Freeze-drying of biological products

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Introduction

1. Instability in aqueous medium: “The major challenge”
2. Stabilization is deeply explored to reach a shelf-life of several years
3. Freeze-drying is considered to be an attractive way to achieve long-term storage stability
4. Lyophilization is a process of drying by freezing and sublimation of ice under vacuum
5. It is used to convert solutions of labile materials into solids of sufficient stability for distribution and storage.

The *steps*

1

Freezing: The first step.

The liquid suspension is cooled, and ice of water are formed. Throughout the freezing process more water contained in the liquid phase freezes. The freezing is considered the most aggressive and stressful step of freeze-drying

2

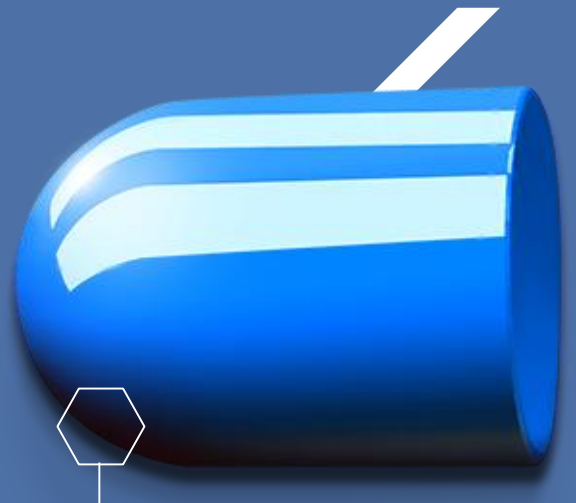
The annealing.

An annealing step is often introduced into the freez- ing protocol to allow larger ice crystals to grow at the expense of small ones in a process denoted as “Ostwald ripening.”

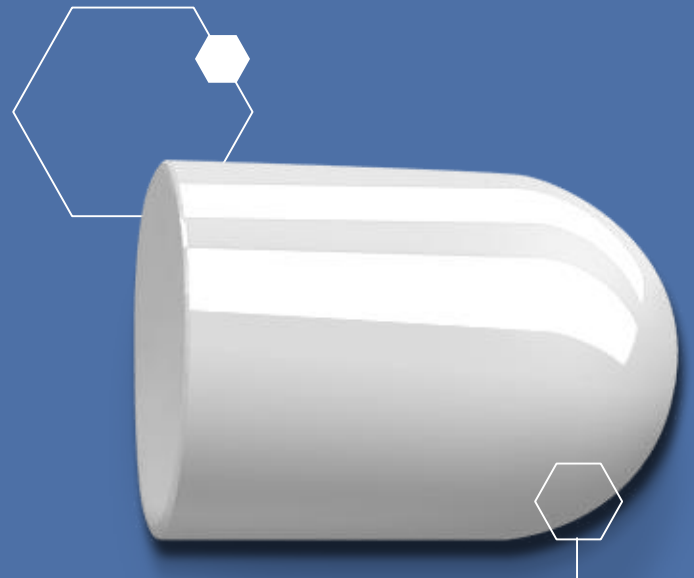
3

The dehydration steps.

The dehydration steps involve the removing of ice and unfrozen water that remains dissolved or adsorbed on the solid phase.Collapse and loosening of porous structure can happen when the product is heated above *Tc*. Bulking agents could serve as tonicifier and are used to achieve an elegant and stable non-collapsed cake.



The *bulking agents*



Sugars or sugar alcohols such as lactose, sorbitol, sucrose and mannitol are typically used as a bulking agents. Non-reducing compounds such as mannitol and sucrose are preferred for protein-based formula- tions to avoid Maillard reaction of the protein with the excipient. Amino acids can also be used as bulking agents, such as histidine, glycine, and arginine.

X

Such compounds are able to form hydrogen bonds at specific sites on the surface of proteins, and water that is lost during drying is “replaced” by these additives. A different theory proposes that stabilization is related to immobilization of the protein in a rigid and inert glass matrix, and therefore the protein molecules would remain preserved in their original state.

