, Germany).

Sucrose stock solution and formulation (Suc) were prepared in 15 mM histidine buffer pH 6.0. After dilution with buffer, Suc contained 7% (w/w) sucrose in 15 mM histidine buffer pH 6.0. Arginine base stock solution was titrated with the acid solution until pH 6.0 was reached. The following acronyms were used for the arginine formulations: ArgCitr, ArgHCl, ArgLacto, ArgPhos, ArgSucc. The final arginine formulations contained 4% (w/w) arginine in HPW.

For the glycation study, a ratio of 4:1 mannitol to sucrose (Man:Suc) and Suc stock solutions in 15 mM phosphate buffer pH 7.2 were used. $ArgCitr_{glyc}$ and $ArgHCl_{glyc}$ were titrated to pH 7.2 as described above. After dilution with phosphate buffer, Suc_{glyc} and Man:Suc contained 7% (w/w) sucrose or mannitol:sucrose 4:1, respectively, in 15 mM phosphate buffer pH 7.2 and $ArgCitr_{glyc}$ and $ArgHCl_{glyc}$ contained 4% (w/w) arginine in HPW.

A monoclonal IgG_1 antibody (MW ca. 148 kDa, ϵ = 1.37 mL mg^{-1} cm^{-1}; mAb) was used at 2 mg/ml as model protein.

Prior to use, all formulations were filtrated with 0.2 µm polyethersulfone membrane syringe filters (VWR International).

2.2 Methods

2.2.1 Lyophilisation

Prior to use, lyophilisation stoppers (B2-TR coating, West Pharmaceuticals Services Deutschland GmbH & Co. KG, Eschweiler, Germany) and DIN 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with HPW and dried at 60°C for 8 h. The vials were filled with 1.5 mL formulation and semi-stoppered. Both placebo and protein formulations were freeze-dried according the protocols in Table 6-1 using a FTS Lyostar™ 3 freeze dryer (SP Scientific, Stone Ridge, NY, USA). Thermocouples, placed in different vials positioned over the shelf, were used for temperature recording. Comparative pressure measurement between Pirani and MKS sensor was used for the end of primary drying detection. The vials were stoppered at 800 mbar after secondary drying and crimped with flip-off seals.

Samples were analysed after lyophilisation and after 1, 3, and 6 months storage at 40°C.

Table 6-1: Freeze drying protocols.

Step	Ramp [°C/min]	Shelf temperature [°C]	Pressure [µbar]	Hold time [h]
Cycle 1: ***High residual moisture***				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	48
Secondary Drying	0.4	20	60	5
Cycle 2: ***Low residual moisture***				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	48
Secondary Drying	0.4	40	60	12
Cycle 3: ***Glycation samples - high residual moisture***				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	48
Secondary Drying	0.4	20	60	5
Cycle 4: ***Glycation samples - low residual moisture***				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	48
Secondary Drying	0.4	20	60	5
	0.4	40	60	10

2.2.2 Cake appearance

To visualise the cake appearance, images of the lyophilised products were taken in front of a black background with a Nikon D5300 camera (Nikon GmbH, Düsseldorf, Germany).

2.2.3 Differential scanning calorimetry (DSC)

The glass transition temperature (T_g) of the lyophilised products was determined with a DSC 821^e (Mettler Toledo, Gießen, Germany). Up to 10 mg crushed cake were filled at < 10% r.h. conditions into 40 µl aluminium crucibles and hermetically sealed. Samples containing Suc or ArgHCl were heated to 80°C at 10°C/min, cooled to 10°C at 10°C/min, and reheated to 120°C at 10°C/min. Other samples were heated to 120°C at 10°C/min, cooled to 10°C at 10°C/min, and reheated to 150°C at 10°C/min. The midpoint of the phase transition in the second heating step was determined as T_g.

2.2.4 Karl-Fischer titration

Residual moisture (RM) analysis was performed by coulometric Karl-Fischer titration (AQUA 40.00, Analytik Jena AG, Halle, Germany) with a headspace module. About 20 mg crushed lyophilised product was prepared under dry atmosphere (< 10% rel. humidity) and heated up to 100°C in the headspace module. The evaporated water was transferred into the titration solution and RM determined.

2.2.5 X-Ray powder diffraction (XRPD)

Powder morphology was analysed with an X-Ray Diffractometer XRD 3000 TT (Rich. Seifert & Co. GmbH & Co. KG, Ahrensberg, Germany) from 5 – 45° 2θ with steps of 0.05° 2θ and 2 seconds per step (Cu Kα, 40 kV, 30 mA, λ = 154.17 pm).

2.2.6 Reconstitution time

Samples were reconstituted with HPW considering the water mass removed by lyophilisation for each formulation. Vials were gently rolled by hand. The time until complete dissolution based on visual inspection was considered as reconstitution time.

2.2.7 Light obscuration

Subvisible particles between 1 and 200 µm were characterised by light obscuration with the PAMAS SVSS-C35 particle counter with an HCB-LD-25/25 sensor (Partikelmess- und Analysensysteme GmbH, Ruthesheim, Germany). After sample pre-rinse with 0.2 ml, each sample was analysed 4 times with 0.2 ml according to USP 787 [22]. Between each sample, the system was rinsed with 0.2 µm filtrated HPW. At least 3 samples/formulation were used for calculating the subvisible particle count of particles ≥ 1 µm, ≥ 10 µm, and ≥ 25 µm per ml.

2.2.8 High performance size exclusion chromatography (HP-SEC)

Higher and lower molecular weight species (HMWS/LMWS) were analysed with size exclusion chromatography using an Agilent 1100 series HPLC system, equipped with an UV/Vis detector (280 nm; Agilent Technologies, Santa Clara, CA, USA). A Waters AQUITY UPLC Protein BEH 200Å column (1.7 µm, 4.6 x 150 mm, Waters GmbH, Eschborn, Germany) was used with the following parameters: 50 mM sodium phosphate with 400 mM sodium perchlorate buffer pH 6.0 as mobile phase with a flow rate of 0.4 ml/min. Prior to analysis, samples were centrifuged at 7000 rpm for 10 min (Force7, Denver Instruments, Bohemia, NY, USA). The integrated peak area was determined after blank subtraction using the ChemStation software (Agilent Technologies) and relative areas were calculated.

2.2.9 Boronate affinity chromatography (BAC)

Glycation was detected with boronate affinity chromatography by using an Agilent 1200 series HPLC system equipped with a UV/Vis detector at 280 nm (Agilent Technologies). A TSK Boronate-5PW column (7.5 x 75 mm, Tosoh Bioscience, Stuttgart, Germany) was used with Buffer A (100 mM HEPES, 200 mM NaCl, 70 mM Tris, pH 8.6) and Buffer B (500 mM sorbitol in Buffer A) [23]. After injection, Buffer A was kept at 100% for 10 min followed by a linear gradient of Buffer B from 0% to 100% in 5 min. Buffer B was kept at 100% for 3 min. Within 1 min, the system was flushed back to Buffer A until the end of the run after 35 min. A flow rate of 1 ml/min was used with a column temperature of 40°C.

The degree of glycation in % was calculated as the ratio of the AUC of the individual curves, integrated and blank subtracted with the ChemStation software (Agilent). The first peak at around 2.5 min was referred to as non-glycated species and the second peak at around 15.5 min was referred to as glycated species.

2.2.10 Circular dichroism (CD)

Mab containing ArgCitr, ArgHCl, and ArgPhos samples were diluted with formulation buffer to 0.4 mg/ml for the near-UV circular dichroic measurements (250 – 320 nm). The spectra were collected at 25°C with a Jasco J-810 spectropolarimeter (JASCO Deutschland GmbH, Pfungstadt, Germany). Quartz cuvettes with 10 mm path length were used at a scan rate of 20 nm/min. The spectrum of the respective buffer was subtracted for each sample and the mean residue ellipticity calculated [24].

3. Results and Discussion

3.1 Investigation of protein stability in low and high residual moisture lyophilisates

3.1.1 Physical chemical characterisation of lyophilisates with low and high residual moisture

The major aim of this study was to identify and understand the influence of residual moisture on the ability of arginine, depending on its counter ion, to stabilise proteins during and after lyophilisation compared to the gold standard sucrose. Therefore, different arginine formulations were lyophilised by different secondary drying conditions to obtain different residual moisture levels.

Arginine containing formulations result in higher RM values compared to the sucrose reference formulation with 0.2% to 0.5% RM compared to 0.2% and 2.2% to 3.2% RM compared to 1.5% for high and low RM samples (Figure 6-1A and B). This may be related to a difference in water affinity, the higher total solid content or microcollapse of the arginine based formulations [15, 20]. Upon storage, arginine formulations showed a more pronounced increase in RM compared to the sugar based formulation [12, 15, 25]. Interestingly, high RM sucrose had a significantly lower RM level compared to the arginine formulations over the whole storage time. Within each set, ArgHCl and ArgSucc consistently showed higher RM values compared to ArgCitr, ArgLacto, and ArgPhos, due to collapse and shrinkage which opposes water removal during lyophilisation.

The differences in RM were reflected in T_g (Figure 6-1C and D) [7]. Within the Low RM lyophilisates, Suc and ArgHCl showed the lowest T_g values with around 65°C, increasing in the order ArgSucc, ArgPhos, ArgLacto, and ArgCitr to 120°C. The same ranking was found for the high RM formulations, but with overall lower T_g values. During storage, the T_g values decreased slightly, which corresponds to the increase in RM [7]. Nevertheless, T_g values of arginine formulations except for ArgHCl remained between 60 and 90 °C despite a high RM level. ArgHCl showed partial crystallisation already after

1 month storage at 40°C for both, high and low RM. All other products remained fully amorphous over the entire time.

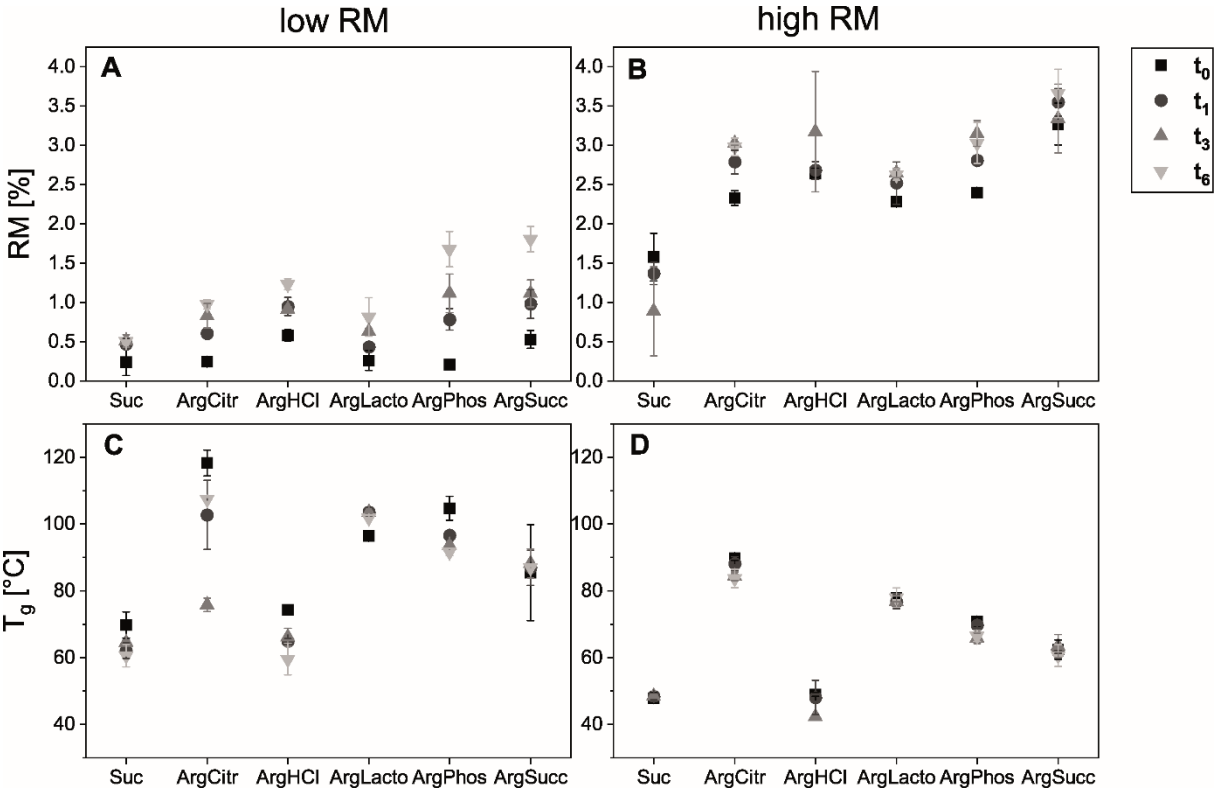

Figure 6-1: RM and T_g results of sucrose and arginine formulations over 6 months at 40°C. A. RM of low RM samples. B. RM of high RM samples. C. T_g of low RM samples. D. T_g of high RM samples. Both Suc and ArgHCl with high RM collapsed during storage and were not analysed after 6 months.

