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Effect of Arginine on the Aggregation of Protein in Freeze-Dried Formulations Containing Sugars and Polyol: II. BSA Reconstitution and Aggregation

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Abstract

The current paper continues our study on the ability of L-arginine to prevent/reduce the aggregation of proteins that results from the various stresses during the lyophilisation and/or storage of lyophilized protein-based products. The first part of our study, *i.e.* formulation development, was devoted to the rational design and optimization of an L-arginine containing lyophilized formulation which can resist the natural tendency of L-arginine to absorb atmosphere moisture. Mannitol and trehalose were chosen among other excipients to be included in the protein-based formulation, as mannitol in a combination with L-arginine has been shown to reduce moisture sorption while trehalose provides a degree of lyoprotection. In the present study, a number of formulations, which comprised bovine serum albumin (BSA) with and without L-arginine, and with five different ratios of trehalose-to-mannitol (from 30:70 to 80:20) were lyophilised and assessed. The internal structures and the moisture sorption/retention of the lyophilized formulations were characterised. To study the effect of L-arginine on BSA solid-phase stability, the lyophilized powder was exposed to accelerated storage conditions (high moisture (75% RH) and temperature (22 or 45 °C)) for up to 24 h. The lyophilized BSA formulations were then reconstituted and solution-state protein aggregation assessed by turbidimetry at 360 nm and fluorescence spectroscopy using the thioflavin T assay. It was demonstrated that L-arginine can be used in protein-based freeze-dried formulations to significantly reduce the aggregation of protein during the manufacturing, storage and subsequent reconstitution. The results also revealed the importance of a sufficient amount of mannitol in the arginine-containing formulations.

KEY WORDS

freeze-drying; protein aggregation; L-arginine; mannitol; trehalose;

Abbreviations

BSA Bovine serum albumin
Arg L-arginine HCl
ThT Thioflavin T

FD Freeze-drying/freeze-dried
RH Relative humidity
OD Optical density
TGA Thermogravimetric analysis
DSC Differential scanning calorimetry
SEM Scanning electron microscopy

INTRODUCTION

Since the late twentieth century, different protein-based therapeutics (bio-pharmaceuticals) and peptide-based products have emerged in the drug market (1–6). Today, approximately 20% of all commercial pharmaceuticals are bio-pharmaceuticals (7 and references therein) and the global biotherapeutic**s** market is expected to reach $386.7 billion by the end of 2019 (8). Monoclonal antibodies represent the majority of bio-pharmaceuticals on the market followed by hormones and growth factors (7 and references therein). Proteins and polypeptides offer the distinct advantages of specific mechanisms of action and are highly potent. However, they also present certain distinct challenges which originate from their immunogenicity, high molecular weights and instability (aggregation/denaturation) (9–12).

Protein destabilisation/denaturation and aggregation during manufacturing and storage is a significant problem in the biopharmaceutical industry as aggregates decrease the activity of a protein and hence lower the efficacy of a protein-based drug product (4,8,9,13,14). Moreover, certain aberrations (*i.e.* abnormalities) in protein structure or the presence of protein aggregates can trigger immunogenic reactions in the patient.
Lyophilisation, or freeze-drying, is often used to increase the stability and shelf life of bio-pharmaceutical protein-containing drug products (1,3,4). However, freezing and dehydration introduce some additional stresses such as cold denaturation, liquid-phase separation, freeze concentration, pH change and structure destabilisation due to the loss of the protein hydration shell, *etc.* (1,4,15,16). Stresses that can compromise the protein structure and efficacy can occur at many stages in the production of a lyophilised product. These include shearing from mixing or shaking, freeze-thawing, drying—as well as during temperature and relative humidity fluctuations during storage and during/after reconstitution (1,14,17–22).

Various types of excipients (sugars, polyols, amino acids, monovalent electrolytes) are used to enhance the physical and chemical stability of peptide and protein pharmaceuticals (5,15,23–29). The mechanisms of protein stabilisation by different sugars are discussed widely in the literature (for instance, see the recent reviews (30,31). Two of the main mechanisms of stabilisation by sugars are (i) water substitution by hydrogen bonded sugar molecules at the surface of protein (thermodynamic stabilisation)

and (ii) vitrification which leads to the suppression of molecular mobility (kinetic stabilisation).

Among the 20 naturally occurring amino acids, L-arginine (ArgHCl) has become recognised as an excipient which increases the solubility of proteins and suppresses their aggregation (24 and references therein, 32–35) and co-aggregation (36) in solution. Recent studies have shown that arginine can also improve the solid state stability of freeze-dried protein pharmaceuticals (28,37). The stabilisation provided by arginine strongly depends on the arginine concentration and the type of counter-ions (36 and references therein).

The present paper continues our study (38) on the effect of arginine on protein aggregation during the manufacturing process and storage. The ultimate aim of this study was to assess the ability of arginine to prevent/reduce protein aggregation caused by the various stresses encountered during lyophilisation, storage and/or reconstitution of a lyophilized product. However, introducing arginine into a protein-based lyophilized product is challenging because of its hygroscopicity that is not considered in some works related to freeze-dried formulations containing L-arginine. It is known that the extent of protein aggregation in the solid state is related to both the residual water, at the end of the process cycle, and to the amount of water absorbed by the protein on storage, and is due to the impact of water on the conformational dynamics of the protein molecule (39,40). Therefore, the first part (38) of our study—excipient selection (formulation development)—was devoted to the rational design and optimization of a formulation that can resist or, at least, significantly reduce the moisture uptake by lyophilized samples containing L-arginine.

Appropriate excipients (sugars/polyols) were selected according to their ability to (i) form elegant cakes and (ii) absorb the least amount of moisture when combined in a binary mixture with L-arginine. The study showed that, among several commonly used sugars/polyols (*i.e.* mannitol, trehalose, sucrose, lactose), mannitol was by far the best in terms of its ability to resist moisture sorption by the freeze-dried cakes containing arginine, even when stored at very harsh conditions. Having selected mannitol as the best moisture protector in combination with arginine, a third excipient (trehalose) was chosen as it provides lyoprotection and allows producing acceptable freeze-dried (FD) cakes in ternary formulations.

Analysis of a number of quality attributes (*i.e.* moisture uptake, cake shrinkage, mechanical properties, internal structure) of the lyophilized 3-excipient formulations (containing L-arginine + mannitol + trehalose) showed that the property dependencies on the trehalose content were non-linear (38). Therefore, in the final part of our study, reported in this article, several trehalose-to-mannitol ratios were selected for test in the

protein-containing formulations. Overall, five excipient compositions with different trehalose-to-mannitol ratios, with or without L-arginine, were used to prepare the protein-based formulations. Lyophilized samples with (Arg+) and without arginine (noArg) were studied comparatively. The internal structures and the moisture sorption/retention abilities of the lyophilized samples were characterised. Since the main emphasis of the present research was on the study of the impact of L-arginine on protein stability during manufacturing and storage of lyophilised product, before reconstitution cakes were exposed to the stressed storage conditions (increased relative humidity (75%) and either room (22 °C) or elevated temperature (45 °C)) which can damage proteins and accelerate protein aggregation. The stability test was followed by protein reconstitution and measurement of the extent of protein aggregation.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), L-arginine hydrochloride (ArgHCl), trehalose dihydrate, D-mannitol and Tris buffer salt (ACS reagent grade) were purchased from Sigma-Aldrich Co, UK. Unless otherwise specified, all materials and reagents were of analytical grade and were used without any further purification.

Methods

Freeze Drying

Stock solutions (10% *w*/*v*) of BSA, trehalose, mannitol and L-arginine hydrochloride in 20 mM Tris-HCl buffer (pH 7.2) were prepared immediately prior to freeze-drying. Samples were mixed accordingly and freeze-dried using Heto FD8 freeze-drier (Germany) in transparent glass vials (one vial contained 2 ml of solution such that the total weight of solids was 0.2 g per vial) using the freeze-drying cycle described in (38). To reduce radiation effects (41), vials were loaded in a close hexagonal packing profile in the centre of a shelf. Product temperature was monitored using a thin wire thermocouple. At the end of the freeze-drying cycle, the vials were stoppered under vacuum and crimped with aluminium seals. Samples were stored in a desiccator in a dry cold place.