Mannitol

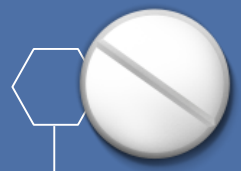
Mannitol may crystallize partially during lyophilization and crystallization may continue after freeze-drying as a result of moisture and heat. Mannitol crystallization may be inhibited by the presence of additives, high cooling rates, or primary drying at low temperature. However, there are situations where it is desirable to have crystalline mannitol as a bulking agent in a freeze-dried product, since the existence of crystalline mannitol in the final lyophile yields elegant cakes.

"The more promising approach is the usage of mannitol in combination with an amorphous lyoprotector."

Only in the amorphous state mannitol can stabilize proteins.

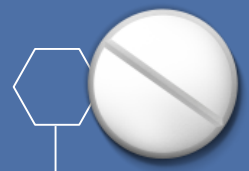
The

freeze-drying cycle



1

Freeze-drying microscopy



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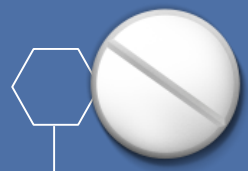
Differential scanning calorimetry



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X-ray diffraction

4

Scanning electronic microscopy

5
Water content analysis

6
Stability study

Freeze-drying of *biological products*

Table 1. Characterization of Ova-6-*O*-CMC nanocapsules suspension before and after freeze-drying in the presence of different cryoprotectants.

Cryoprotectant	Concentration (%)	Size ± SD (nm)		PI ± SD		S_F/S_I			
		Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying	After freeze-drying	Aspect	Time (s)	RH (%)
Without cryoprotectant	–	169.0 ± 1.0	***	0.126 ± 0.020	***	***	Collapsed	***	4.10
Mannitol	5	167.9 ± 1.9	238.9 ± 10.1	0.171 ± 0.003	0.240 ± 0.010	1.40	Correct	Immediate	1.13
Mannitol	10	195.4 ± 1.2	212.2 ± 2.6	0.176 ± 0.020	0.207 ± 0.007	1.08	Correct	Immediate	1.30
Sucrose	5	160.9 ± 0.5	203.2 ± 0.8	0.149 ± 0.006	0.178 ± 0.014	1.26	Partially collapsed	Immediate	1.01
Sucrose	10	182.0 ± 1.8	262.0 ± 0.4	0.151 ± 0.005	0.228 ± 0.014	1.44	Partially collapsed	Immediate	2.31
Glucose	5	161.2 ± 1.6	212.9 ± 1.8	0.164 ± 0.013	0.296 ± 0.018	1.32	Collapsed	60 s	1.21
Glucose	10	180.9 ± 1.2	201.1 ± 11.3	0.169 ± 0.013	0.252 ± 0.023	1.11	Collapsed	240 s	2.63
Lactose	5	160.9 ± 1.6	253.6 ± 1.9	0.152 ± 0.019	0.188 ± 0.014	1.58	Partially collapsed	Immediate	4.09
Lactose	10	181.1 ± 0.8	203.3 ± 1.1	0.152 ± 0.008	0.154 ± 0.013	1.12	Partially collapsed	Immediate	5.98
Mannitol-PVP	10–1.25	865.5 ± 55.1	1355.3 ± 24.0	0.511 ± 0.025	0.377 ± 0.077	1.56	Collapsed	Immediate	0.64
Mannitol-PVA	10–1.25	483.9 ± 8.8	384.6 ± 19.1	0.575 ± 0.053	0.727 ± 0.060	0.79	Correct	Immediate	11.60
Mannitol-Poloxamer	10–1.25	141.7 ± 2.7	104.6 ± 6.7	0.752 ± 0.015	0.998 ± 0.002	0.74	Correct	Immediate	1.92
Mannitol-PEG 4000	10–1.25	291.2 ± 4.6	562.7 ± 7.5	0.175 ± 0.020	0.294 ± 0.008	1.93	Correct	Immediate	12.62

***Aggregated suspension.
The results of nanocapsules size and polydispersity index are means of three measurements ± SD.

Freeze-dried cake with mannitol 10% (w/v) observed by SEM showed the conservation of porous structures and demonstrated a successful freeze-drying of nanocapsules suspension.