Right after lyophilisation, samples with both high and low RM showed the same macroscopic appearance. Sucrose and ArgSucc were slightly shrunken, whereas ArgHCl had partially collapsed (Figure 6-2). ArgHCl is prone for collapse due to its low T_g' [15, 20, 25]. After 6 months storage at 40°C, the formulations with low RM did not change. Within the high RM samples, Suc and ArgHCl showed complete collapse and approx. one third of the Suc and Suc placebo vials showed discolouration.

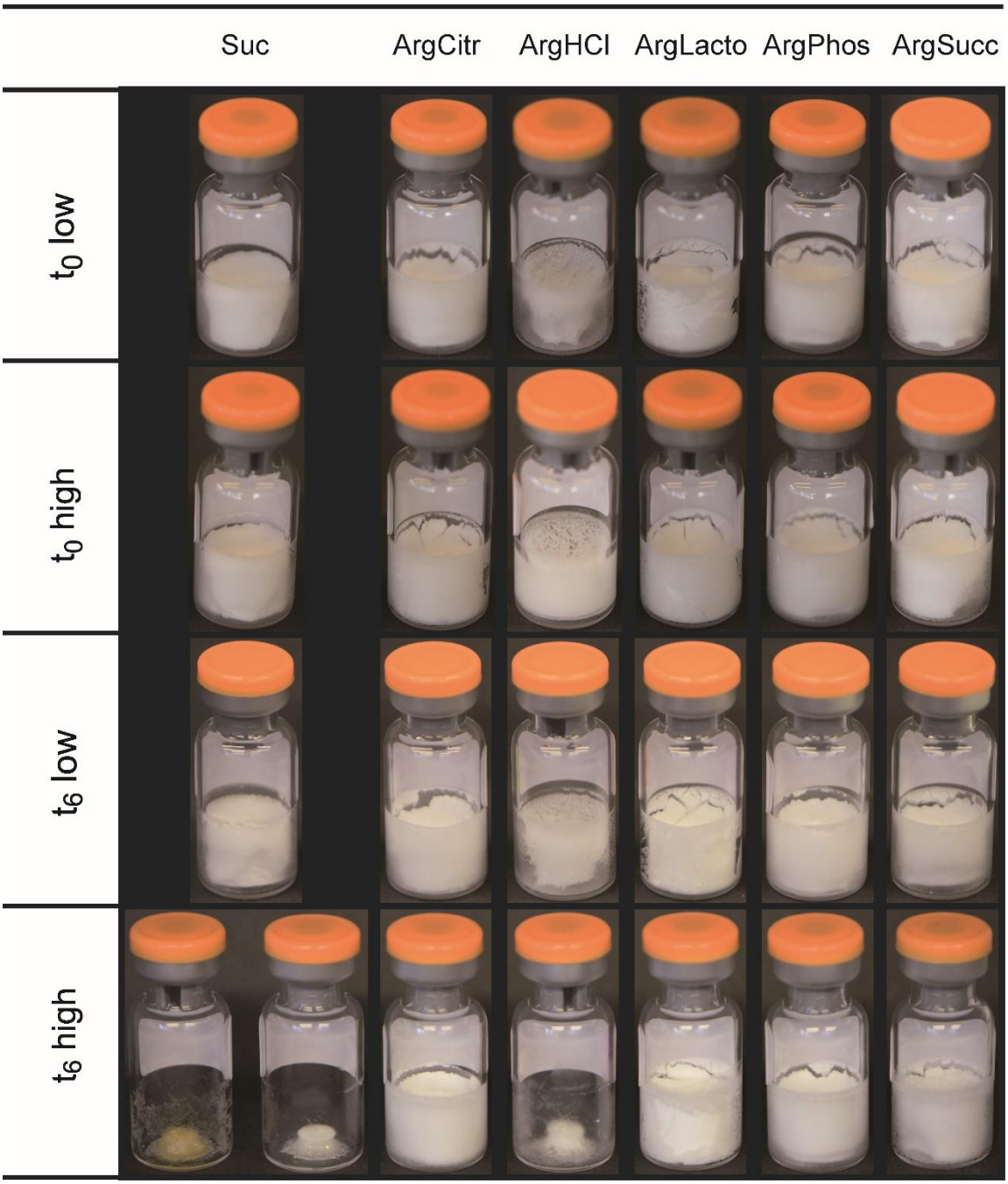

Figure 6-2: Macroscopic appearance of sucrose and arginine formulations after lyophilisation and after 6 months storage at 40°C.

All lyophilisates reconstituted within 30 seconds at t_0. The reconstitution time did not change except for the fully collapsed ArgHCl and Suc formulations with high RM which required up to 6 min and 16 min, respectively.

3.1.2 mAb stability with low and high residual moisture during lyophilisation and upon storage

The effect of residual moisture level on protein aggregation was studied considering formation of subvisible particles (Figure 6-3) and HMWS in SEC (Figure 6-4). Sucrose low RM was set as benchmark with approx. 25,000 particles ≥ 1 µm/ml, approx. 300 particles ≥ 10µm/ml, and approx. 10 particles ≥ 25 µm/ml after lyophilisation and over 6 months storage at 40°C. The HMWS level of 2% HMWS after lyophilisation did not change over 6 months storage. In contrast, Suc high RM, starting at the benchmark level after lyophilisation, showed an enormous increase to around 300,000 particles ≥ 1 µm/ml and 1,400 particles ≥ 10 µm/ml after 6 months storage at 40°C going along with collapse and independent of whether the vials showed discolouration. Visual inspection showed that the reconstituted products exhibited flocculated visible particles. Collapse and partial decolouration led to an increase in HMWS formation already after 3 months, increasing up to 10% HMWS after 6 months at 40°C.

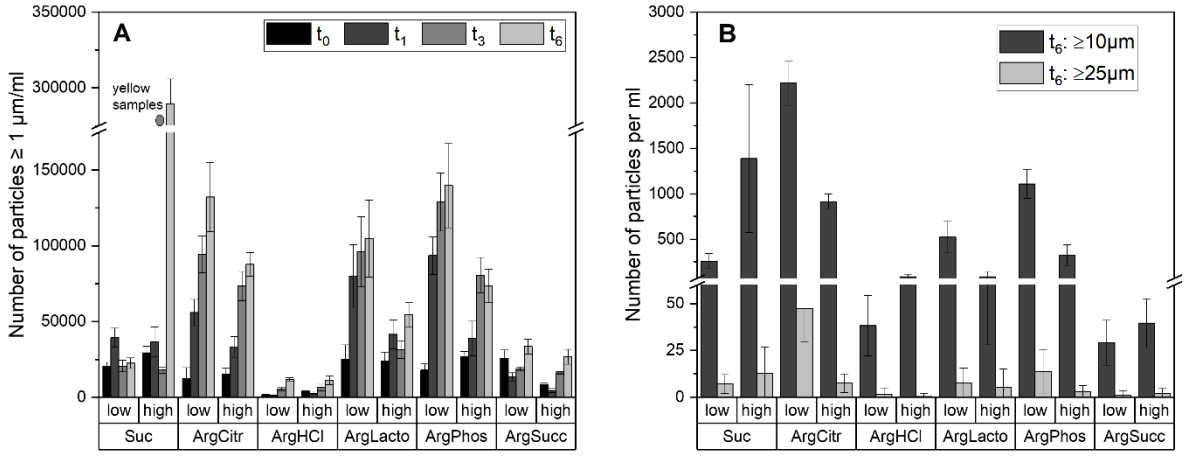

Figure 6-3: Number of subvisible particles (A) ≥ 1 µm/ml of sucrose and arginine formulations with high and low RM over 6 months at 40°C and (B) ≥ 10 µm and 25 µm after 60 months at 40°C.

All arginine based formulations showed comparable or lower particle levels after lyophilisation to the sucrose benchmark. Both ArgCitr and ArgPhos formulations resulted in an increase in particle count over 6 months up to approx. 140,000 particles ≥ 1 µm/ml and 80,000 particles ≥ 1 µm/ml with low and high RM, respectively. ArgCitr low RM exhibited around 2,000 particles ≥ 10 µm/ml and a few visible particles. SEC of ArgCitr and ArgPhos revealed an increase from 2% HMWS after lyophilisation to 5 – 7% HMWS after 6 months at 40°C. In ArgLacto low RM the particle count increased up to 100,000 particles ≥ 1 µm/ml and the HMWS to 2.5% after 6 months. ArgLacto high RM tended towards low particle counts with approx. 50,000 particles ≥ 1 µm/ml after storage and constant HWMS levels

of 2.5%. Interestingly, both RM levels of ArgSucc were comparable with Suc low RM. Particle counts and HWMS formation did not exceed Suc low RM values. Therefore, ArgSucc showed a clear benefit to Suc resulting in a similar protein stability but neither collapse nor RM dependent protein stability. Interestingly, both ArgHCl formulations were clearly superior to Suc resulting in approx. 10,000 particles ≥ 1 µm/ml after storage independent of the collapse level. HMWS level did not increase over 6 months storage.

LMWS were between 0.3 – 0.7% for all samples, independent of storage except for collapsed and discoloured Suc high RM lyophilisates which exhibited up to 3% LMWS which led to an overall increase with high standard deviation. The discolouration and mAb instability of the collapsed Suc high RM lyophilisates was evaluated further. Boronate affinity chromatography (BAC) identified approx. 28% glycated mAb in these samples whereas glycated species were absent in any other formulation.

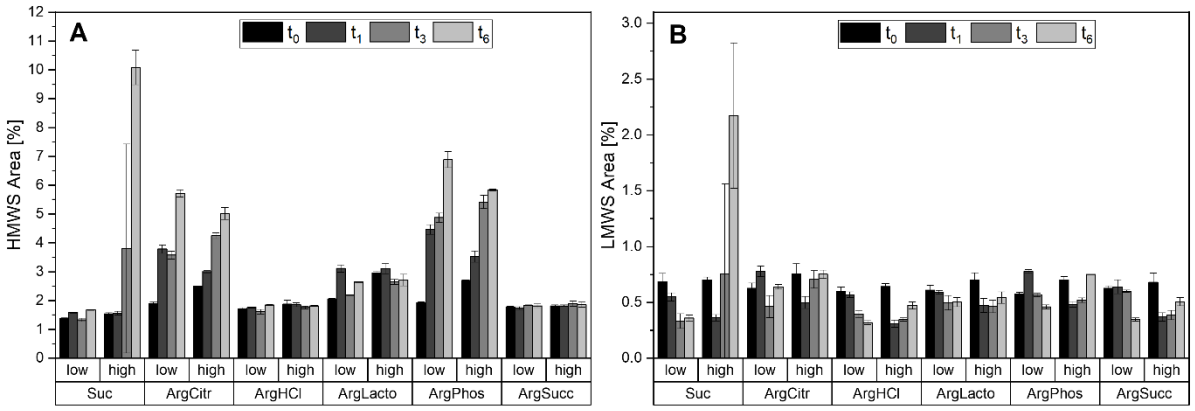

Figure 6-4: HMWS (A) and LMWS (B) results for sucrose and arginine formulations with high and low RM over 6 months at 40°C.