Through-Vial Impedance Spectroscopy

Aliquots (2 or 3 ml) of freshly prepared 10 wt% BSA solution with and without arginine were added to the specially designed impedance measurement vials connected to the data-acquisition system (42), placed on a freeze-dryer shelf and exposed to the same freeze-drying cycle used to prepare FD samples. The impedance measurements were performed over a frequency range of $10–10^6$ Hz with a scan interval of 3 min as described previously (43).

Moisture Sorption

Freeze-dried samples were placed in aluminium foil boats and incubated in a desiccator at 75% relative humidity (RH) at either 22 or 45 °C. Incubation of the lyophilized powders at constant RH was achieved by vapour equilibration in desiccators over NaCl saturated solution. Weight of the samples over time was measured gravimetrically and moisture uptake was calculated as

where W_m and W_d are the weight of moist and dry powder, correspondently.

Thermogravimetric Analysis

A Perkin-Elmer Pyris 1 thermogravimetric analyser (USA) was used to record the water evaporation profiles and to assess the moisture content in freeze-dried powder samples. Five to fifteen milligrams of powder was placed into a thermogravimetric analysis (TGA) aluminium pan; the pan was sealed and a hole was made using a needle. Samples were heated from 25 to 300 °C at a rate of 10 °C/min under nitrogen atmosphere and the decrease in sample weight was monitored. Onset and endpoint temperatures were calculated using Pyrus software.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were carried out using a Perkin-Elmer Jade DSC (USA). Between 5 and 10 mg of the FD powder was placed into an aluminium DSC pan and crimped with vented lid. Samples were heated either (i) from 0 to 180 °C at 1 °C/min; or (ii) from − 20 to 300 °C at 10 °C/min. Melting temperatures and the enthalpy of melting were calculated using the Pyris software.

Scanning Electron Microscopy

The internal structures of the BSA-containing freeze-dried cakes at different magnifications (× 250, × 500, × 1000, × 5000) were visualised using a Carl Zeiss EVO HD scanning electron microscope (UK). Powder samples were mounted on 12.5 scanning electron microscopy (SEM) pin metal stubs using 12 mm carbon tabs (Agar scientific).

Protein Reconstitution and Aggregation Assay

Prior to reconstitution, the freeze-dried cakes were incubated for 24 h at 75% RH and 22 or 45 °C. Proteins were reconstituted by adding double distilled water to the FD cakes at 4 °C, until complete dissolution of the powder was achieved. The protein concentration was measured using a UV-Vis spectrophotometer with a 1-cm path-length quartz cuvette. The percent solution extinction coefficient was taken as 6.67 E1% for BSA at 280 nm (44). Protein concentration was adjusted to keep it constant in solutions with and without arginine. The UV-Vis spectra of the reconstituted proteins were recorded in the range 240–400 nm and the A_{260}/A_{280} ratios calculated.

Reconstituted protein solutions were incubated at room temperature (22 °C) and the formation of aggregates was assessed by measuring the apparent absorbance at 360 nm caused by increased turbidity (turbidimetry assay), using a Thermo-Scientific UV-Vis Evolution 60S spectrophotometer. Before the optical density measurements, the protein solution was mixed in order to re-suspend any aggregates that may have precipitated on standing. The formation of aggregates was also confirmed by light microscopy.

Fluorescence Spectroscopy (ThT Assay)

ThT stock solution (1 mM) was prepared using double-distilled water and filtered using a 0.2 μm syringe filter. The stock solution was stored at 4 °C for no longer than 1 month. All samples containing ThT were kept in the dark to minimise photobleaching. In a typical experiment, 300 μl of reconstituted protein solution was mixed with 1.7 ml of Tris-buffer followed by the addition of a small aliquot of ThT stock solution to the working reaction, immediately before the measurement. The final ThT concentration in solution was 10 μM. The pHs of the buffer and the protein solutions were kept at 7.2 as both acidic and basic pHs are known to decrease the ThT fluorescence emission (45). Fluorescence measurements were carried out using a LS55 spectrofluorometer (Perkin-Elmer, UK) in 1-cm quartz cuvettes containing 2 ml of unstirred solution. The temperature inside the cuvette was maintained at 25 °C by a thermostatic cell holder. ThT emission spectra were recorded in 0.5 nm increments by exciting the sample at 445 nm and collecting the emission between 455 and 650 nm; the excitation and emission slit widths were set at 2.5 and 5 nm, respectively.

RESULTS AND DISCUSSION

Freeze-Dried Formulations Containing BSA, Mannitol, Trehalose and L-Arginine

Five formulations containing protein (BSA, 50 wt%), D-mannitol, trehalose and L-arginine (10 wt%) with different trehalose:mannitol ratios were prepared (Table I). Five control formulations contained the same trehalose:mannitol ratios but without L-arginine. The ratios between protein, mannitol and trehalose were kept the same for arginine-containing and arginine-free samples. Hereinafter, the formulations containing L-arginine will be named as Arg+ and those without arginine—noArg. After freeze-drying, all formulations formed well-shaped non-collapsed white FD cakes without significant cracks.

Table I
Compositions of the Freeze-Dried Formulations Containing Protein (BSA), Mannitol, Trehalose and L-arginine

Formulation ID	Trehalose:Mannitol	Protein, %	Mannitol, %	Trehalose, %	L-arginine, %
T12	30:70	50%	28	12	10
T16	40:60	50%	24	16	10
T20	50:50	50%	20	20	10
T24	60:40	50%	16	24	10
T32	80:20	50%	8	32	10

Arginine Effect on Freeze-Drying of the Protein Formulation

Through-vial impedance spectroscopy (42,43) was used to monitor the effect of arginine on the rate of primary drying of the protein-based formulations. This technique is based on the measurements of impedance (or electric capacitance) over a wide frequency range using an electrode system affixed to the outside of a standard 10-ml glass lyophilisation vial filled with the BSA solution to be freeze-dried (41). During freezing and drying stages the temperature, physical state and composition of the sample are changing dramatically due to the aqueous phase freezing and the sublimation of ice, causing significant changes in impedance (42).

Figure 1a presents 3D plots of dielectric loss as a function of time and frequency for two 10 wt% protein solutions without and with Arg (55% BSA, 22.5% trehalose, 22.5% mannitol and 50% BSA, 20% trehalose, 20% mannitol, 10% arginine). The proportion between protein, trehalose and mannitol was kept constant in both samples. The capacitance values C″ of the relaxation peaks calculated using a bespoke peak funding algorithm (42) are presented in Fig. 1b. The capacitance is proportional to the dielectric permittivity that differs significantly for ice (> 90) and for dry excipients (< 5) (46). As the height of the ice layer decreases due to water sublimation, the dielectric loss peak decreases over time (Fig. 1b). The initial rate of C″ decrease can be associated directly with the rate of water sublimation. Once the ice layer reaches the bottom edge of the electrode then the magnitude of the ice peak (C″) decreases to zero and hence the time to that point, $t(C''_{PEAK} = 0)$, can be taken as a relative measure of the drying times between each formulation.