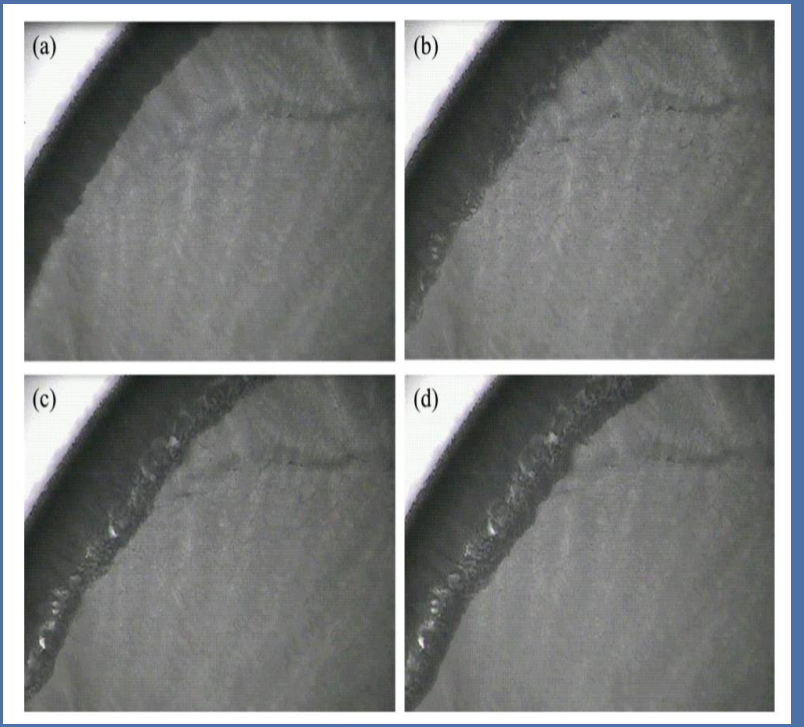
Stabilization during *dehydration*

The comparison between glass transition temperature *T'g* and *Tc* values for differ ent formulations pointed out two behaviors depending on the nature of the bulking agent used.

Table 2. Experimental determinations of collapse temperature (T_c), glass transition temperature (T'_g), and crystallization temperature (T_{cr}) of Ova-6-*O*-CMC nanocapsules suspension in presence of different cryoprotectants.

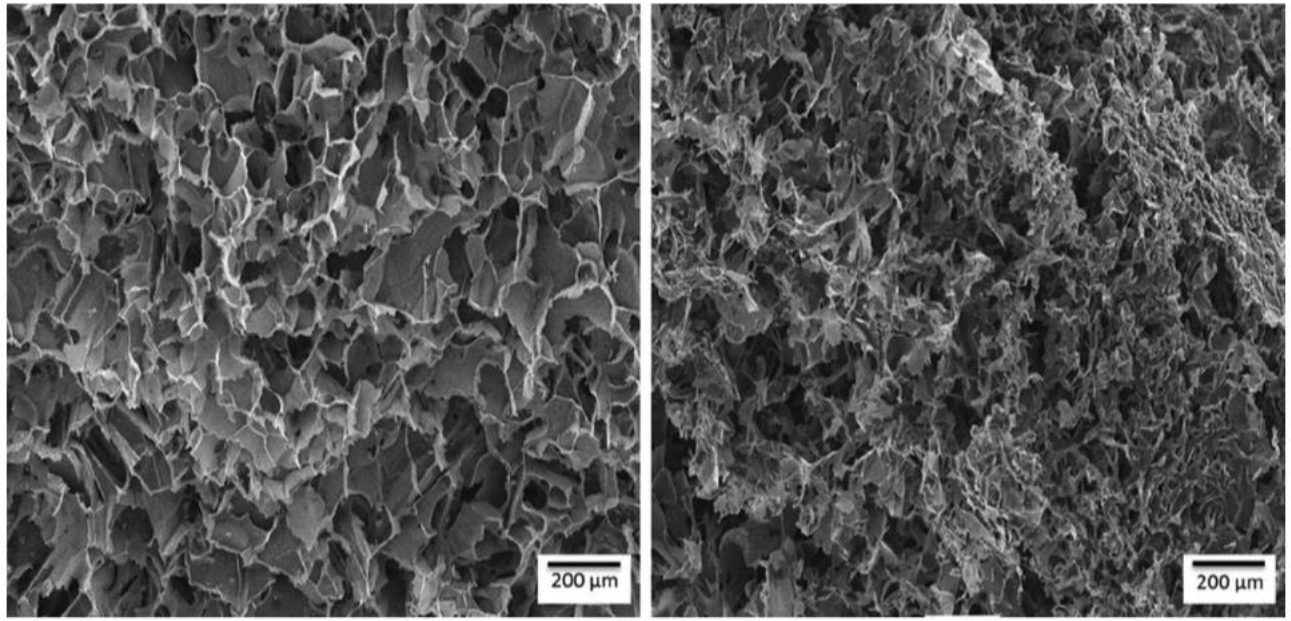
Cryoprotectant	Concentration (%)	T_c[a] (°C)	T'_g[b] (°C)	T_c[b] (°C)
Without cryoprotectant	–	−53.50	−55.13	–
Mannitol	5	−27.90	−34.90	−21.10
Mannitol	10	−26.10	−33.94	−19.50
Sucrose	5	−26.40	−35.89	–
Sucrose	10	−26.60	−34.77	–
Glucose	5	−41.80	−39.53	–
Glucose	10	−39.18	−38.83	–
Lactose	5	−31.40	−43.84	–
Lactose	10	−31.80	−37.62	–
Poloxamer	2.5	−30.80	−21.54	–
PEG 4000	2.5	−34.90	−20.20	–
PVP	2.5	−30.80	−30.45	–
PVA	2.5	−10.50	−27.00	–
Mannitol-PVP	10–2.5	−5.10	−33.70	−20.80
Mannitol-PVA	10–2.5	−2.40	−32.93	−21.00
Mannitol-Poloxamer	10–2.5	−26.00	−34.52	−23.70
Mannitol-PEG 4000	10–2.5	−26.00	−34.58	−25.80