The integrity of the mAb's tertiary structure was exemplarily evaluated for ArgCitr, ArgHCl, and ArgPhos formulations of both RM levels after 6 months storage at 40°C with near-UV CD (Figure 6-5). No changes were detectable comparing the formulations with the samples before lyophilisation.

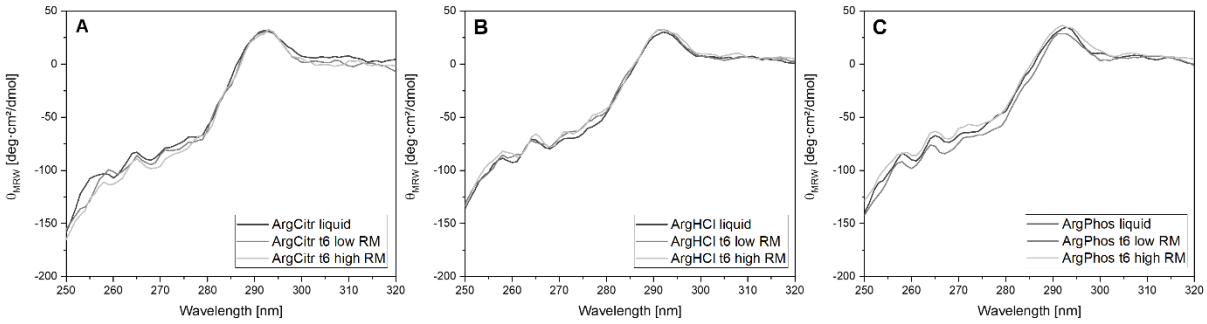

Figure 6-5: Near-UV CD spectra for (A) ArgCitr, (B) ArgHCl, and (C) ArgPhos formulations before lyophilisation and after storage for 6 months at 40°C.

Thus, overall mAb aggregation was more pronounced in ArgPhos, ArgCitr and to a lesser extend increased in ArgLacto compared to Suc. The Suc high RM exhibited collapse and discolouration after 6 months and after 3 months already an increase in HMWS was observed. ArgSucc was comparable to sucrose. ArgHCl resulted in lower subvisible particles levels compared to sucrose even though the lyophilisates collapsed. RM did not influence protein stability in Arg formulations with a trend to better stability at higher RM values. Suc lyophilisates resulted in less protein stability with increased RM upon storage at 40°C. Comparing the individual counter ions, trivalent acids such as citric acid and phosphoric acid exhibited the worst protein stability. No clear conclusion could be drawn for mono- and bivalent acids. While ArgSucc was comparable to Suc, ArgHCl resulted in better protein stability but with a high risk of cake collapse.

3.2 Glycation challenge study at higher temperature

To further study the observed protein glycation and moisture dependent protein stability we additionally compared sucrose (Suc_{glyc}), mannitol and sucrose mixture (Man:Suc 4:1), $ArgCitr_{glyc}$, and $ArgHCl_{glyc}$ with low and high RM upon storage at 25°C, 40°C, and 60°C over 9 months, assessing protein stability with light obscuration, SEC, and BAC.

Suc_{glyc}, Man:Suc, and $ArgCitr_{glyc}$ resulted in pharmaceutically elegant cakes for both RM levels, whereas $ArgHCl_{glyc}$ exhibited a loss in cake structure. Low and high RM lyophilisates showed 0.1 - 0.5% RM and 1.2 – 2.3% RM, respectively (Table 6-2). The Suc_{glyc} formulation exhibited a T_g of 74°C and 50°C for low and high RM, respectively. The Man:Suc mixture resulted in the δ-Mannitol modification and a T_g of sucrose of 77°C for both RM levels. The T_g of $ArgHCl_{glyc}$ was comparable to the Suc_{glyc} lyophilisates, whereas ArgCitr had significantly higher T_g values.

Table 6-2: T_g and RM of formulations for glycation challenge study.

	T_g [°C]		RM [%]	
	low RM	**high RM**	**low RM**	**high RM**
Suc_{glyc}	73.6 ± 1.1	49.6 ± 2.1	0.3 ± 0.1	1.8 ± 0.2
Man:Suc 4:1	77.0 ± 2.6	76.0 ± 1.8	0.3 ± 0.1	1.2 ± 0.1
$ArgCitr_{glyc}$	122.8 ± 0.9	97.7 ± 1.8	0.1 ± 0.1	1.6 ± 0.1
$ArgHCl_{glyc}$	76.7 ± 7.7	48.8 ± 2.9	0.5 ± 0.3	2.3 ± 0.2

Man:Suc and $ArgCitr_{glyc}$ cakes did not show a change in their appearance upon storage. Suc_{glyc} high and low RM lyophilisates were collapsed after 9 months stored at 40°C and 60°C and both showed discolouration when stored at 60°C. $ArgHCl_{glyc}$ cakes completely collapsed when stored at 40°C and 60°C and $ArgHCl_{glyc}$ high RM collapsed also at 25°C storage temperature. All products reconstituted within 1 minute except for collapsed Suc_{glyc} low and high RM and $ArgHCl_{glyc}$ high RM stored at 60°C over 9 months which required more than 10 minutes for complete dissolution and the collapsed discoloured Suc_{glyc} high RM samples stored at 60°C did not dissolve completely.

A storage temperature of 60°C fostered discolouration in sucrose samples in an extreme manner. Due to its high T_g values, $ArgCitr_{glyc}$ can act as amorphous matrix at high storage temperatures, comparable to mannitol lyophilisates. $ArgHCl_{glyc}$ high RM showed already at 25°C storage temperature collapse as well as the overall difficulties in freeze drying ArgHCl by cause of its low T_g' values.

With respect to protein stability, subvisible particle formation was not observed for any samples upon storage at 25°C (Figure 6-6). The particle counts remained below 36,000 particles ≥ 1 µm/ml. Suc_{glyc} high RM resulted in increased particle counts over 1 month at 60°C already showing visible flocculated particles. After 9 months, less subvisible particles were found since large visible particles had formed. Furthermore, discoloured 60°C Suc_{glyc} lyophilisates did not dissolve and were not analysed. $ArgCitr_{glyc}$ low RM showed a slight increase in subvisible particles at 25°C over 9 months. Storage at 40°C over 9 months resulted in increased particle counts for $ArgCitr_{glyc}$ low RM, but not for high RM. This behaviour was also observed in the first study (see above) and more pronounced at 60°C storage. Man:Suc resulted in a drastic increase in particles over 9 months at 60°C only for low RM. Interestingly, collapsed $ArgHCl_{glyc}$ preserved a low level of particles with less than 10,000 particles ≥ 1 µm/ml over 9 months at 40°C. Only in low RM $ArgHCl_{glyc}$ particle count increased to 72,000 particles ≥ 1 µm/ml over 9 months at 60°C.

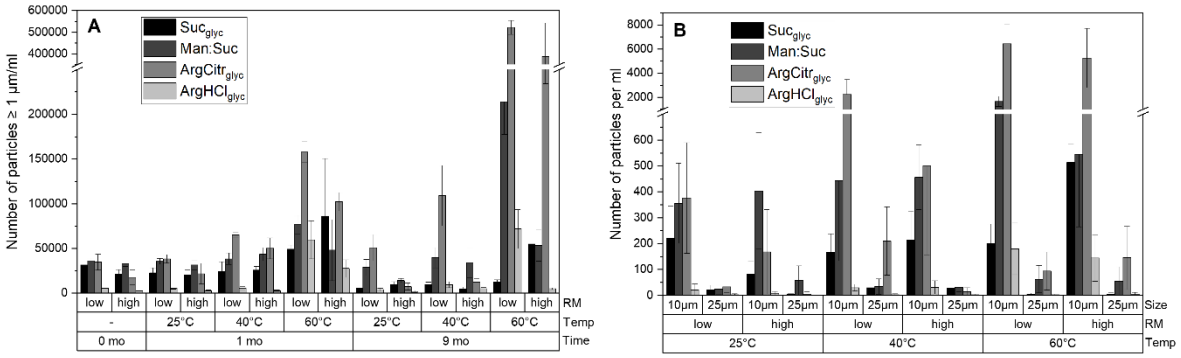

Figure 6-6: Number of particles (A) ≥ 1 µm/ml of glycation challenge study over 9 months at 25°C, 40°C, and 60°C and (B) ≥ 10 µm and ≥ 25 µm of glycation challenge study after 9 months at 25°C, 40°C, and 60°C.

While Suc$_{glyc}$, Man:Suc, and ArgHCl$_{glyc}$ did not reveal a RM dependence in particle formation over storage, ArgCitr$_{glyc}$ showed lower particle counts in formulations with higher RM. Suc$_{glyc}$ and Man:Suc resulted in increased particle formation, especially upon storage at 60°C. While Suc$_{glyc}$ high RM collapsed upon storage at 40°C, Man:Suc was superior due to the crystalline matrix. Nevertheless, storage at 40°C did not increase particle formation.

The starting level of 2% HMWS in Suc$_{glyc}$ remained constant over 9 months storage at 25°C and 40°C (Figure 6-7). Suc$_{glyc}$ low RM revealed only a slight increase in HWMS over 9 months at 60°C, whereas Suc$_{glyc}$ high RM showed a drastic increase, partially due to the reduced total AUC of the chromatographs related to the formation of larger insoluble particles. Furthermore, glycated species exhibit a higher absorbance spectra leading to higher HMWS values [26]. Within one month at 60°C, both low and high RM Suc$_{glyc}$ samples resulted in more than 25% glycated species (Table 6-3). This level was after 9 months increased up to 60% in low RM samples. Although more glycated species should form in high RM samples, the high amount of insoluble particles and the poor reconstitution resulted in many covalent bound aggregates. These aggregates were not recorded by BAC leading to an overrepresenting of the non-glycated species. Compared to Suc$_{glyc}$, Man:Suc started with 2.2% HMWS, showing an increase to after 9 months at 40°C and 60°C up to 5% and 40%, respectively. The HMWS level after 9 months at 60°C was overestimated due to a higher absorbance spectra of the glycated species [26]. Man:Suc formulations exhibited glycated species already after 1 month storage. Only in Man:Suc high RM glycation was detectable after 9 months storage at 40°C, whereas no glycation was detectable in the corresponding Suc$_{glyc}$ formulations. ArgCitr$_{glyc}$ already started with a higher HMWS level after lyophilisation compared to Suc$_{glyc}$ and resulted in increased soluble aggregates at all storage temperatures. A slight tend towards lower HMWS formation was found for the high RM samples. After 9 months, ArgCitr$_{glyc}$ exhibited around 5%, 8%, and 13% stored at 25°C, 40°C, and 60°C, respectively.

Among the different formulations, $ArgHCl_{glyc}$ was clearly superior due to the constant HWMS levels over storage. The HMWS level of 1.8% of $ArgHCl_{glyc}$ did not increase over 9 months storage at 25°C, 40°C, and 60°C. Compared to sucrose based cakes, no glycation was observed for the arginine based formulations.

Overall, LMWS were low at 0.3 to 0.6 % in all samples, except for Man:Suc and $ArgCitr_{glyc}$ stored at 60°C which showed an increase to approx. 1% as well as Suc_{glyc} high RM after 60°C storage but for latter the total AUC was reduced whereas the AUC of LMWS was unchanged. Interestingly $ArgHCl_{glyc}$ did not exhibit an increase in LMWS over storage.