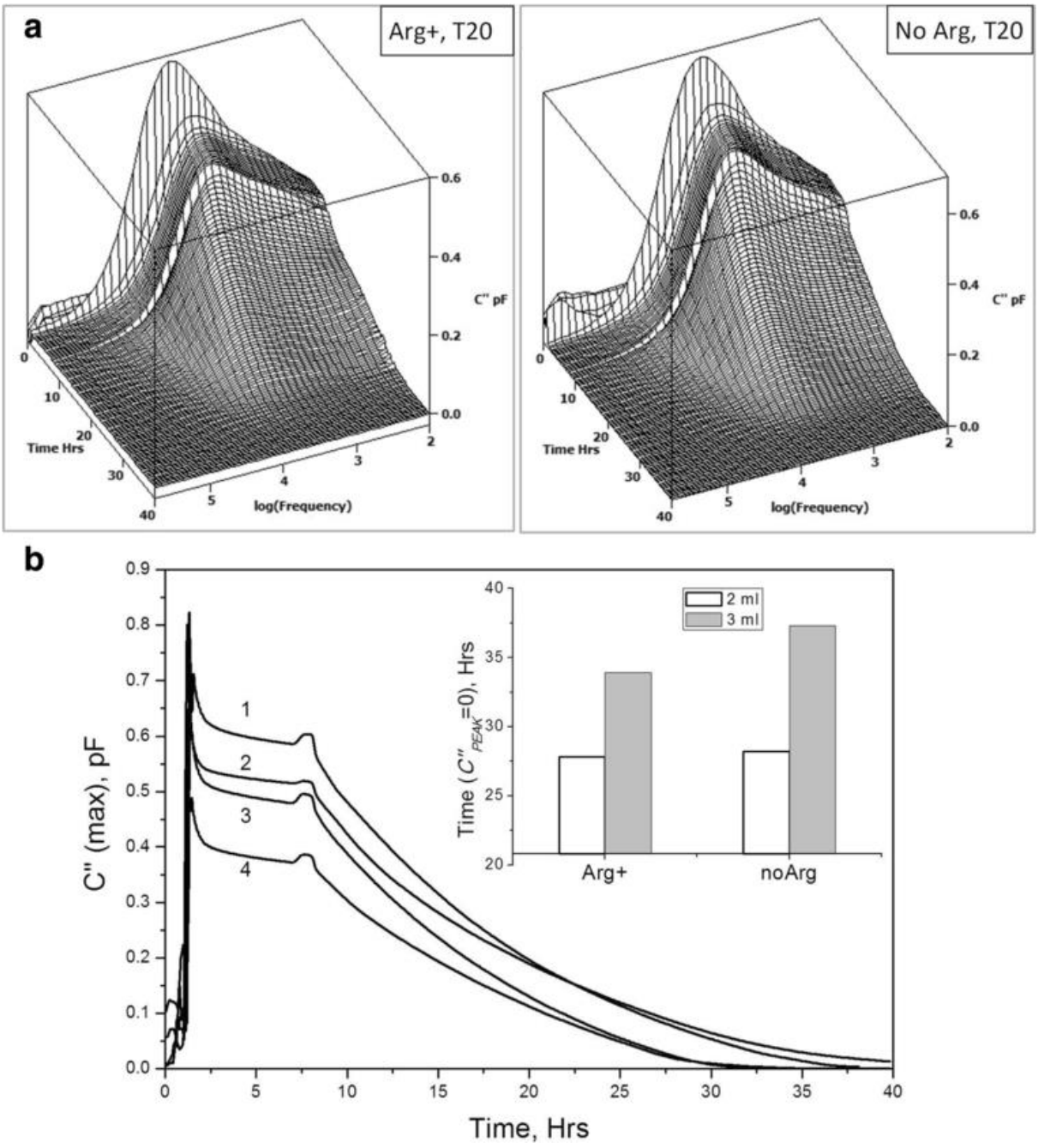

Fig. 1

Monitoring of freeze-drying process using TVIS system. **a** 3D dielectric loss spectra (imaginary part of capacitance, C″ *vs.* time and frequency) monitored during the freeze-drying process of T20 samples including 10 wt% L-arginine (left) and without L-arginine (right). **b** The magnitude of the spectrum peak of the imaginary part of dielectric capacitance C″ *vs.* time for T20 samples with (1, 4) and without L-arginine (2, 3). Total volume of liquid in a vial: 2 ml (3, 4) and 3 ml (1, 2). Insert: end set of the primary drying time for T20 samples with/without arginine

As expected, the time when C''_{PEAK} approaches zero for the 3 ml sample occurred 8–10 h later than for the 2 ml sample. Arg presence had no significant effect on the end point time for the 2-ml sample; 3-ml sample containing Arg dried *ca.* 4 h faster than the sample without arginine. This result coincide with our previous finding for the protein-free two- and three-component systems containing mannitol and trehalose (38), where noArg samples dried slower than Arg+. The arginine effect on the ice sublimation time was more pronounced in the case of protein-free samples described in our Part I paper (38) as they contained higher percentage of arginine (20 wt%).

In (38), we have suggested that the increased sublimation rate for the arginine-containing formulation is probably related to the facilitated diffusion of water in this system. Formation of a surface layer on the top of a sample during freezing process can also contribute to the slower rate of sublimation in the case of noArg formulations.

Effect of Arginine on the Internal Structure of Freeze-Dried Products

SEM images (Fig. 2) illustrating the internal structures of lyophilized powders reveal that dry BSA-sugar samples, both with and without L-arginine, resemble hexagonal honey-comb structures typical for many freeze-dried products. The presence of arginine in the formulation does not have a significant impact on the type of the structure and on the size of hexagonal cells. However, there is a small tendency towards decrease in the pore sizes and the wall thicknesses in the FD samples containing arginine (in comparison to the arginine-free FD samples). As arginine comprises only 10 wt% of a sample, it is expected that the morphology of the samples is mainly determined by protein and sugar/polyol composites.

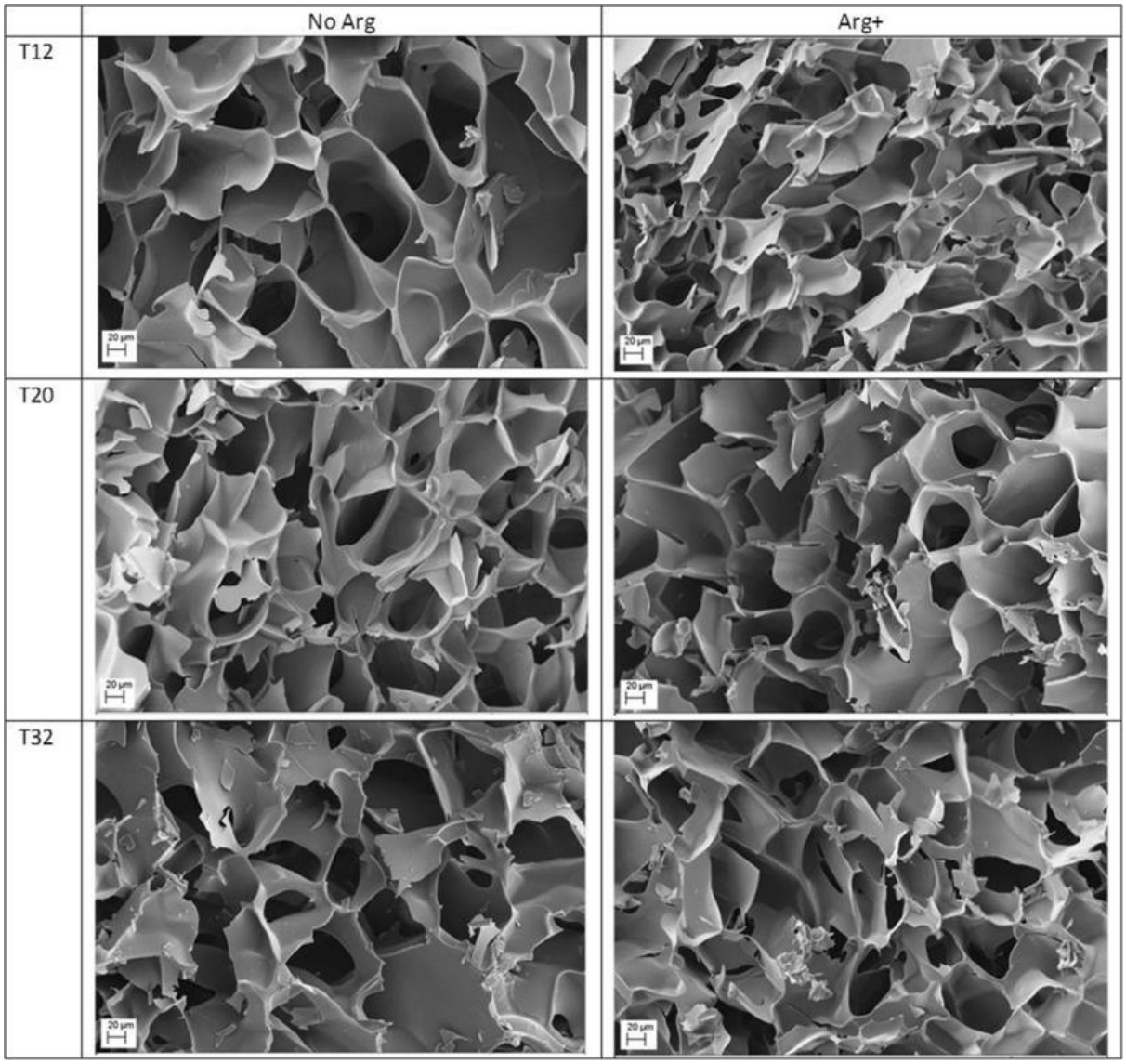

Fig. 2
SEM images (× 500) of lyophilized samples (T12, T20 and T32, see Table I) with (Arg+) and without (noArg) L-arginine (10 wt%)