[a]Determined by freeze-drying microscopy.
[b]Determined by DSC.



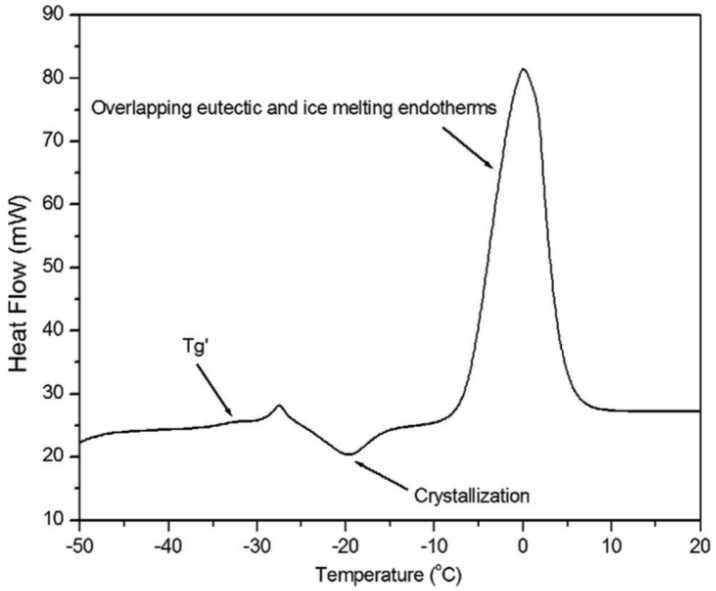
The collapse temperature can be defined as a more relevant parameter than *T'g* for freeze-drying development and optimization

Mannitol formulations had a leafy amorphous appearance with an irregular array of pores whereas formulations without cryoprotectant presents holes in the structure which indicates the collapse of the dried product.



Collapse increased the residual humidity for formulations freeze-dried with sucrose, glucose, and lactose which can impact the product quality.

Thermal analysis of mannitol frozen formulations: Influence of rates of cooling and ***temperatures of annealing***