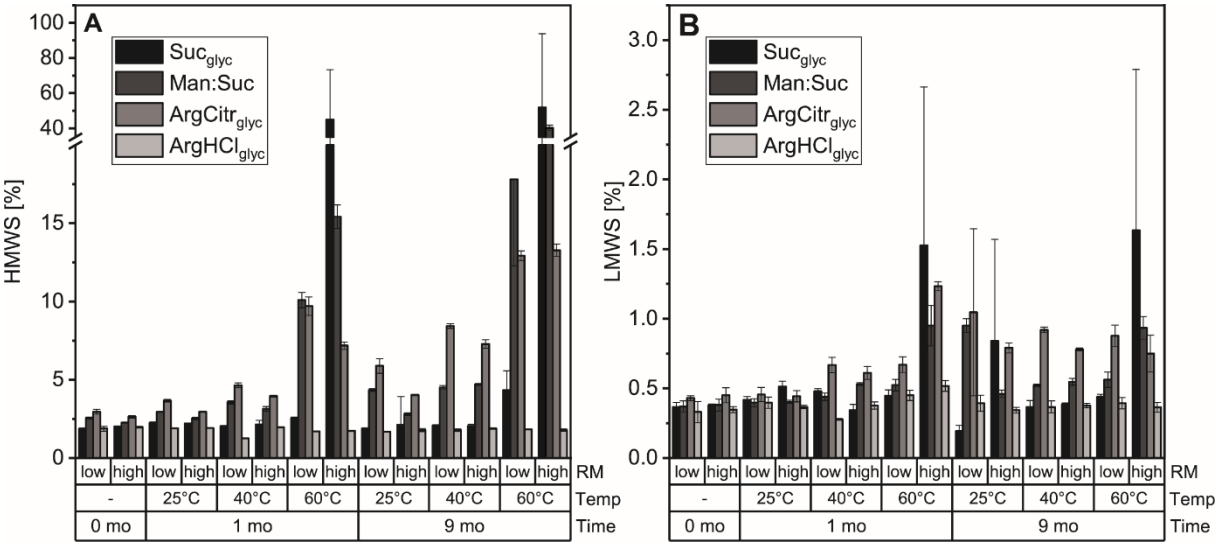

Figure 6-7: HMWS (A) and LMWS (B) results for glycation challenge study over 9 months at 25°C, 40°C, and 60°C.

Table 6-3: Glycated species. (n.d. = not detectable)

	RM	Glycated species						
		t_0	1 month			9 months		
			25°C	40°C	60°C	25°C	40°C	60°C
Suc_{glyc}	low	n.d.	n.d.	n.d.	40.6 ± 5.9 %	n.d.	n.d.	61.0 ± 17.5 %
	high				26.7 ± 9.2 %			41.5 ± 6.1 %
Man:Suc 4:1	low				23.8 ± 0.5 %			41.5 ± 7.0 %
	high				22.3 ± 0.4 %		4.1 ± 0.6 %	58.5 ± 1.0 %
$ArgCitr_{glyc}$	low	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	high							
$ArgHCl_{glyc}$	low							
	high							

Overall, collapsed Suc_{glyc} and Man:Suc showed increased HMWS levels as well as glycation at 60°C with some effects noticeable also at 40°C and reduced protein stability with higher RM levels. Mannitol was able to form a crystalline matrix to inhibit cake macro-collapse but did not prevent glycation. $ArgCitr_{glyc}$ resulted in increased HMWS levels over storage with a slight RM dependence towards less protein instabilities at high RM. Again, $ArgHCl_{glyc}$ despite collapse showed consistently low HMWS levels over 9 months and now glycation.

4. Conclusion

The aim of this study was to investigate the influence of different residual moisture levels on protein stability of a model mAb in arginine based lyophilisates in comparison to sucrose based lyophilisates. Too low as well as too high RM levels can potentially destabilise the protein in amorphous sugar matrices [7-9]. Arginine lyophilisates with citrate, hydrochloride, lactobionate, phosphate, and succinate as counter ions were compared to a sucrose formulation, all freeze dried to RM levels of less than 0.5% and 2.5%.

At low RM levels of 0.5%, T_g values of arginine based formulations were equal or higher than that of the sucrose based reference formulation. The increased RM level resulted in about 20°C lower T_g values. At high RM, Suc and ArgHCl showed the lowest T_g of all tested formulations at around 50°C, which may cause instabilities during accelerated storage conditions at 40°C but not at 2°C – 8°C. Proteins are stated to be well immobilised with a T_g at least 10°C – 20°C above the intended storage temperature [14, 27, 28]. According to the low T_g values, Suc and ArgHCl high RM resulted in macro-collapse upon storage at 40°C and 60°C and ArgHCl high RM resulted in macro-collapse upon storage even at 25°C.

Interestingly, ArgCitr, ArgLacto, and ArgPhos lyophilisates with high RM levels showed a trend to better protein stability over storage compared to their low RM equivalents. ArgHCl and ArgSucc low and high RM exhibited superior protein stability compared to Suc low RM over 6 months at 40°C. Suc high RM collapsed and discoloured after 6 months at 40°C and showed higher particle and HWMS levels compared to high RM arginine products.

Additionally, glycation was detected in collapsed discoloured Suc_{glyc} lyophilisates but not seen for arginine based formulations. Also, non macro-collapsed cakes achieved by adding mannitol to sucrose still revealed glycation. Glycation was not detected in arginine based lyophilisates. Formation of soluble mAb aggregates was observed in $ArgCitr_{glyc}$ formulations, more pronounced with higher RM.

$ArgHCl_{glyc}$ did not show no protein instability, independent of RM, although they collapsed at higher storage temperature.

This study presents ArgHCl and ArgSucc as potential alternatives to improve the physical stability of mAb formulations to sucrose based formulations. Additionally, glycation was eliminated using arginine based formulations. ArgHCl lyophilisates were superior to Suc regarding the protein stability of the investigated mAb, but need to be combined with excipients to overcome cake collapse while maintaining protein stability.

Acknowledgement

We would like to thank Carolin Berner, LMU, Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, for performing the Near-UV CD measurements.

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Chapter 7

Improvement of Arginine Hydrochloride Based Antibody Lyophilisates

The following chapter has been published as research article in the International Journal of Pharmaceutics and appears in this thesis with the journal's permission:

Ivonne Seifert and Wolfgang Frieß
Improvement of Arginine Hydrochloride Based Antibody Lyophilisates
Int. J. Pharm. 589 (2020), 119859

The supplementary data was inserted into this chapter.

Abstract

Arginine hydrochloride (ArgHCl) can be used as stabiliser of protein drugs in both liquid and freeze dried formulations reducing aggregation. But ArgHCl exhibits a low T_g' value which can lead to cake collapse during lyophilisation. We analysed arginine (Arg) citrate, hydrochloride and lactobionate based lyophilisates aiming for elegant cake appearance in combination with minimal protein aggregation using 2 mg/ml monoclonal antibody (mAb) without additional excipients and in combination with sucrose (Suc) as amorphous stabiliser as well as mannitol (Man) and phenylalanine (Phe) as crystalline bulking agents. Furthermore, 50 mg/ml mAb was also used. Based on a process which resulted in poor cake appearance for the ArgHCl based cake with 2 mg/ml mAb, the combination with sucrose, mannitol (Man:Arg 4:1) and phenylalanine (Phe:Arg 1:4, 1.5:3.5, and 2:3) as well as a high mAb concentration were able to improve cake appearance. Overall, the crystalline bulking agent Phe in combination with ArgHCl renders elegant cakes with minimal protein aggregation. The alternative Arg salts did not require cake quality enhancement, but mAb stability was inferior to ArgHCl. The combination with mannitol showed reduced stability due to a substantially lower amount of amorphous matrix. Thus, the combination of ArgHCl with phenylalanine was superior to any other formulation tested including the sucrose based and may allow for substantial process time reduction due to the crystalline scaffold delivering pharmaceutically elegant cakes.

Keywords

Freeze-drying, arginine, phenylalanine, hydrochloride, collapse, antibody, protein stability

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1. Introduction

Freeze drying of biopharmaceuticals is frequently used to achieve adequate storage stability [1]. The protein molecules are stabilized due to a reduced mobility in a glassy amorphous excipient matrix and by formation of hydrogen bonds with excipient molecules which thus replace the water molecules surrounding and stabilizing the protein molecules in solution [2, 3]. The most used stabilizing excipient with this respect is sucrose (Suc). Trehalose is another preferred non-reducing disaccharide for this purpose. In order to obtain pharmaceutically elegance of the cakes which is important for market and patient acceptance, freeze drying below the glass transition temperature of the maximally freeze concentrated solution T_g' or at least below the collapse temperature T_c is usually required [4-6]. A collapsed amorphous matrix itself is not necessarily detrimental for protein stability [7-9]. But keeping the product temperature T_p below T_g' or T_c during primary drying leads to extended process times coming along with higher costs [4, 10].

Besides sugars, amino acids are able to form amorphous matrices. They are already used in commercially available products and in a few cases completely sugar-free amino acid based formulations are used [11-15]. Especially L-arginine is of highest interest due to its ability to reduce protein-protein interactions and aggregation in liquid formulations and reconstituted lyophilisates [16, 17]. This effect is driven by an interaction of arginine with the protein surface via hydrogen bond formation and ion-dipole interactions [18-20]. Furthermore, arginine (Arg) is able to form amorphous matrices in combination with different counter ions and can strongly reduce protein aggregation [21-23]. The stabilising effect and the glass properties depend on the counter ion and whereas typically the best stability is achieved with the hydrochloride formulations, higher T_g' and T_g values of the lyophilisates result with citrate, phosphate, or succinate salts [22, 23]. Arginine hydrochloride (ArgHCl) based cakes are prone to collapse during primary drying due to very low T_g' of around -42.5°C compared to Suc with -32°C [23-26]. In comparison, the T_g' of arginine citrate at pH 6.0 of approx. -28°C makes a suitable lyophilisation process easy [21].

There are different approaches to achieve a higher T_g' or T_c to achieve fast primary drying without product collapse. This positive effect comes along with the use of high protein drug concentrations [4, 5]. The T_g' onset temperature of a 1:4 Suc to ArgHCl mixture went up from -40°C in placebo to -32.5°C at 50 mg/ml mAb [23]. Furthermore, a low T_g' value can be increased via mixtures with other excipients that form amorphous systems with a higher T_g'. A combined T_g' value based on the Gordon-Taylor equation results [27]. In case of ArgHCl the T_g' of a mAb formulation can be increased from -42.7°C to -34°C by using a Suc to ArgHCl 4:1 mix [22, 23, 28]. The mostly used approach to achieve an elegant cake and potentially run primary drying at a product temperature above the critical temperature of

the amorphous matrix is the addition of a crystalline bulking agent [2, 12, 29]. Since the crystallising excipient has no protein stabilising abilities, its concentrations should be kept low to allow for enough amorphous cryo- and lyoprotectant considering the concentration limitation due to the isotonicity requirement of parenterals [30-32]. The most prominent bulking agent mannitol (Man) requires a high ratio of mannitol to sugar, typically 4:1 to ensure crystallising matrix formation [33]. Incomplete Man crystallisation can lead to lowered T_g of the lyophilisates and crystallisation events upon storage which reduces protein stability. The combination of Man with ArgHCl at ratios of 2:1 and 4:1 resulted in elegant cakes, making Man a promising candidate for ArgHCl formulations for lyophilisation [34], but protein stability was not assessed. Phenylalanine (Phe) was recently found to serve as crystallising bulking agent at lower concentrations leaving more room for the amorphous stabilising matrix. Avoiding Man by Phe resulted in lyophilisates with elegant appearance and good physical stability of a mAb at 5:45 and 10:40 Phe:Suc ratios [13].

Within this study, the main focus was on the improvement of the cake quality of an ArgHCl formulation for lyophilisation of biopharmaceuticals. To achieve this goal, i) the counter ion was varied from hydrochloride to citrate and lactobionate, ii) formulations with a high mAb concentration were evaluated and iii) mixtures with Suc, Man, and Phe were tested. We analysed mAb aggregation during the process as well as in the different formulations upon 6 months storage at 40°C.

2. Materials and Methods

2.1 Materials

Stock solutions of L-arginine base (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), citric acid anhydrous (VWR International, Ismaning, Germany), lactobionic acid (Acros Organics, Geel, Belgium), sucrose (Merck KGaA, Darmstadt, Germany), and D-(-)-mannitol (Merck) were prepared with highly purified water (HPW). Furthermore, 1 M hydrochloric acid (VWR International) and L-phenylalanine (Merck) were used.