Moisture Uptake by FD Formulations Containing BSA With/Without L-Arginine

Freeze-dried cakes were subjected to a humidified environment in order to assess their moisture sorption properties. Cakes were incubated at 75% RH at room temperature (22 °C) during 24 h and the weight gain over time was monitored. The total moisture uptake after 24 h was assessed gravimetrically (TGA and analytical balances).

Kinetic measurements of the moisture sorption show that the weight of FD cakes increases with time (Fig. 3a). For all samples, the first hour of moisture treatment had the biggest impact on the total amount of moisture uptake with between 55 and 65% of the total moisture being accumulated after 1 h of incubation at high relative humidity. In

addition, during the first hour of moisture treatment no significant difference was observed between the moisture accumulation by the noArg and the Arg + FD formulations (Fig. 3a). Figure 3b shows that the total moisture content in the FD cakes after moisture treatment increases when the trehalose content increases (and, consequently, mannitol content decreases). For all trehalose-to-mannitol ratios, those FD formulations containing arginine (Arg+) accumulated more moisture than those without arginine (noArg) (Fig. 3b).

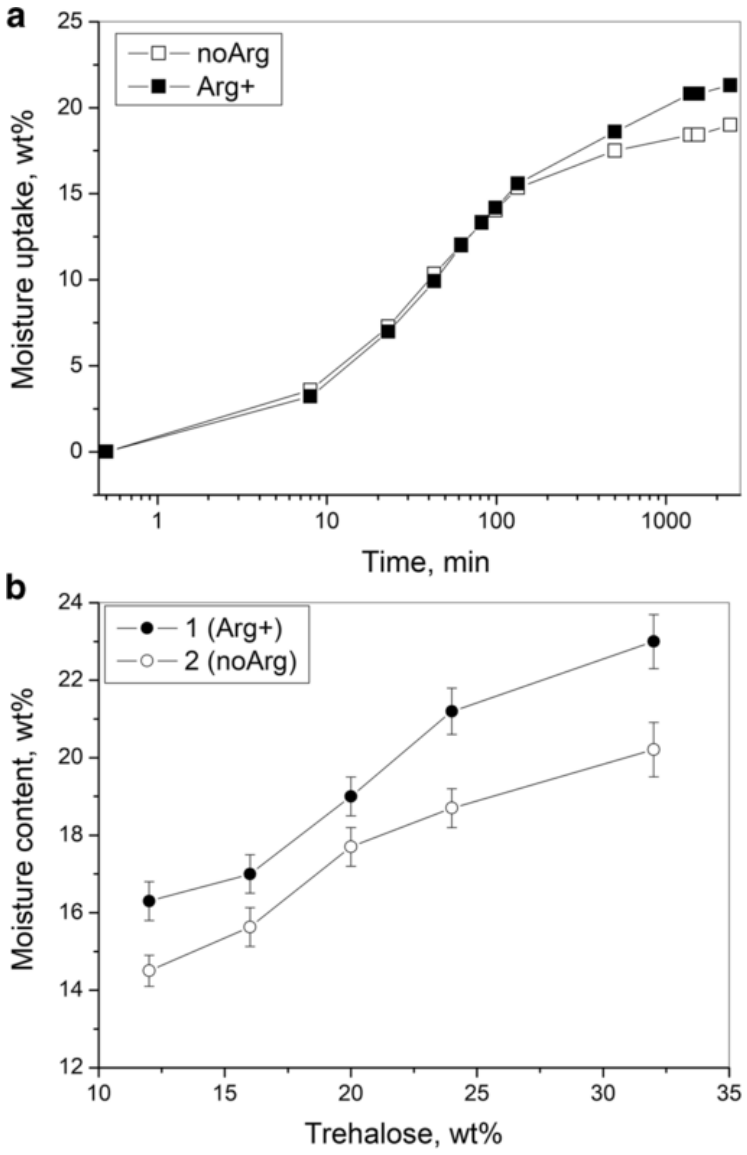

Fig. 3

Moisture uptake by FD formulations containing BSA, trehalose, mannitol with or without L-arginine during moisture treatment at 75% RH and room temperature. **a** Kinetics of moisture sorption by lyophilised T16 formulations without (noArg) and with (Arg+) L-arginine. **b** Total moisture uptake by formulations after 24 h of incubation at 75% RH. Formulation compositions are presented in Table I

The presence of arginine in the formulation also changed the moisture release profiles. Figure 4 shows the TGA thermograms illustrating the weight loss by moisture-treated FD cakes comprising albumin (50%), trehalose and mannitol with/without Arg. A typical TGA thermogram shows two steps, where the first step is mainly the result of the weight loss due to moisture evaporation and the second step is due to material decomposition at higher temperatures. For the cakes containing no Arg (Fig. 4a, curve "1"), the moisture evaporation profiles are almost independent of the trehalose and mannitol content. In the case of Arg-containing formulations, the TGA profiles strongly depend on trehalose-to-mannitol ratio. Low-to-average trehalose content formulations containing Arg (T12-T24, see Table I) show only minor differences from the non-Arg formulations (Fig. 4a, top). The presence of Arg in formulations with high trehalose/low mannitol content (T32, Fig. 4a, bottom left) leads to a strong alteration of the TGA profiles, in that there is a significant increase in the end temperature of the moisture evaporation while the onset temperature remains almost unchanged. This results in a strong increase in the temperature interval (ΔT) of the moisture evaporation (Fig. 4a, bottom right) from the moisturised formulations containing Arg.