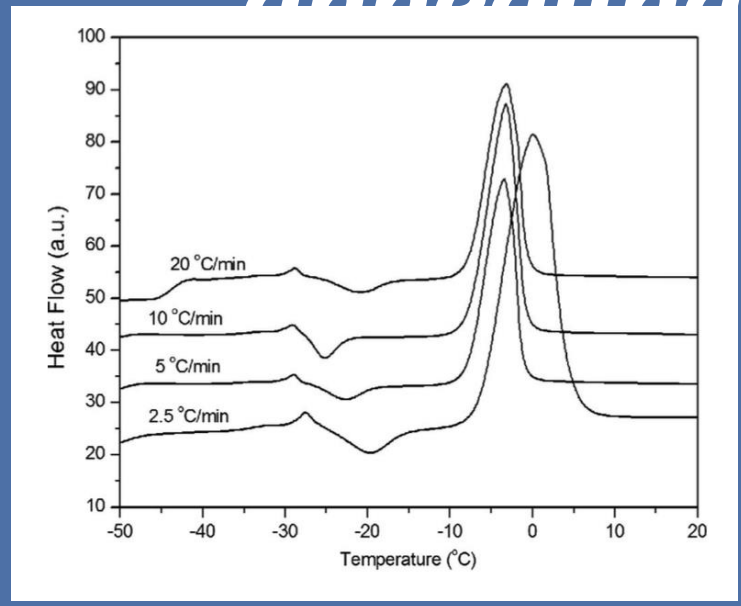


Table 3. Experimental determinations of collapse temperature (T_c), glass transition temperature (T'_g), and crystallization temperature (T_{cr}) of Ova-6-*O*-CMC nanocapsules prepared with mannitol 10% (w/v) with different rates of cooling.

Rates of cooling (°C/min)	T_c [a] (°C)	T'_g [b] (°C)	T_{cr} [b] (°C)	ΔH (J/g)
2.5	−26.10	−35.13	−19.00	6.68
5.0	−25.80	−35.24	−22.68	7.97
10.0	−25.90	−35.05	−25.31	8.95
20.0	−25.90	−35.65	−21.03	10.95

[a]Determined by freeze-drying microscopy.
[b]Determined by DSC.

"During freeze-drying mannitol tends to crystallize in the α, β, δ, or as mannitol hydrate depending on the process conditions applied during freezing, primary and secondary drying and thereby looses the ability to stabilize the protein or nanocapsules within the glassy matrix."

"Annealing facilitates ice crystallization resulting in supersaturation of mannitol followed by its nucleation. Therefore, the presence of nuclei in the annealed samples would be responsible for the crystallization exothermic of mannitol at a lower temperature."

"Annealing procedure resulted in ice crystallization, decreased the unfrozen water content in the amorphous freeze-concentrate, and increased the glass transition temperatures."

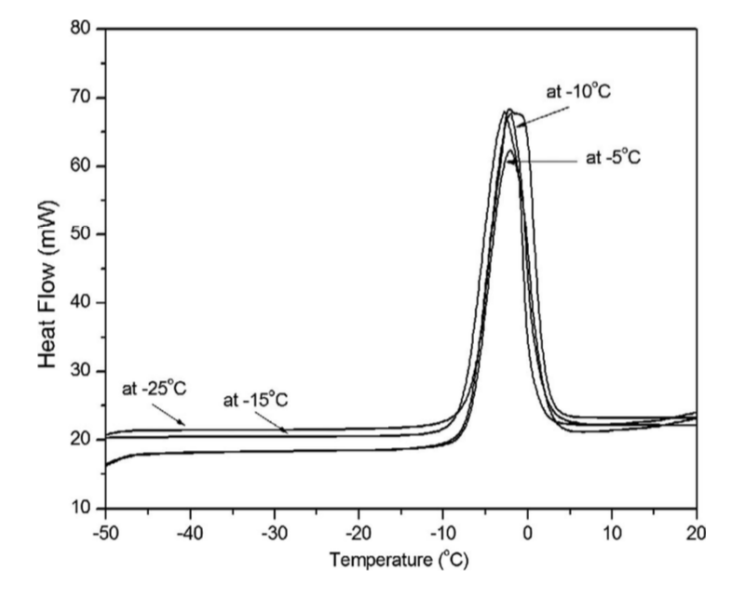


Table 4. Experimental determinations of collapse temperature (T_c), glass transition temperature (T'_g), and crystallization temperature (T_{cr}) of Ova-6-*O*-CMC nanocapsules prepared with 10% (w/v) of mannitol with different temperatures of annealing.

Temperature of annealing (°C)	T_c [a] (°C)	$T'_{g\,1}$ [b] (°C)	$T'_{g\,2}$ [b] (°C)	T_{cr} [b] (°C)	ΔH (J/g)
−5.0	−9.20	−33.95	−25.96	−23.88	−11.07
−10.0	−9.10	−34.60	−23.88	−23.23	−11.96
−15.0	−8.80	−34.95	−29.33	–	–
−25.0	−8.60	−34.89	−32.18	–	–
No annealing	−26.10	−35.13	−35.13	−19.00	−6.68

[a]Determined by freeze-drying microscopy.
[b]Determined by DSC.