A monoclonal IgG_1 antibody (94 mg/ml, MW ca. 148 kDa, ϵ = 1.37 mL/mg·cm, referred to as mAb) in 15 mM histidine buffer pH 5.3 was used as model protein. Formulations contained 2 mg/ml mAb, formulations marked with HC contained 50 mg/ml mAb. For HC formulations, the mAb was dialysed against HPW with a 10-fold buffer exchange in Vivaspin® 20 Ultrafiltration Uni (30 kDa, Sartorius, Goettingen, Germany) and concentration checked after dialysis and formulation preparation at 280 nm (NanoDrop™ 2000, Thermo Fisher Scientific, Waltham, MA, USA).

The pH of the arginine base stock solution (8% w/w) was titrated to pH 6.0 with hydrochloric acid (ArgHCl), citric acid (ArgCitr), or lactobionic acid (ArgLacto). The weight was recorded for further sample dilution with mAb and HPW until the final concentrations were reached. Suc, Man, or Phe were subsequently added as solid if required. Table 7-1 represents the formulation compositions. A solid content of around 5% was chosen to gain isotonic compositions. Thereby, 2% arginine and 3.5% sucrose turned out to be isotonic, whereas less mannitol and phenylalanine were necessary and therefore used on 5% solid content basis.

The formulations were filtered with 0.2 µm polyethersulfone membrane syringe filters (VWR International) prior to filling.

Table 7-1: Excipient solid content ratios of formulations tested. *Content of counter ion (hydrochloric acid, citric acid, and lactobionic acid) required for pH adjustment not involved in ratio description.

Acronym	Arg base [% w/w]*	Suc [% w/w]	Man [% w/w]	Phe [% w/w]
-- / HC	5.0	--	--	--
Suc	2.0	3.5	--	--
Man 4.5	0.5	--	4.5	--
Man 4	1.0	--	4.0	--
Man 3	2.0	--	3.0	--
Man 2	3.0	--	2.0	--
Man 1	4.0	--	1.0	--
Phe 0.5	4.5	--	--	0.5
Phe 1	4.0	--	--	1.0
Phe 1.5	3.5	--	--	1.5
Phe 2	3.0	--	--	2.0

2.2 Methods

2.2.1 Lyophilisation

Lyophilisation stoppers (B2-TR coating, West Pharmaceuticals Services Deutschland GmbH & Co. KG, Eschweiler, Germany) and DIN 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with HPW prior to use and dried at 60°C for 8 h. The vials were filled with 1.5 mL formulation and semi-stoppered subsequently. An FTS Lyostar™ 3 freeze dryer (SP Scientific, Stone Ridge, NY, USA) was used with the protocols provided in Table 7-2. The product temperature was recorded with thermocouples and the

end of primary drying was followed by comparative pressure measurement between Pirani and capacitance gauge. The vials were stoppered at 800 mbar after secondary drying and crimped with flip-off seals. Samples were analysed after lyophilisation and after 1, 3, and 6 months storage at 40°C.

Table 7-2: Freeze drying protocols. * End of primary drying detected by comparative pressure measurement.

Step	**Ramp [°C/min]**	**T_{Shelf} [°C]**	**Pressure [µbar]**	**Hold time [h]**
Freezing	1	-50	-	1.5
Primary Drying	0.5	-20	60	47 – 65*
Secondary Drying	0.4	40	60	8.3

2.2.2 Cake appearance

Images of the lyophilised products were taken in front of a black background with a Nikon D5300 camera (Nikon GmbH, Düsseldorf, Germany) to visualise cake appearance.

2.2.3 Differential scanning calorimetry (DSC)

The glass transition temperature of the maximally freeze concentrated solution (T_g') was determined with a DSC 821^e (Mettler Toledo, Gießen, Germany). 25 µl liquid sample were filled into 40 µl aluminium crucibles and hermetically sealed. The samples were cooled to -60°C at 10°C/min and heated to 25°C at 10°C/min. The midpoint of the phase transition was determined as T_g'. To analyse the glass transition (T_g) of the lyophilised products approx. 10 mg crushed cake were filled at ≤ 10% r.h. conditions into 40 µl aluminium crucibles and hermetically sealed. The samples were heated to 80°C at 10°C/min, cooled to 10°C at 10°C/min, and reheated to 120°C. The midpoint of the phase transition in the second heating was determined as T_g.

2.2.4 Karl-Fischer titration

Coulometric Karl-Fischer titration with a headspace module (AQUA 40.00, Analytik Jena AG, Halle, Germany) was used for residual moisture (RM) analysis. About 20 mg crushed lyophilised powder were transferred into a fresh 2R vial at ≤ 10% r.h. and heated up to 100°C in the headspace module. The evaporated water was transferred into the titration solution and RM determined.

2.2.5 X-Ray powder diffraction (XRPD)

An X-Ray Diffractometer XRD 3000 TT (Rich. Seifert & Co. GmbH & Co. KG, Ahrensberg, Germany) equipped with a Cu Kα (40 kV, 30 mA, λ = 154.17 pm) was used to analyse powder morphology from 5 – 45° 2θ with steps of 0.05° 2θ and 2 seconds per step.

2.2.6 Reconstitution time

Samples were reconstituted with HPW, vials gently rolled by hand and the time until complete dissolution based on visual inspection was considered as reconstitution time.

2.2.7 Light obscuration

A PAMAS SVSS-C35 particle counter with an HCB-LD-25/25 sensor (Partikelmess- und Analysensysteme GmbH, Ruthesheim, Germany) was used for characterising subvisible particles between 1 µm and 200 µm by light obscuration. After sample pre-rinse with 0.2 mL, each sample was analysed 4 times with 0.2 mL according to USP 787 [35]. Between each sample, the system was rinsed with 0.2 µm filtered HPW. At least 3 samples per formulation were used for the subvisible particle count calculation of particles ≥ 1 µm per mL.

2.2.8 High performance size exclusion chromatography (HP-SEC)

An Agilent 1100 series HPLC system equipped with an UV/Vis detector (280 nm; Agilent Technologies, Santa Clara, CA, USA) was used for analysing higher and lower molecular weight species (HMWS/LMWS) by size exclusion chromatography. A Waters AQUITY UPLC Protein BEH 200Å column was used with 50 mM sodium phosphate pH 6.0 with 400 mM sodium perchlorate at 0.4 mL/min. All samples were centrifuged at 7000 rpm for 10 min prior to use (Force7, Denver Instruments, Bohemia, NY, USA). The integrated peak area was determined after blank subtraction and relative areas calculated with the ChemStation software (Agilent Technologies).

3. Results and Discussion

3.1 Screening of arginine salts with different excipients

Aim of this study was to achieve good cake appearance and protein stability using arginine based lyophilisates. For this purpose, the counter ion was varied and the standard hydrochloride replaced by citrate and lactobionate. These salts were combined with either a higher mAb concentration of 50 mg/ml compared to 2 mg/ml standard, Suc as amorphous cryo- and lyoprotectant, and Man and Phe as crystalline bulking agents.

Arginine salts vary substantially in T_g' depending on pH and valency [28]. Whereas the hydrochloride form rendered a T_g' value of -46.3°C at pH 6.0, the citrate and the lactobionate forms showed markedly higher values of -27.1°C and -25.6°C indicating a higher probability of good cake appearance. With a higher mAb concentration T_g' was increased, corresponding to literature [4, 31, 36], to -23.1°C, -21.4°C, and -32.5°C for ArgCitr, ArgLacto, and ArgHCl respectively. A Suc:Arg 3.5:2 mixture exhibited decreased T_g' values of -30.4°C for ArgCitr and -28.8°C for ArgLacto and an increased value of -38.2°C for ArgHCl according to the Gordon-Taylor equation stating a mixed T_g' based on the individual mass fraction and T_g' values (Suc T_g' = -32°C [26]) [37]. Mixtures with the crystallising bulking agents Phe:Arg 1:4 and Man:Arg 4:1 did show the T_g' values of the Arg salts.

3.1.1 Characteristics of the arginine based products

Both pure ArgCitr and ArgLacto resulted in pharmaceutically elegant lyophilisates, whereas ArgHCl products showed pronounced collapse and major structural defects. At 50 mg/ml mAb pharmaceutically elegant cakes could be obtained in all cases which reflects the rise in T_g' and collapse temperature with higher protein concentration. The mixture of ArgHCl with sucrose (Suc:Arg 3.5:2) resulted in elegant macroscopic appearance with some shrinkage and detachment from the vial wall due to the increase in T_g'. The combination of sucrose with the other Arg salts did not lead to change of appearance. Both Phe:Arg 1:4 and Man:Arg 4:1 cakes were pharmaceutically elegant for all arginine salts due to the crystalline scaffold. Man:Arg ratios of less than 4:1 were not sufficient to form pharmaceutically elegant products (Figure 7-1). The macroscopic appearance of all lyophilisates did not change upon 6 months storage at 40°C.

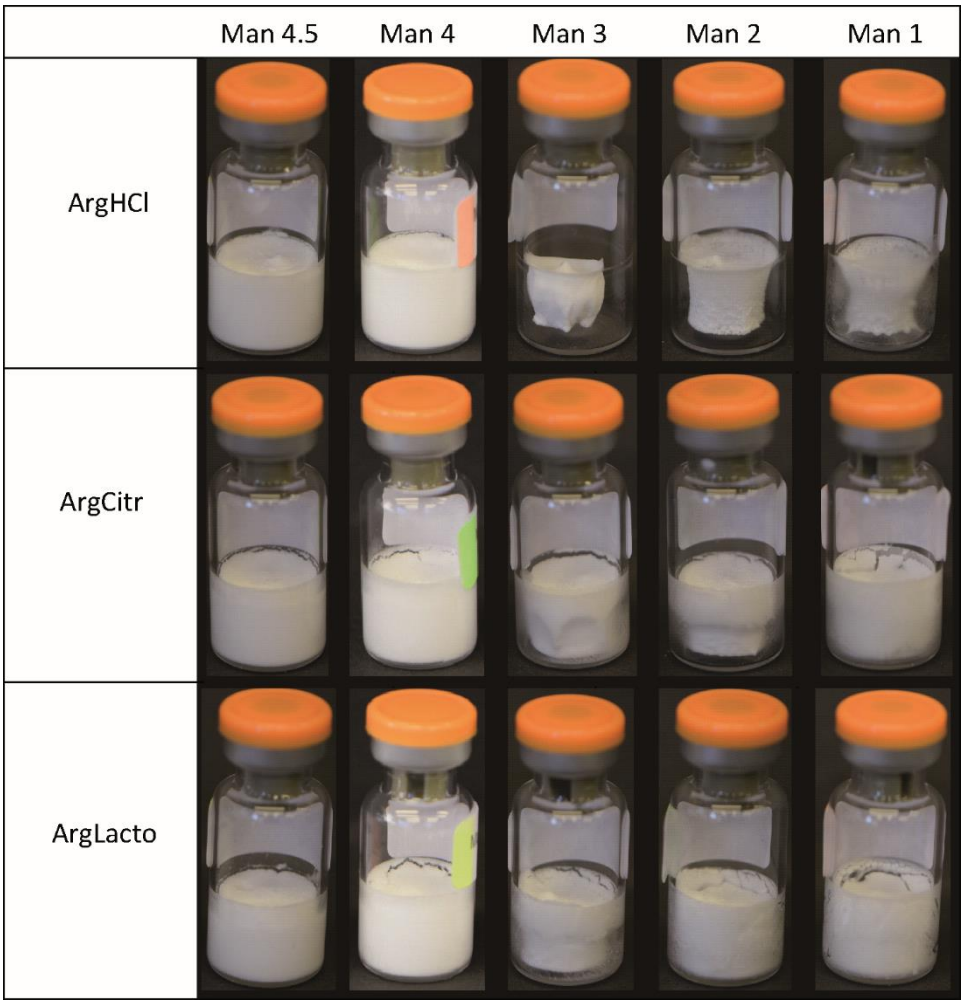

Figure 7-1: Macroscopic appearance of arginine lyophilisates mixed with mannitol at ratios of Man:Arg 4.5:0.5, 4:1, 3:2, 2:3, and 1:4 after lyophilisation.