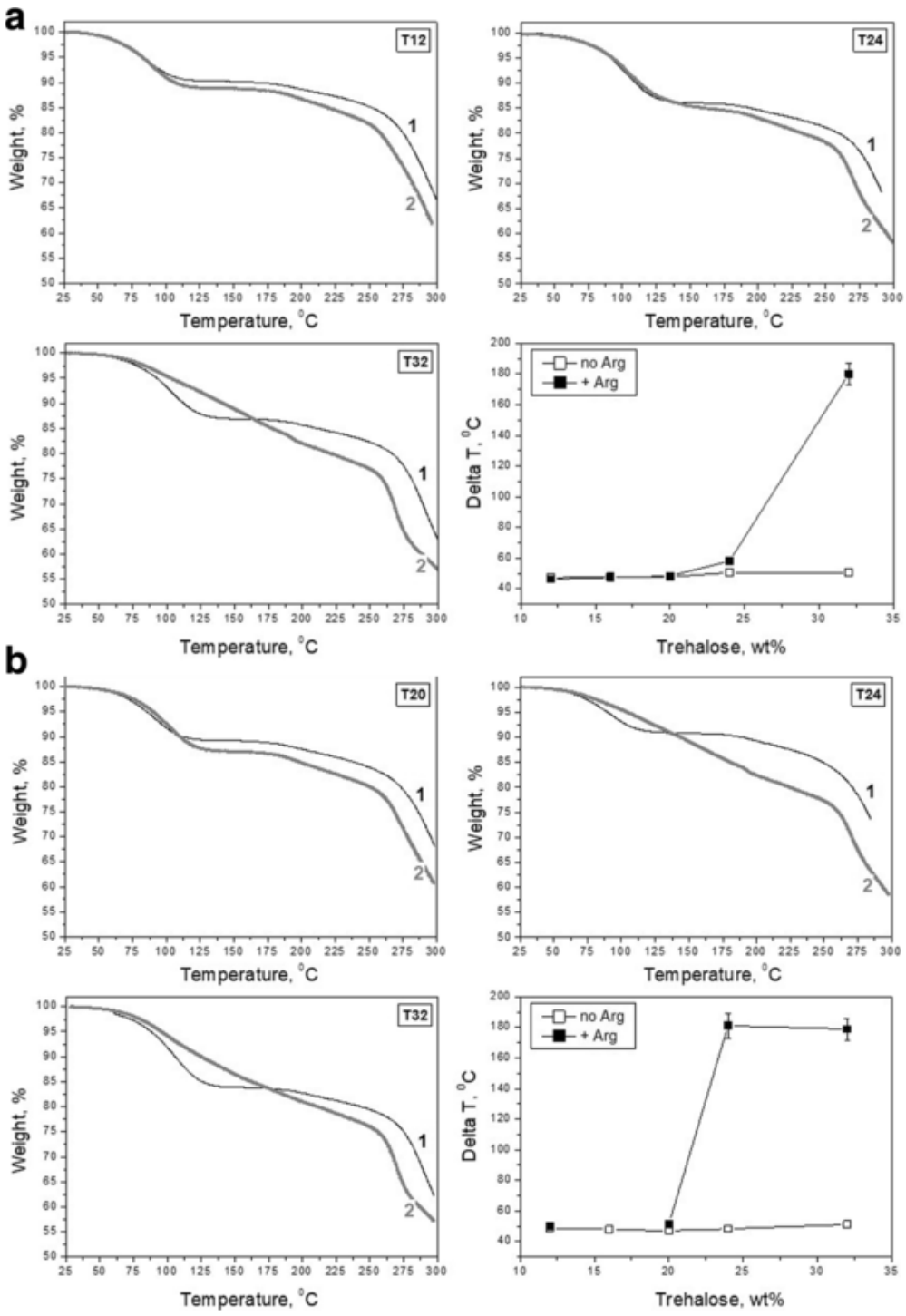

Fig. 4

TGA study of moisture release by FD formulations containing BSA, trehalose, mannitol with/without L-arginine. Prior to TGA, all FD formulations were moisture-treated by incubating at 75% RH, 24 h, 22 °C (**a**) or 45 °C (**b**). **a**, **b** Top and bottom left—TGA thermograms recorded from 25 to 300 °C at 10 °C/min illustrating the weight loss by moisture-treated formulations T12, T24 and T32 (see Table I). **a**, **b** Bottom right—dependencies of the temperature interval of moisture release ΔT (where $\Delta T = T_{\text{endset}} - T_{\text{onset}}$) on the trehalose content in formulation

Moisture treatment (75% RH) at elevated temperature (45 °C) for 24 h resulted in more pronounced differences between the noArg and the Arg+ formulations at lower trehalose concentrations. As Fig. 4b shows, both T24 and T32 Arg+ formulations possess altered TGA moisture evaporation profiles in comparison to noArg formulations. Moreover, elevated temperature in combination with increased moisture resulted in a colour change from white to light brownish in the case of Arg+ formulations.

Overall, the moisture treatment results show that the presence of arginine in BSA-based FD formulations increases the moisture uptake by approximately 10–15% after 24 h of incubation at 75% RH. Arginine also makes the process of water evaporation more gradual by increasing the endpoint temperature. At the same time, a high content of mannitol in a formulation diminishes/compensates the effect of Arg on moisture sorption.

Thermal Stability of Freeze-Dried BSA Formulations With/Without L-Arginine

DSC thermograms (Fig. 5a) of formulations comprising BSA (50 wt%), trehalose (24 wt%), mannitol (16 wt%) with/without Arg (10 wt%) after moisture treatment (75% RH, 24 h, room temperature) were recorded to assess the impact of arginine on the thermal stability of the protein. Both thermograms have a thermal peak around 66–68 °C which corresponds to the BSA melting temperature (47). The presence of Arg does not shift the peak temperature. This result is in accordance with the known fact that arginine, being an aggregation suppressor, does not enhance the protein thermal stability (48,49). Arginine also has a minimal effect on the enthalpy of the endothermic transition, ΔH, equal to 279 ± 20 J/g (since the samples were moisturised, the water evaporation might contribute to this value).

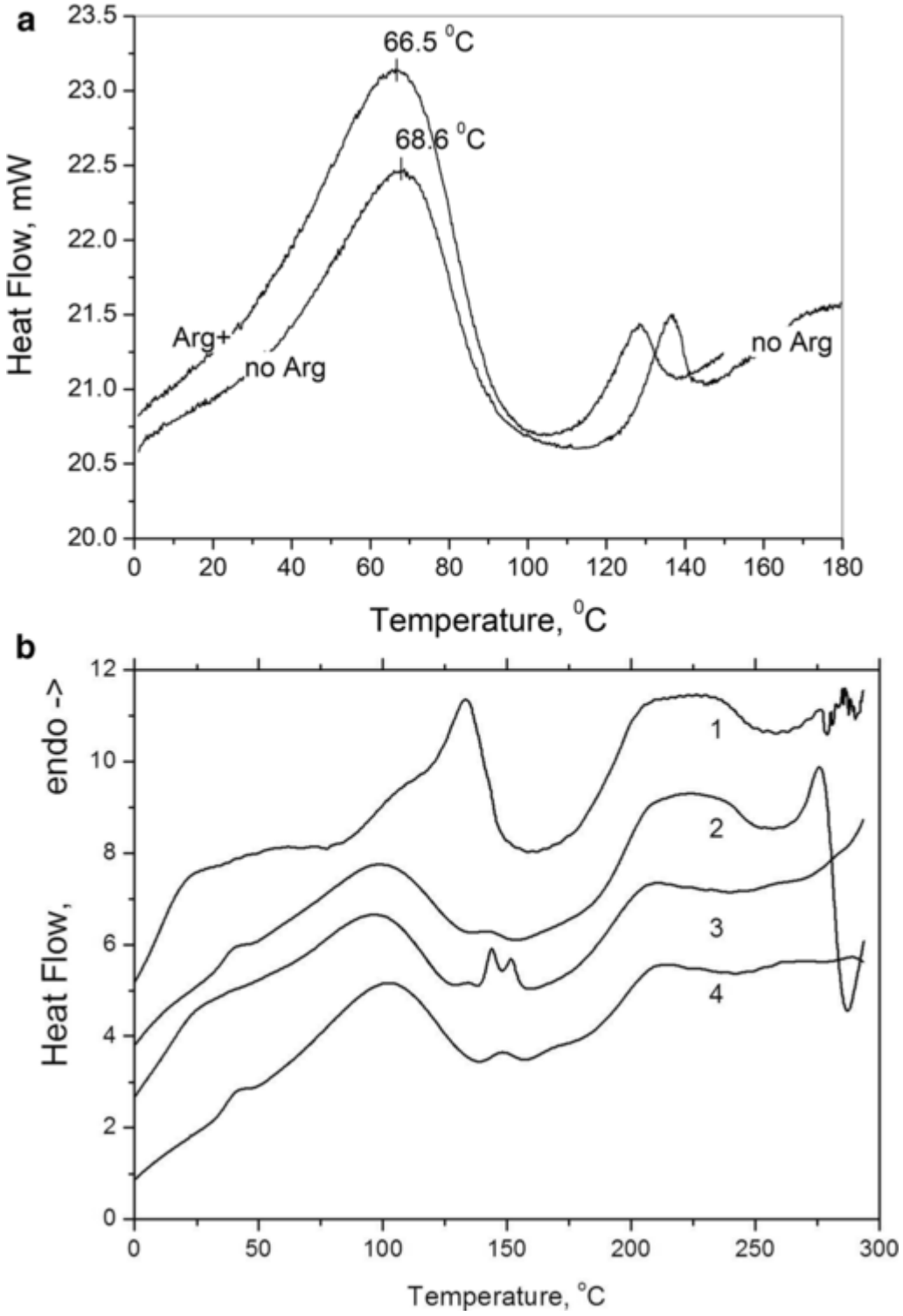

Fig. 5

DSC thermograms of FD formulations containing BSA, trehalose, mannitol with/without L-arginine. **a** Thermograms for formulation T24 (see Table I) recorded from 0 to 180 °C at 1 °C/min. Samples were pre-treated at 75% RH, room temperature, during 24 h prior to measurements. **b** Thermograms recorded from 0 to 300 °C at 10 °C/min for samples: 1—T12 Arg+; 2—T32 Arg+; 3—T12 noArg; 4—T32 noArg L-arginine