Characterization of the freeze-dried formulations with mannitol: influence of ***rates of cooling and temperatures of annealing***

"A suggested stabilization mechanism by lyoprotectants is the water replacement hypothesis. This mechanism supposes the formation of hydrogen bonds between polar groups at the nanoparticles surface and a lyoprotectant at the end of the drying process."

"If the annealing temperature is above *T'g*, ice will melt and smaller ice crystals will melt faster than larger one. It has been found that aboveT0g molecular relaxation timesdecrease exponentially with (*T* – *T'g*)."

Table 5. Freeze-drying study of Ova-6-*O*-CMC nanocapsules prepared at mannitol 10% (w/v) with different rates of cooling.

		Size ± SD (nm)		PI ± SD		S_F/S_I
Cooling procedure	Final temperature (°C)	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying	After freeze-drying
In liquid nitrogen	−196	213.2 ± 1.4	222.5 ± 1.6	0.133 ± 0.011	0.184 ± 0.018	1.04
Acetone/dry ice bath	−78	210.1 ± 2.8	223.7 ± 2.9	0.141 ± 0.006	0.176 ± 0.004	1.06
Freeze dryer shelf	Ramp at 2.5°C/min to −50°C	205.7 ± 1.3	220.5 ± 1.5	0.126 ± 0.010	0.168 ± 0.010	1.07
	Ramp at 1.0°C/min to −50°C	202.4 ± 1.4	217.5 ± 1.9	0.132 ± 0.014	0.177 ± 0.017	1.07

The results of nanocapsules size and polydispersity index are means of three measurements ± SD.

Table 6. Freeze-drying study of Ova-6-*O*-CMC nanocapsules prepared at mannitol 10% (w/v) with different annealing temperatures.

Temperature of annealing (°C)	Size ± SD (nm)		PI ± SD		S_F/S_I
	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying	After freeze-drying
−25.0	204.5 ± 1.0	232.5 ± 2.5	0.117 ± 0.012	0.135 ± 0.010	1.14
−15.0	208.4 ± 1.1	212.4 ± 1.2	0.106 ± 0.010	0.178 ± 0.010	1.01
−10.0	210.3 ± 1.1	256.5 ± 3.7	0.125 ± 0.012	0.240 ± 0.014	1.22
−5.0	208.0 ± 1.2	261.8 ± 9.9	0.119 ± 0.017	0.276 ± 0.060	1.26

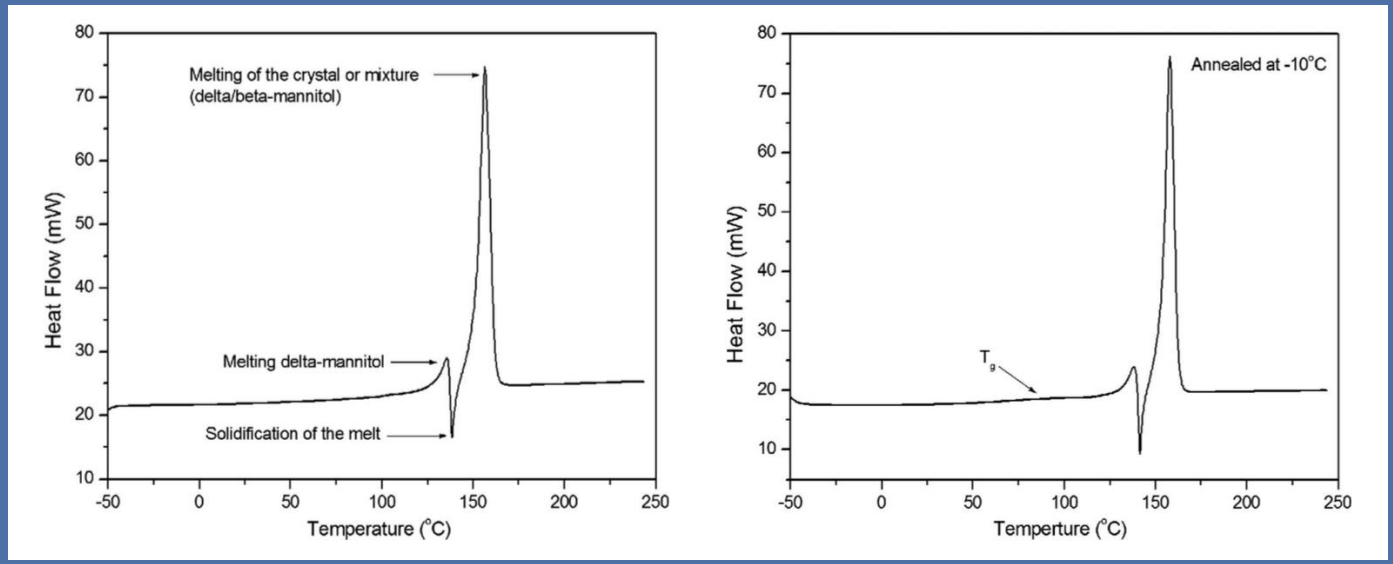
The results of nanocapsules size and polydispersity index are means of three measurements ± SD.