All lyophilisates resulted in less than 1.2% RM. The T_g of ArgHCl cakes of 74°C, independent of the protein concentration, decreased with addition of Suc and Phe to 55°C and 69°C, respectively. A similar trend was found for ArgCitr and ArgLacto based products. They exhibited a T_g of 120°C and 100°C, respectively, decreasing with Suc and Phe. The lowest T_g was 80°C with the addition of Suc to both formulations. Still this T_g was more than 20°C above storage temperature and therefore not considered critical for collapse upon storage [38].

Man containing formulations resulted in δ-mannitol after freeze drying (Figure 7-2). Peaks of crystalline Phe could not be detected although appearance and DSC indicated Phe crystallisation. The high content of amorphous matrix appears to cover the crystalline structures and shields the signal of Phe in the XRPD [12]. The morphology of the formulations did not change upon storage, except for ArgHCl which partially crystallised upon storage for 3 months at 40°C. Crystallisation of ArgHCl was inhibited by the high mAb concentration and in the mixture with sucrose or phenylalanine.

Reconstitution of all samples was completed within 10 seconds which extended to 3 min for ArgCitr HC and ArgLacto HC upon storage.

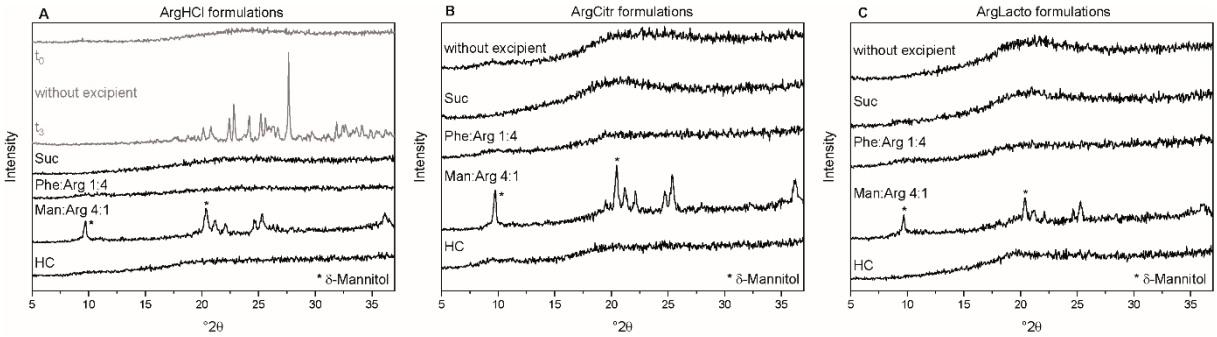

Figure 7-2: XRPD of (A) ArgHCl, (B) ArgCitr, and (C) ArgLacto lyophilisates with 2 mg/ml mAb without excipient and as Suc:Arg 3.5:2, Phe:Arg 1:4, and Man:Arg 4:1 mixtures and with 50 mg/ml mAb (HC) at t0. In (A), crystallisation of ArgHCl 2 mg/ml mAb lyophilisates is depicted after 3 months at 40°C (t3).

3.1.2 mAb process and storage stability

The effect of the Arg salts pure and in combination with the other excipients was studied considering the colloidal stability based on formation of subvisible particles (Figure 7-3) and soluble aggregates (Figure 7-4A). Pure ArgHCl as benchmark resulted in approx. 10,000 particles ≥ 1 µm/ml and remained at this t_0-level after 6 months storage at 40°C. An increase in protein concentration did not affect particle levels but resulted in marked formation of HMWS upon storage. The addition of Suc to ArgHCl did not change mAb stability. The Man:Arg 4:1 mixture showed substantial aggregate formation after 6 months storage at 40°C with around 6% soluble aggregates, 140,000 particles ≥ 1 µm/ml, up to 1000 particles ≥ 10 µm and a few visible particles. Varying the Man:Arg ratios did not improve protein stability (Figure 7-5 and 7-6). The addition of Phe to ArgHCl formulations at a 1:4 ratio did not compromise protein stability compared to ArgHCl without additional excipients.

Both alternative arginine salts ArgCitr and ArgLacto provided less mAb stabilisation during both the process and upon storage compared to ArgHCl. Sucrose had a positive effect on these formulations decreasing particle and HMWS formation. Nevertheless, the Suc mixtures with ArgCitr or ArgLacto were inferior to the ArgHCl-Suc mixture and this was still slightly inferior to the pure ArgHCl and the ArgHCl Arg:Phe 4:1 mixture.

In all formulations, LMWS levels remained between 0.2 – 0.8% upon storage and only mixtures with sucrose exhibited around 1.4% LMWS after 6 months storage at 40°C (Figure 7-4B).

Thus, the Arg salt alternatives ArgCitr and ArgLacto resulted in better cake appearance than ArgHCl but were inferior with respect to process and storage stability. Furthermore, high mAb concentrations provided pharmaceutically elegant lyophilisates in all Arg salts but exhibited less protein stability especially pronounced HMWS formation. Mixtures with Man did not provide process and storage stability. The addition of Suc and Phe improved the cake appearance of ArgHCl lyophilisates and the mixture with Phe provided outstanding process and storage stability. Due to the high amount of the amorphous Arg salt compared to Phe, different ratios of Phe:Arg were analysed in the following section.

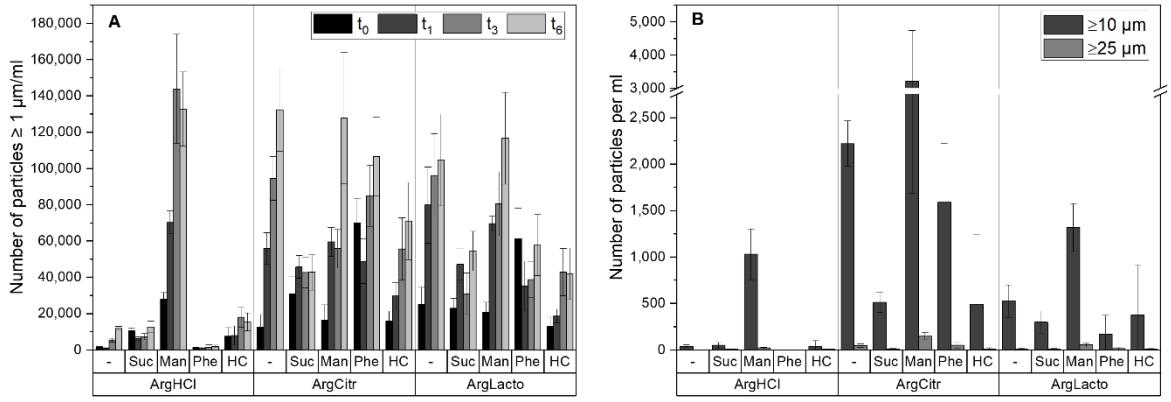

Figure 7-3: (A) Number of subvisible particles ≥ 1 µm/ml of arginine based 2 mg/ml mAb lyophilisates without further excipient (-), as mix with Suc, Man, Phe or 50 mg/ml mAb (HC) after lyophilisation (t0) and upon storage for 1 (t1), 3 (t3), and 6 months (t6) at 40°C. (B) Number of subvisible particles ≥ 10 µm/ml and ≥ 25 µm/ml after 6 months at 40°C.

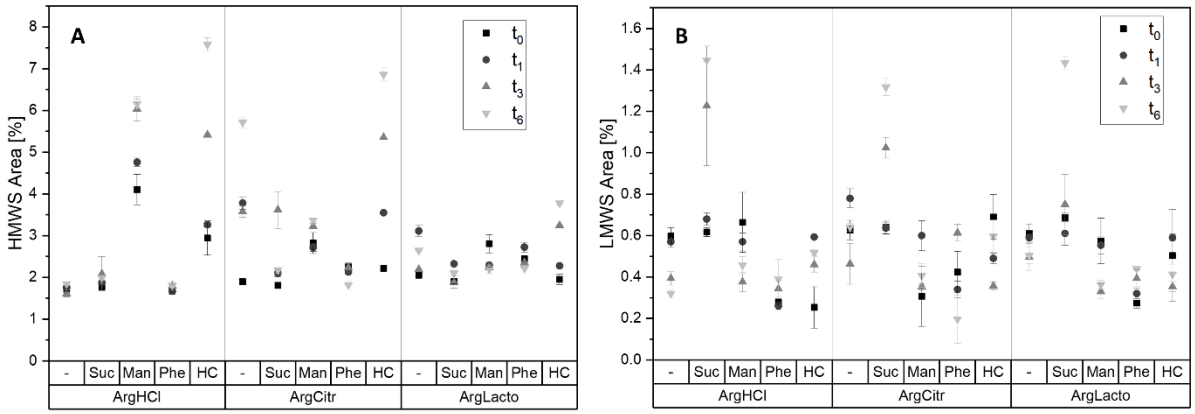

Figure 7-4: (A) Higher molecular weight species (HMWS) and (B) lower molecular weight species (LMWS) of arginine based lyophilisates with 2 mg/ml mAb (-), as a mix with Suc, Man, and Phe, and with 50 mg/ml mAb (HC) after lyophilisation (t0) and upon storage for 1 (t1), 3 (t3) and 6 months (t6).

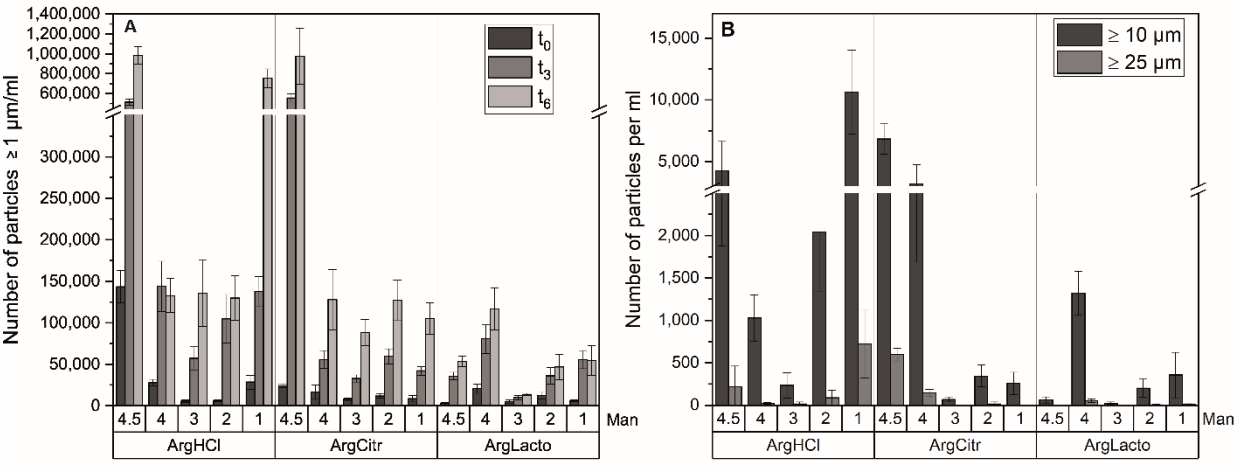

Figure 7-5: Subvisible particle count in arginine based 2 mg/ml mAb lyophilisates in Man:Arg ratios of 4.5:0.5, 4:1, 3:2, 2:3, and 1:4 after lyophilisation (t0) and upon storage for 3 (t3) and 6 (t6) months.