DSC thermograms for BSA-containing freeze-dried noArg/Arg+ formulations with different trehalose-to-mannitol ratios recorded in the wide range of the temperatures (at 10 °C/min) are shown in Fig. 5b. The endothermal peak in the 140–150 °C region (Fig. 5b) is associated with melting of mannitol (38,50). The presence of a melting peak confirms that mannitol is, at least partially, in the crystalline state in the freeze-dried samples containing BSA. It is known that D-mannitol has three common polymorphic forms and (partial) crystallisation of mannitol during the freeze-drying process can produce different anhydrous polymorphs (α, β and δ) (51) and a hemihydrate form (52,53). Three polymorphs do not undergo transformations under mechanical stress or long periods of storage (52 and references therein). The α and β polymorphs have very similar crystal structures and nearly identical physical properties including melting points (52). As is seen in Fig. 5b, in noArg protein-containing freeze-dried samples, mannitol predominantly forms one polymorph if a formulation contains low amount of mannitol and high amount of trehalose (T32 formulation in Fig. 5b) and forms two polymorphs if the amount of mannitol is increased (T12 in Fig. 5b).

Low melting enthalpy values (11 ± 5 J/g) reflect the fact that only a small part of mannitol is in crystalline form in the protein-containing noArg formulations. Perhaps, the overall low percentage of mannitol in the samples (from 8 wt% in T32 to 28 wt% in T12, Table I) makes it difficult for mannitol to crystallise. Besides, the presence of a high amount of a protein (BSA) in formulations can potentially delay and reduce the amount of mannitol crystallisation as it has been showed recently (54).

The presence of arginine had no significant effect on the amount of crystalline mannitol and its polymorphs in the protein formulations with low amount of mannitol (T32 sample, Fig. 5b). However, when the amount of mannitol increased (T12 sample), the presence of arginine resulted in the mannitol conversion into a single polymorph form (Fig. 5b). At the same time, the total amount of crystalline mannitol in the sample increased almost four times as revealed by increased value of the melting enthalpy (ΔH values were ~15 J/g for noArg T12 sample and ~58 J/g for Arg+ T12 sample). Thus, the presence of L-arginine increases mannitol crystallisation in protein-containing formulation, however, only if the mannitol content is higher than the trehalose content. It was shown recently that in the mixture of mannitol and trehalose, the crystallisation behaviour of both solutes, after freeze-drying, depends on the mannitol to trehalose ratio (R): when $R \geq 1$, the extent of mannitol crystallisation was directly proportional to the value of R, when $R < 1$, trehalose completely suppresses mannitol crystallisation (55). Our results also show that the amount of crystalline mannitol is lower in formulations with mannitol-to-trehalose ratios < 1. Thus, it cannot be excluded that high trehalose and/or BSA content (but not arginine alone) determine the low amount of crystalline mannitol in the formulations.

It is of interest to note that the effect of arginine on the polymorph forms of crystalline mannitol was very similar in the protein-free formulations containing mannitol, trehalose and L-arginine, as reported in our first paper (38). On decreasing the mannitol content (and, consequently, increasing the trehalose content with fixed Arg concentration), an endothermic double peak was observed close to 150 °C which is consistent with the peaks from the β and δ forms of mannitol being shifted towards lower temperatures and transformed into a broad single asymmetric peak with a shoulder. This result shows that the effect of arginine on the mannitol crystallinity in freeze-dried products is not significantly altered by a presence of even a large amount of BSA in a sample.

The endothermic peak close to 220 °C (Fig. 5b) can be ascribed to trehalose melting, after first devitrification during DSC run. Melting of freeze-dried trehalose at these temperatures was observed previously (for example, see (56,57)). The presence of arginine does not alter this peak significantly. It is often expected that after freeze-drying, trehalose is in amorphous state and undergoes a glass transition in the temperature interval 80–120 °C (according to different literature data and depending on the sample content, method of preparation, moisture, *etc*.; for instance, authors of (57) observed trehalose glass transition at 117–118 °C). In the current study, a number of overlapping peaks in this temperature interval makes it impossible to observe a trehalose glass transition.

In the presence of L-arginine, the endothermic peak around 220 °C (Fig. 5b) becomes broader, perhaps, due to the arginine contribution. It was shown previously that, unlike most amino acids (*e.g.* glycine, alanine, valine), arginine is at least partially amorphous after freeze-drying (58,59); crystallisation could be significantly or completely supressed in the presence of HCl when the amino acid salt is formed (see review (37) and references therein). It is possible that in our four-component system, L-arginine is, at least partially, in a crystalline form.

The presence of the decomposition peak (60) at high temperatures (> ~280 °C) on the DSC thermograms of Arg+ samples evidences that sample degradation occurs at these temperatures (Fig. 5b). The thermal decomposition was stronger in the presence of Arg+. Comparison of the Arg-containing samples shows that the decomposition peak was more expressed and shifted towards lower temperatures in the case of the formulations containing more trehalose than mannitol. This result is in accordance with our finding (described below) that the presence of arginine in formulations with lower levels of mannitol facilitates the aggregation of protein at high temperatures.

Reconstitution of FD Formulations Containing BSA With and Without L-Arginine

To enhance the stresses caused by sample storage and to accelerate protein denaturation/aggregation, freeze-dried cakes were pre-incubated for 24 h at 75% RH and

either 22 or 45 °C. After incubation under these stressed storage conditions, the protein samples were reconstituted by adding double-distilled water followed by gentle agitation to ensure complete dissolution. UV-Vis spectra of the freshly reconstituted albumin samples followed the pattern of a typical native protein spectrum. Control experiments showed that immediately after reconstitution, and during the first hour following reconstitution, the optical densities at 360 nm of all solutions were very low (< 0.01) with no signs of protein aggregation.

Aggregation of the Reconstituted BSA in Solution

After moisture treatment and reconstitution, the samples were kept at room temperature and BSA aggregation in solution was assessed by turbidimetric assay at 360 nm as a function of time. Generally, native proteins show no absorbance at wavelengths greater than 310 nm. However, when protein aggregates are formed, the optical density at 360 nm (OD_{360}) increases due to light scattering by the aggregates.

Figure 6a shows the typical time dependencies of optical density at 360 nm for reconstituted protein solutions with/without arginine for different trehalose-to-mannitol ratios in formulations (Treh:Man 30:70, 40:60, 50:50, 60:40, 80:20). Before reconstitution, the samples were moisture treated for 24 h at 75% RH and room temperature. For all samples, the optical density at 360 nm (OD_{360}) increases with time due to formation of the protein aggregates. The appearance of aggregates was also confirmed by light microscopy. The aggregation ratios between noArg and Arg+ samples are summarised in Fig. 6b (the aggregation ratios were calculated as ratios between OD_{360} for a Arg+ sample to OD_{360} for a noArg sample with the same trehalose-to-mannitol content after 14 days of storage). The data shows that for all five formulations containing different trehalose-to-mannitol contents, the degree of protein aggregation is 2–2.5 times lower in the presence of L-arginine. Besides, the aggregation kinetic curves in Fig. 6a show a lag-period during which no aggregation was detected. For all five samples examined, the lag-periods are longer in the case of Arg+ sample. Overall, the results show that in the case of FD formulations pre-moisturised at room temperature, the presence of L-arginine in formulations leads to a significant decrease in the degree of BSA aggregation.