Effect of ***annealing procedure*** on termal properties of the freeze-dried formulations with mannitol

Table 7. Glass transition temperatures (T_g) of freeze-dried Ova-6-*O*-CMC nanocapsules prepared with mannitol 10% (w/v) with different annealing temperatures measured by DSC at 20°C/min.

Process	Annealing procedure	T_g (°C)[a]
I	Without annealing	58.84
II	−25.0	68.48
III	−15.0	68.35
IV	−10.0	72.81
V	−5.0	61.02

[a]Determined by DSC.



1 It was reported that the glass transition decreases markedly as the relative concentration of mannitol increases demonstrating the plasticizer effect of mannitol.

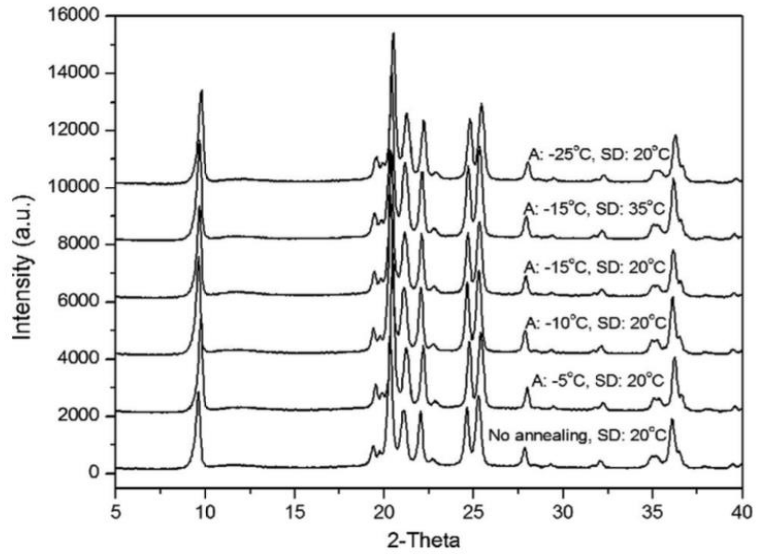
2 For soluble interleukin receptor (sIL-13r) formulations from 0 to 9.2 mg/ml the glass transition temperature of the mobile phase increases as a function of protein concentration. Polymorphic crystalline mannitol (a mixture of mannitol hydrate, β-, and δ-mannitol) converts to δ-mannitol for sIL-13r.