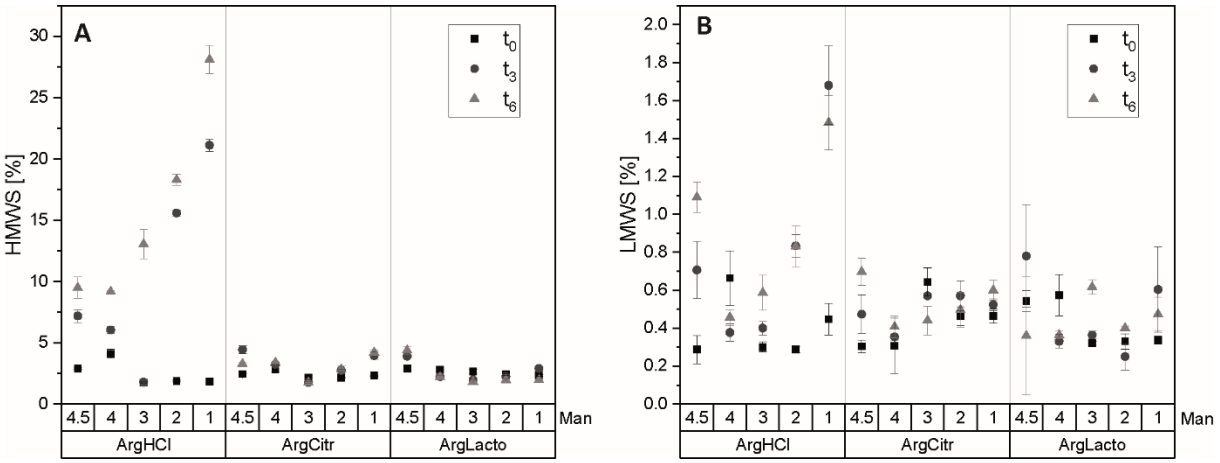

Figure 7-6: (A) Higher molecular weight species (HMWS) and (B) lower molecular weight species (LMWS) of arginine based 2 mg/ml mAb lyophilisates in Man:Arg ratios of 4.5:0.5, 4:1, 3:2, 2:3, and 1:4 after lyophilisation (t_0) and upon storage for 3 (t_3) and 6 (t_6) months.

3.2 Effect of Phe:Arg ratio on mAb lyophilisates

In a next step, the Phe:Arg ratio was varied to study the impact of a level of crystalline bulking agent which may result in more elegant cakes at the expense of protein stability. ArgCitr and ArgLacto lyophilisates showed an adequate appearance compared to ArgHCl lyophilisates. But their stabilization potential is lower, potentially enhancing the effect of a decrease in amount of amorphous matrix formed and thus making the impact of the Phe:Arg ratio more clear.

3.2.1 Powder characteristics of Arg based lyophilisates with different Phe:Arg ratios

A Phe:Arg ratio of 0.5:4.5 was not sufficient to avoid the collapse of ArgHCl based cakes whereas all other mixtures rendered lyophilisates of pharmaceutical elegance that did not change upon storage (Figure 7-7). At Phe:Arg 1:4 slight shrinkage and detachment of the cake from the vial wall was noticeable. All ArgHCl based lyophilisates with Phe showed a T_g of 60°C to 70°C and 0.6 – 1% RM which changed slightly to 60°C and 1.3 – 1.6%, respectively, upon 6 months storage at 40°C (Figure 7-8). A higher increase in RM with almost no decrease in T_g for vacuum- and freeze-dried sugar-free arginine formulations was also observed previously compared to sucrose containing formulations [13], but the mechanistics behind are unclear.

Both ArgCitr and ArgLacto did not require the Phe addition to achieve pharmaceutically elegant cakes in the first place. Accordingly, pharmaceutically elegant cakes were obtained in all mixtures with Phe and the appearance did not change upon storage. Both exhibited high T_g values above 80°C at 0.4 – 1.0% RM after lyophilisation. Upon storage, the T_g values decreased by 10°C at most accompanied by a slight increase in RM. Overall, the formulations tend towards higher RM levels and lower T_g values with higher Phe fraction.

Crystallisation of phenylalanine could not be proven by XRPD in any of the lyophilisates, but crystallisation of ArgHCl was observed in Phe:Arg 0.5:4.5 products after 3 months storage at 40°C. All samples reconstituted within 10 seconds but after 6 months at 40°C reconstitution of lyophilisates with the highest Phe fraction, Phe:Arg 2:3, took 1, 3 and 6 min, respectively, for ArgCitr, ArgLacto, and ArgHCl.

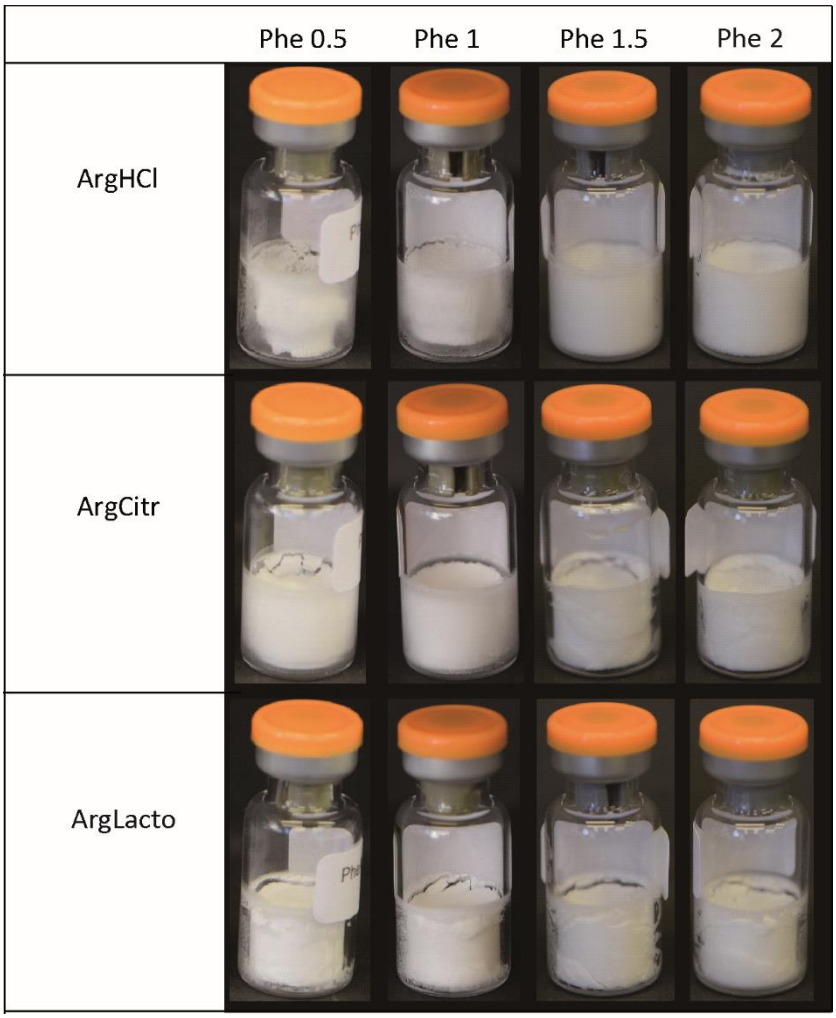

Figure 7-7: Macroscopic appearance of arginine based lyophilisates with 2 mg/ml mAb mixed with Phe:Arg ratios of 0.5:4.5, 1:4, 1.5:3.5, and 2:3 after lyophilisation.

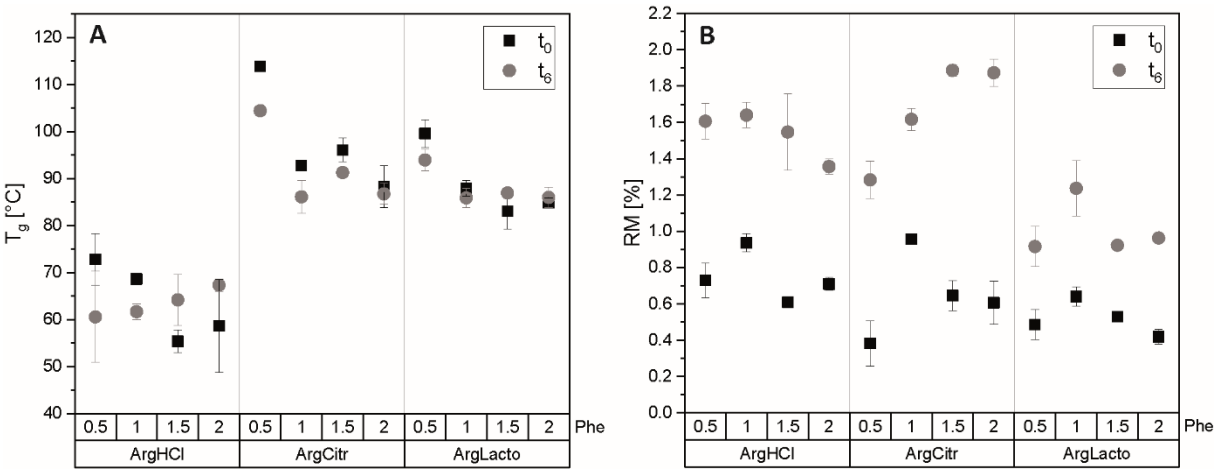

Figure 7-8: (A) T_g and (B) RM of arginine based lyophilisates with 2 mg/ml mAb mixed with Phe:Arg ratios of 0.5:4.5, 1:4, 1.5:3.4, and 2:3 after lyophilisation (t_0) and upon storage for 6 months (t_6).

3.2.2 mAb process and storage stability in different Phe:Arg ratio lyophilisates

The mAb stability in the lyophilisates was evaluated based on subvisible particle analysis by light obscuration (Figure 7-9) and SEC (Figure 7-10A). The ArgHCl based formulations resulted in less than 10,000 particles ≥ 1 µm/ml after lyophilisation, independent of the Phe concentration, and remained at this t_0-level upon storage for 6 months at 40°C in case of an Phe:Arg ratio of 0.5:4.5 or 1:4. Higher Phe:Arg ratios exhibited a slight increase in subvisible particles up to 40,000 particles ≥ 1 µm/ml upon storage. Formation of HMWS was not observed upon storage.

Both process and storage stability were reduced in ArgCitr containing Phe. A substantial increase in particles ≥ 1 µm and ≥ 10 µm was observed. This was accompanied by a trend towards higher particle levels with increasing Phe:Arg ratio potentially due to a reduction of the amount of amorphous Arg matrix. The 0.5:4.5 and 1.5:3.5 Phe:Arg formulations tended towards HMWS formation upon storage.

Process stability of ArgLacto with Phe was reduced as particle formation was already observed for all Phe concentrations after lyophilisation. The formulations did not show mAb aggregation upon storage for 6 months at 40°C based on HMWS and subvisible particle levels, with an overall trend towards less particles ≥ 10 µm with higher Phe concentrations.

Fragmentation of the mAb was not observed during lyophilisation or upon storage (Figure 7-10B).

Thus, ArgHCl formulations with Phe:Arg ratios of 1:4, 1.5:3.5, and 2:3 form elegant cakes with outstanding protein stability. The higher ratio of Phe:Arg 2:3 showed a slightly decreased protein stability with minor increase in particle formation and slower reconstitution. This ratio provides sufficient ArgHCl stabiliser, but the high amount of crystallising Phe appears to have a negative impact on process stability. Phe:Arg 0.5:4.5 was not sufficient to form elegant lyophilisates and ArgHCl partially crystallised. The alternative Arg salts were not able to stabilise the protein in a similar manner as ArgHCl. ArgCitr exhibited a substantial increase in particle formation with an increase in Phe content. ArgLacto resulted in elegant products already without the need for a bulking agent, which makes handling convenient. The addition of Phe did not change protein stability except for a decrease in ≥ 10 µm particle levels with higher Phe concentrations. Both HCl and Lacto are monovalent counter ions to Arg with Lacto as sugar acid potentially showing different interactions with Arg. Therefore, monovalent acids might be beneficial for the protein stabilising effects of the Arg salts. Citr as tricarboxylic acid showed different glass forming abilities with Arg, which were not suitable for sufficient protein stability [21]. The interaction of Phe in combination with Arg salts is so far not understood. Regardless of the valency of the counter ion, Phe containing lyophilisates showed improved protein storage stability compared to the Arg salts without further excipient. The hydrophobic nature of Phe exposed to the protein surface in the reconstituted state could potentially

inhibit protein self-association, which was observed for an antibody with highly solvent-exposed Phe residues [39].