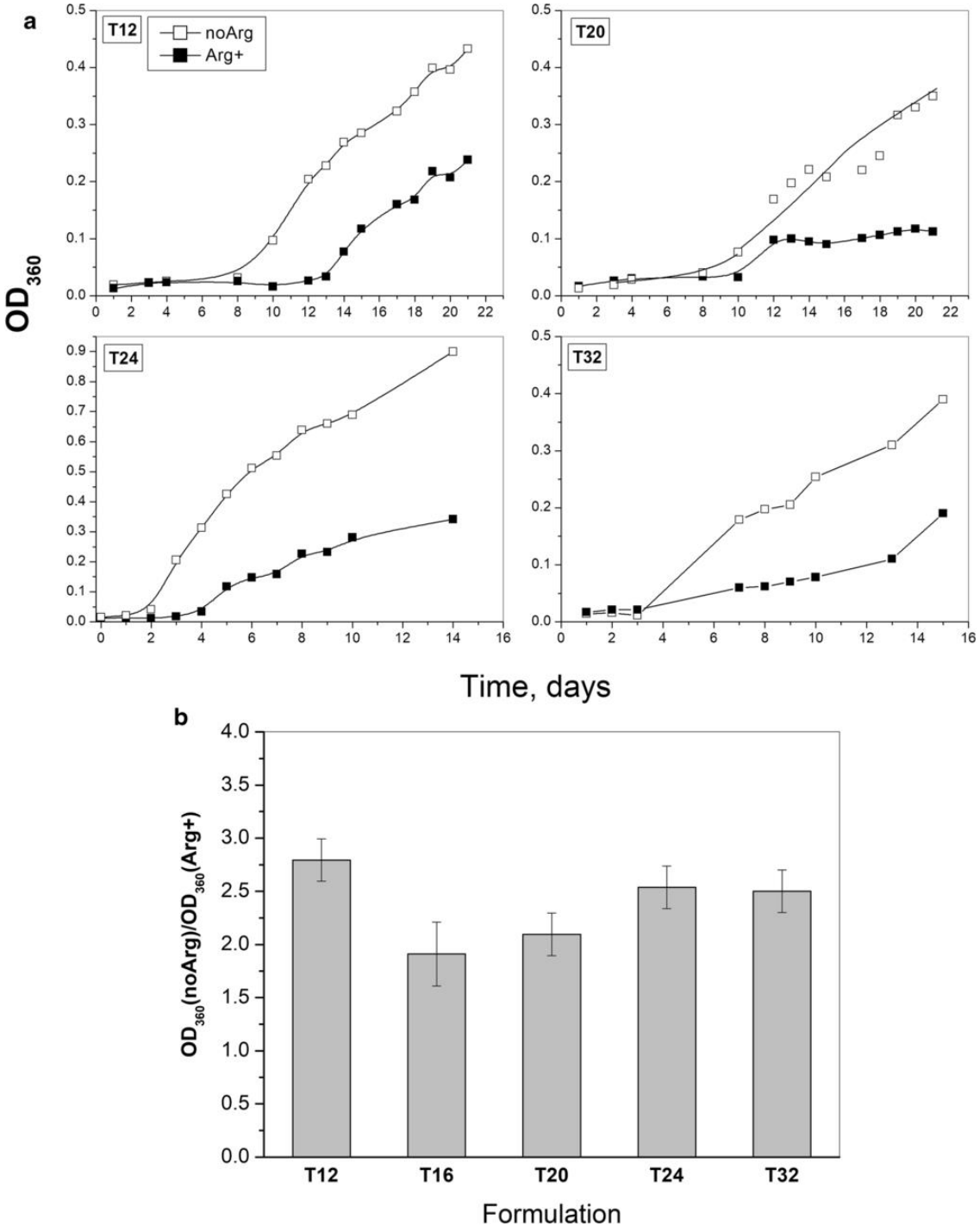

Fig. 6

Aggregation of reconstituted protein in solution. **a** Dependencies of optical density at 360 nm over time for reconstituted BSA in Tris-buffered solutions with/without l-arginine. Prior to protein reconstitution FD formulations were pre-incubated at 75% RH and 45 °C for 24 h. Formulation compositions (T12, T20, T24 and T32) are given in Table I. **b** The aggregation ratios between noArg and Arg + formulations, where OD_{360}(noArg) and OD_{360}(Arg+) are optical densities at 360 nm of the BSA-containing formulations without and with l-arginine. Reconstituted protein solutions were incubated at room temperature for 14 days before the final level of protein aggregation was taken

The presence of protein aggregates in the reconstituted samples was confirmed by fluorescence spectroscopy using Thioflavin T (ThT) assay. ThT, a fluorescent dye, binds to protein aggregates but not to protein monomer and upon binding displays an enhanced fluorescence emission and a characteristic red shift of the emission maximum (61). Typical fluorescence emission spectra of ThT alone and in the presence of native protein (BSA) in solution are shown in Fig. 7 (curves 1 and 2, 3). The intensity of the fluorescence emission of ThT is very low when no protein aggregates are present in solution. In the presence of protein aggregates, the emission intensity increases remarkably (Fig. 7, curves 4 and 5). ThT fluorescence emission is higher in the case of noArg (curve 5) than Arg+ samples (curve 4) indicating higher level of protein aggregation in the noArg formulation. The increase in the intensity of ThT emission correlates with increase in optical density at 360 nm. Thus, the ThT assay results confirm that the presence of L-arginine in a formulation decreases the level of protein aggregation in reconstituted protein-based products**.**

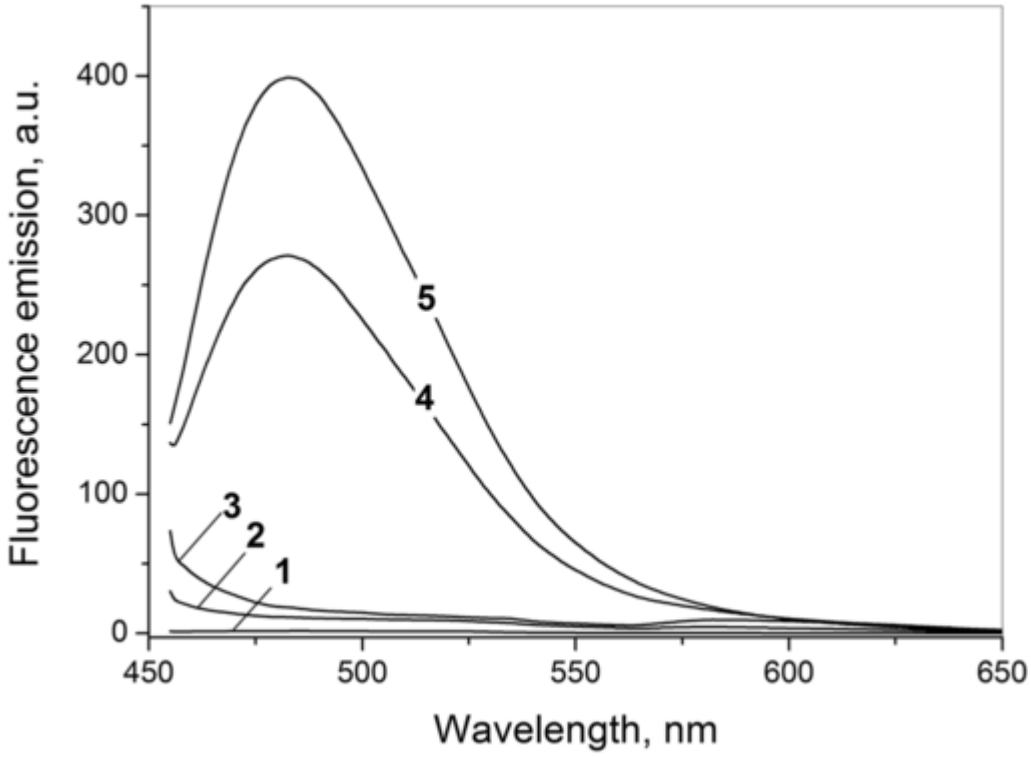

Fig. 7

Steady-state fluorescence emission spectra of ThT: (1)—alone in Tris-HCl buffer; (2)—in the presence of freshly prepared BSA; (3)—in the presence of freshly reconstituted T24 formulation; (4), (5)—in the presence of T24 Arg+ (4) and T24 noArg (5) samples incubated during 7 days at room temperature after reconstitution. All samples were prepared in 20 mM Tris-HCl buffer (pH 7.2). ThT aliquot from stock solution (1 mM) was added to the working reaction immediately before the experiment

In the case of FD formulations pre-incubated at elevated temperature (45 °C) and high relative humidity (75%) prior to reconstitution, no significant difference between BSA aggregation in the presence or absence of L-arginine in the formulations was found for four (T12, T16, T20 and T24 samples) out of five formulations studied (Fig. 8). In the case of the T32 formulation with the highest trehalose content (80:20 trehalose-to-mannitol ratio), the degree of protein aggregation was significantly higher in the presence of L-arginine (Fig. 8, bottom right). The lag-periods for T32 formulations pre-treated at 45 °C were also shorter than for other formulations.

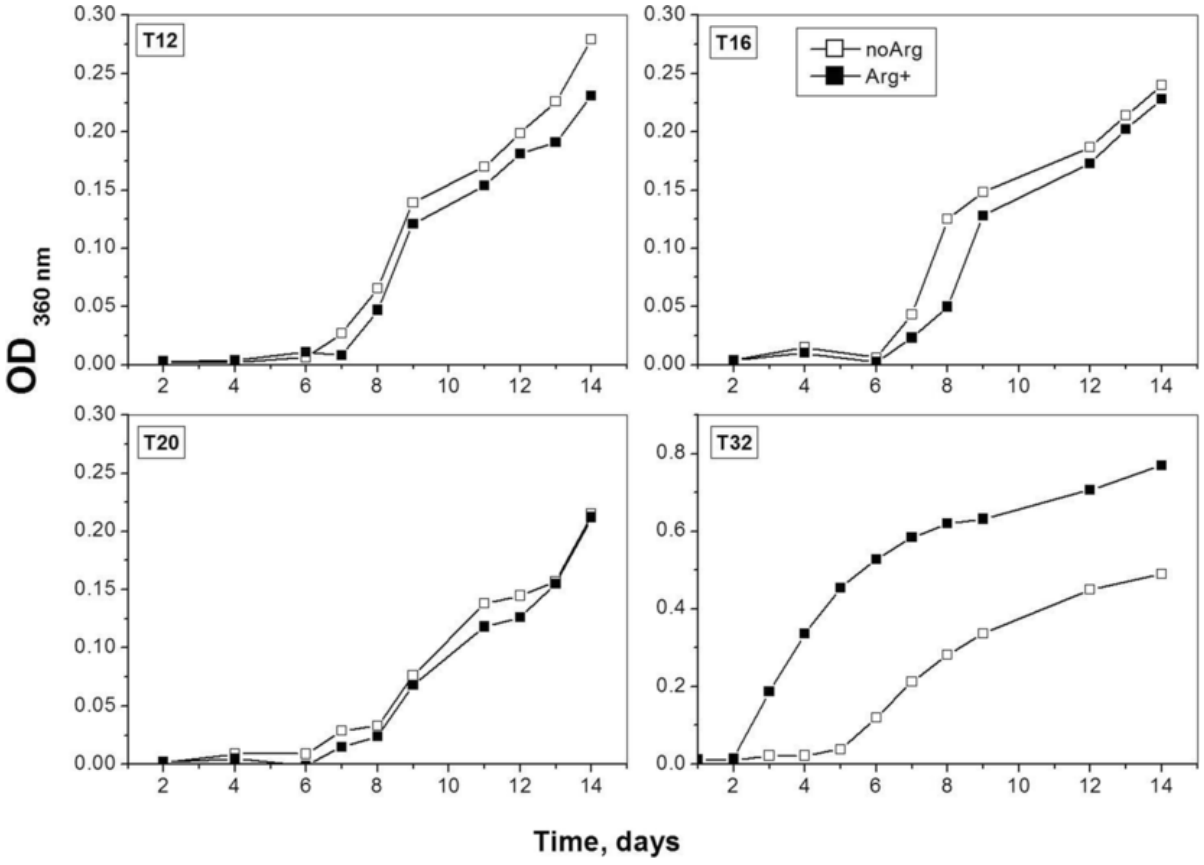

Fig. 8

Dependencies of optical density at 360 nm over time for reconstituted BSA in Tris-buffered solutions with/without L-arginine. Prior to protein reconstitution, FD formulations were pre-incubated at 75% RH and 45 °C for 24 h. Formulation compositions (T12, T20, T24 and T32) are given in Table I

Moisture treatment at elevated temperature, as we showed earlier, also caused significant alteration in the TGA moisture release profiles (Fig. 4b) and a change in the colour of the powder from white to light brown. This evidences significantly stronger changes in the state of protein as a result of pre-treatment with harsher conditions (moisture in combination with elevated temperature). In the latter case, hygroscopic L-arginine causes more damage to the protein during pre-incubation and is unable to reduce protein

aggregation (Fig. 8). Therefore, it can be concluded that the presence of L-arginine does not always reduce the extent of protein aggregation and under certain conditions arginine causes no effect or even increases aggregation. To prevent from this, high mannitol content in product is required.

L-Arginine is the most hydrophilic of the protein-building amino acids, which is able to form up to seven hydrogen bonds per molecule (37 and references therein). Being introduced into a lyophilized formulation, L-arginine somehow increases the moisture uptake by the FD powder thus raising the stress on a protein. At the same time, the arginine's ability to form hydrogen bonds is, perhaps, critical for L-arginine-induced protein stabilisation and protein aggregation suppression: according to the water replacement theory of protein thermodynamic stabilisation during lyophilisation, the stabiliser molecules replace water molecules by hydrogen bonding to the protein. One can suggest that the balance between these two effects (as well as the presence of other excipients that can diminish the moisture attraction by arginine, in our case mannitol) defines the final impact of L-arginine on the protein aggregation.

The results obtained also illustrate the importance of moisture-resistant excipients (*i.e.* mannitol) which can be combined with L-arginine in order to develop a successful protein-based pharmaceutical. Formulations containing relatively large amount of trehalose (~30–40 wt%) and small amount of mannitol (with mannitol-to-trehalose ratios below 1) were shown to be unable to reduce protein aggregation when exposed to high humidity and elevated temperatures, during pre-reconstitution storage. Large amounts of trehalose in formulations also resulted in the inhibition of mannitol crystallisation after freeze-drying and a decrease in the protein degradation temperature. However, it should be noted that in the last experiment (Fig. 8, T80 formulation (bottom right)), the lyophilized formulations were treated with the extremely severe conditions (a combination of high temperature and high humidity) which are very unlikely to be encountered in normal storage conditions. Under less harsh conditions, arginine has been found to be able to protect proteins in the dried state and to reduce the protein aggregation after reconstitution.

In the present work, a relatively wide range of trehalose concentrations were assessed; in future work, the trehalose concentration can be further adjusted with a particular protein in mind as trehalose may affect the activity of the protein-based drugs. For instance, the recovered activity of phosphofructokinase after freeze-drying was shown to be dependent on the trehalose concentration (62).

CONCLUSION

The current study has demonstrated that L-arginine included in freeze-dried formulations leads to a significant decrease in the BSA aggregation. The presence of L-arginine in freeze-dried BSA-containing cakes does not compromise the cake physical appearance.

High hygroscopicity of L-arginine can be significantly compensated by introducing mannitol into a protein formulation, as mannitol in a combination with L-arginine can strongly resist the moisture sorption.

Trehalose was introduced into the final formulation as a lyoprotectant. As a non-reducing sugar, trehalose (as well as mannitol) does not *react* with amino acids or proteins in the Maillard reaction and can be safely used in protein-based formulations. Formulations containing relatively large amount of trehalose (~30–40 wt%) were shown to be unable to reduce protein aggregation when exposed to high humidity and temperature during pre-reconstitution storage. Therefore, to develop a successful protein formulation containing L-arginine, the trehalose concentration needs to be reduced while mannitol concentration is increased, and keeping the mannitol-to-trehalose ratio above 1.

The results obtained in the present study enable the rational design and development of the future formulations suitable to protect protein-based pharmaceutics in solid state during the manufacturing and storage.

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