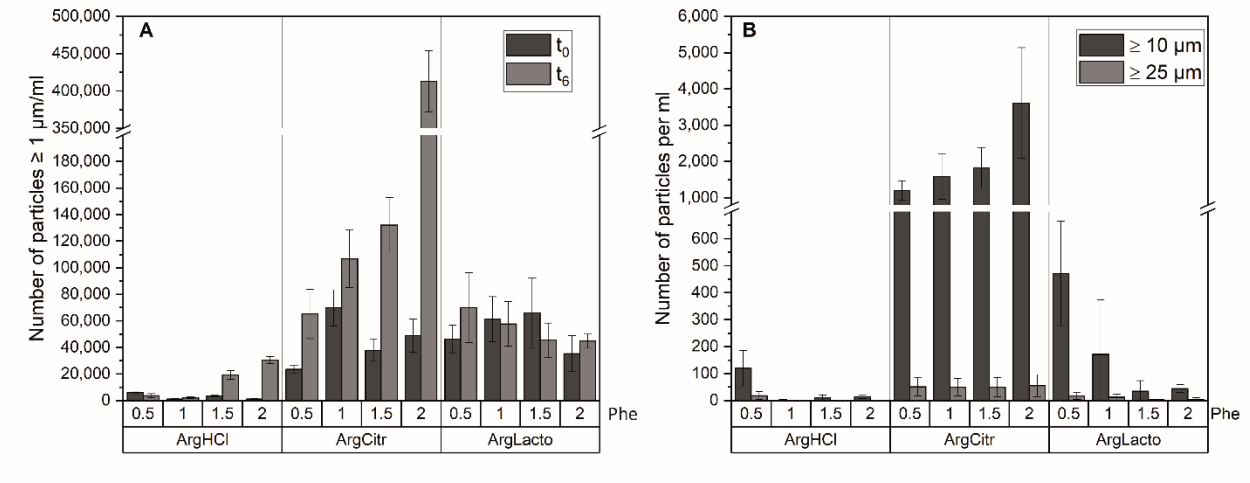

Figure 7-9: (A) Subvisible particle count in arginine based 2 mg/ml mAb lyophilisates mixed with Phe:Arg ratios of 0.5:4.5, 1:$, 1.5:3.5, and 2:3 after lyophilisation (t_0) and upon storage for 6 months (t_6). (B) Number of subvisible particles ≥ 10 µm/ml and ≥ 25 µm/ml after 6 months at 40°C.

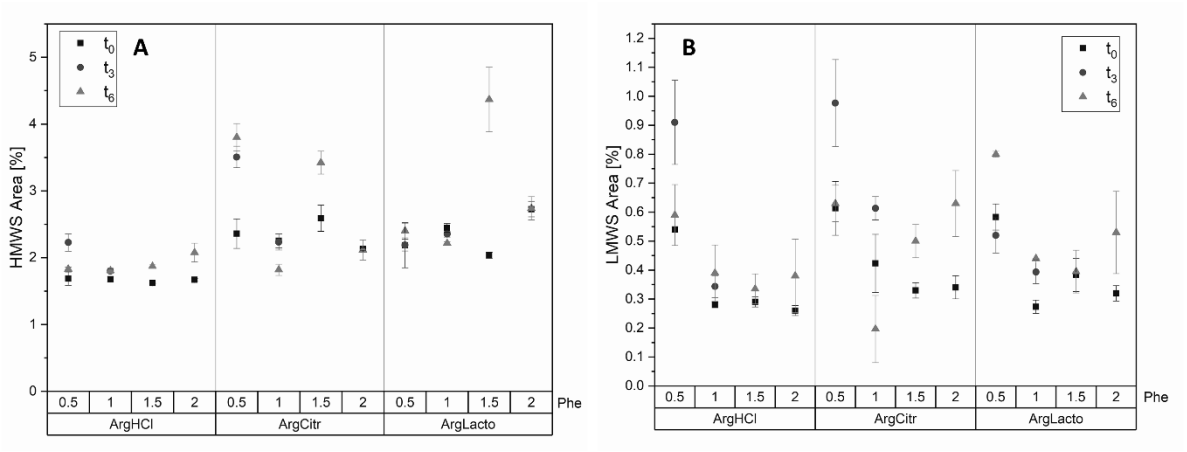

Figure 7-10: (A) Higher molecular weight species (HMWS) and (B) lower molecular weight species (LMWS) of arginine based 2 mg/ml mAb lyophilisates mixed with Phe:Arg ratios of 0.5:4.5, 1:4, 1.5:3.5, and 2:3 after lyophilisation (t_0) and upon storage for 3 (t_3) and 6 (t_6) months.

4. Conclusion

The aim of this study was to find formulations based on Arg, which render both pharmaceutically elegant cakes and outstanding protein stability. Arg formulations can overcome potential drawbacks of sucrose-based formulations for lyophilisation, such as cleavage of sucrose eventually causing glycation of the mAb. Pure ArgHCl lyophilisates exhibit very good protein stability but at least partially collapse during freeze-drying because of the low T_g'. We used 2 mg/ml mAb as standard in our study and investigated the effect of i) a higher mAb concentration of 50 mg/ml, ii) the addition of sucrose as amorphous stabiliser with a higher T_g', iii) mannitol and phenylalanine as crystalline bulking agents, and iv) citrate and lactobionate as arginine counter ion alternatives to hydrochloride.

A high mAb concentration of 50 mg/ml mAb rendered pharmaceutically elegant cakes due a significant increase of T_g', to -32.5°C, but protein stability was compromised upon storage for 6 months at 40°C as indicated by the formation of HMWS. The addition of sucrose to ArgHCl also increased T_g', but only to -38°C, which is still critical with respect to the lyophilisation process. The combination of ArgHCl with sucrose improved cake appearance and protein stability compared to a pure sucrose formulation [22]. With respect to the crystalline bulking agents, a high amount of Man was required to improve cake appearance, which reduced the amount of ArgHCl as stabiliser and therefore caused mAb perturbations. Phenylalanine on the other hand was able to form pharmaceutically elegant cakes even at low concentrations (Phe:Arg 1:4, 1.5:3.5, and 2:3). Furthermore, protein stability during both lyophilisation and upon storage was comparable or even superior to a pure ArgHCl formulation. Using a ratio of Phe:Arg 1:4 or Phe 1.5:3.5 provided the best results regarding cake appearance and protein stability. At these concentrations, reconstitution times were not affected by the hydrophobic amino acid Phe [12].

The alternative Arg salts ArgCitr and ArgLacto already showed T_g' values high enough to avoid collapse in a conventional lyophilisation cycle yielding elegant cake appearance without the addition of crystallising bulking agents. However, ArgCitr lyophilisates showed markedly reduced protein stability with pronounced particle formation compared to ArgHCl. ArgLacto formulations were better than ArgCitr but inferior to ArgHCl. Thus, depending on the sensitivity of the protein, ArgLacto may be a suitable candidate next to ArgHCl for the sugar-free formulation of biopharmaceuticals.

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Chapter 8

Final Summary

The present thesis focused mainly on two aspects in lyophilization of biopharmaceuticals which have not been explored yet in detail: i) determining the water content of the maximally freeze concentrated solution (FC) (Chapter 3) with understanding of the protein stability in the FC (Chapter 4) and ii) the use of arginine (Arg) in combinations with different counter ions as amorphous matrix for lyophilisation of a monoclonal antibody (mAb) (Chapter 5), the effect of residual moisture on Arg based lyophilisates and mAb stability (Chapter 6), and elegant cake appearance with high protein stability based on Arg hydrochloride (Chapter 7).

In order to understand protein stability in the FC it is at first important to know its exact composition, i.e. the water content. A linear regression method taking solid content and change in the enthalpy measured by DSC into account was developed (Chapter 3). Various systems containing sugar (sucrose, trehalose) and other excipients (histidine buffer, phosphate buffer, NaCl, Arg hydrochloride, Arg citrate) with different mAb concentrations exhibited a solid content of 70 – 80% and 20 – 30% unfrozen water. The type of excipient had slight influence but not the mAb concentration. In the next step, a method to generate the extremely viscous FCs was developed based on partial freeze-drying and adjustment of the water content (Chapter 4). By this, we prepared different FCs containing mAb, sucrose, histidine or phosphate buffer, and NaCl. An increase in sugar or buffer concentration resulted in a positive influence on both mAb melting and aggregation temperature as well as on mAb self-interaction. Storage of the FCs above T_g' for up to 1 month did not affect protein integrity. Thus, exceeding T_g' during freeze drying, e.g. upon annealing or during primary drying, is not expected to negatively impact mAb stability. This important finding result also applies, beyond just freeze-drying, to storage of frozen bulk material.

Sucrose based formulations are the gold-standard in freeze-drying of biologics. They may however still require further improvement when it comes to protein stability and could potentially lead to glycation after sucrose cleavage. Arg is known to stabilise proteins due to its ability to bind to the protein surface as well as charge shielding effects. We formulated a mAb in Arg with the citrate, hydrochloride, lactobionate, phosphate, and succinate as counter-ion (Chapter 5) and studied the performance upon freeze-drying as well as the physicochemical characteristics and the mAb stability of the lyophilisates. Arg hydrochloride collapsed and partially crystallised upon storage but provided best protein stability. The citrate, lactobionate, and phosphate salts formed elegant cakes but had deficits in protein stability. Addition of sucrose could on the one hand improve the cake appearance for Arg hydrochloride and on

the other hand improve mAb stability for the other counter-ions. At higher mAb concentration, only the hydrochloride and the lactobionate provided sufficient protein stability next to sucrose.

The same arginine salts were analysed regarding their ability to stabilise a mAb at different residual moisture levels (Chapter 6). Lyophilisates with two different residual moisture levels of less than 0.5% and of approx. 2.5% were stored for up to 6 months at 40°C. The mAb aggregation in Arg hydrochloride and succinate was similar or even less compared to a sucrose reference formulation, while Arg citrate, lactobionate and phosphate resulted in lower protein stability. Despite decreased T_g values, which is still above 50°C, Arg formulations with high RM levels exhibited better protein stability. Glycation of the mAb was detected in sucrose based formulations, but did not occur in Arg based formulations.

Since Arg hydrochloride resulted in very good protein stability but poor cake appearance with partial collapse, as presented in Chapters 5 and 6, ways to improve the cake appearance were investigated (Chapter 7). Arg hydrochloride and, for comparison, citrate and lactobionate were studied alone and in combination with i) a higher mAb concentration, ii) sucrose as an amorphous matrix former with higher T_g' and iii) in combination with mannitol and phenylalanine as crystalline bulking agents. At least a 4:1 ratio of mannitol to Arg or a 1:4 ratio of phenylalanine to Arg were required to improve cake appearance. Only phenylalanine containing formulations exhibited a very good protein stabilising effect similar to pure ArgHCl. Addition of mannitol led to markedly reduced amount of the stabilising amorphous matrix former Arg hydrochloride, considering the solute limitations given by isotonicity, and in consequence to drastically reduced mAb stability. ArgCitr and ArgLacto did not require cake improvement but again, mAb stability is clearly inferior. Higher mAb concentrations also resulted in adequate cake appearance but came at the expense of reduced stability. Thus, we could demonstrate that Arg is a highly valuable amorphous matrix former for lyophilisation of proteins. Using the hydrochloride, a combination with phenylalanine is recommended in order to achieve adequate cake appearance. The crystalline bulking agent also allows for faster processing as it enables a higher product temperature during primary drying. A higher residual moisture is well tolerated by Arg based products which could be an important benefit over sucrose based lyophilisates.

In summary, this thesis provided several new findings regarding the freeze drying of mAb. We established new tools for characterisation and preparing the freeze concentrate. Protein stability is not negatively affected in this concentrated state even when exceeding T_g' for up to 1 month. We also demonstrated that Arg is a highly suitable stabiliser in protein lyophilisates, especially in combination with phenylalanine as crystalline bulking agent.