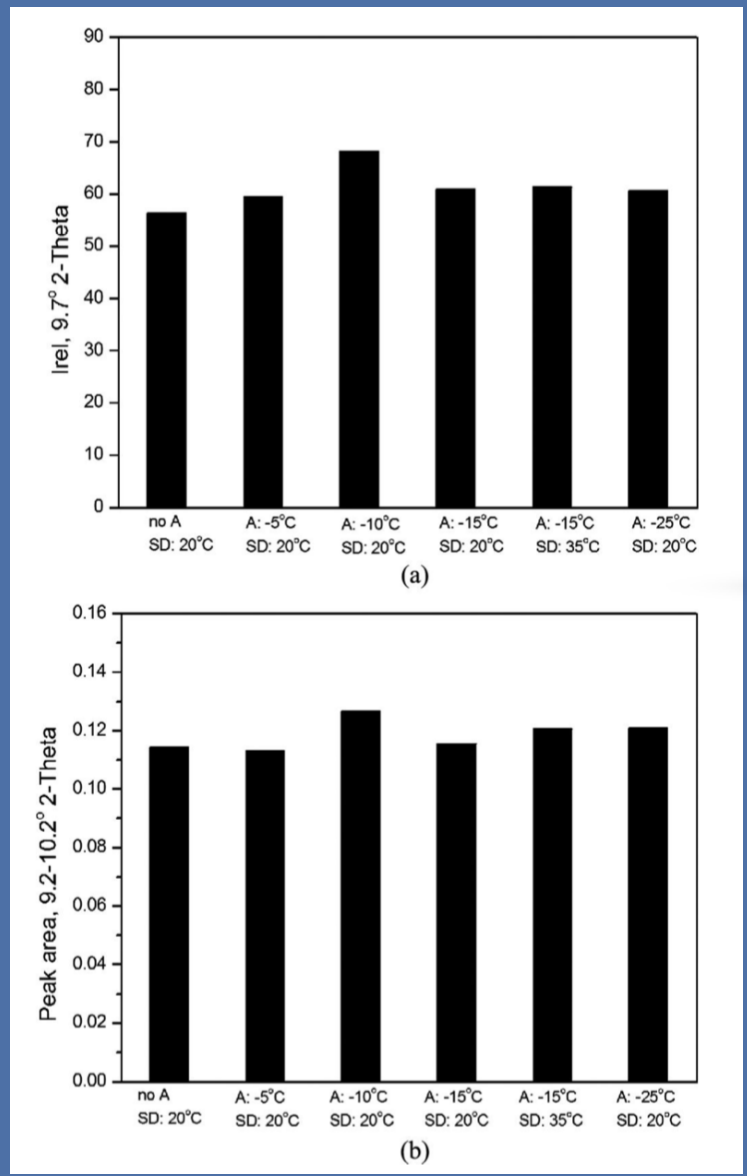
3 Comparable results have been identified for human serum albumin (HAS). It was showed that a stable amorphous state can be achieved by increasing the fraction of stabilized-HSA to 1.5%, where mannitol remains amorphous after freeze-drying and 24 months at 25°C/60% RH.

XRD of lyophilized formulations produced with different ***annealing temperatures***

Table 9. Freeze-drying conditions used for the production of Ova-6-*O*-CMC nanocapsules formulations prepared at mannitol 10% (w/v).

Process	Annealing procedure	Secondary drying shelf temperature (°C)
I	Without annealing	20°C
II	−25.0	20°C
III	−15.0	20°C
IIIa	−15.0	35°C
IV	−10.0	20°C
V	−5.0	20°C





XRD of lyophilized formulations produced with different ***annealing temperatures***

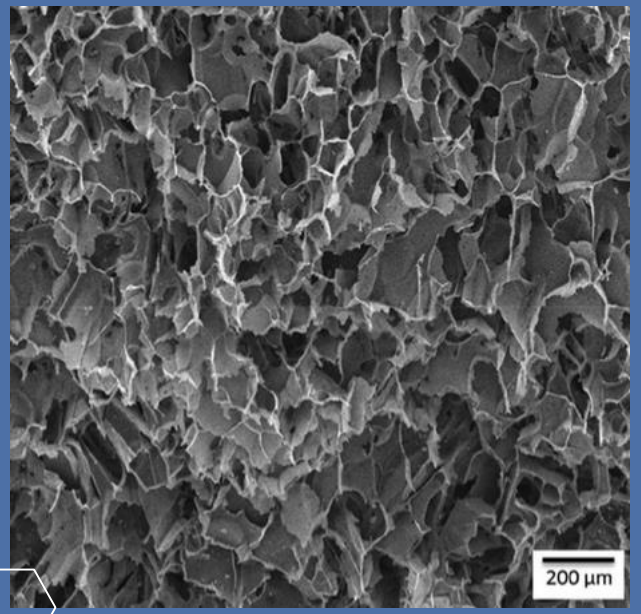
In case of mannitol it can crystallize in the α-, β-, δ-modification or as mannitol hydrate depending on the applied freezing protocol and the process conditions during primary and secondary drying. It was reported a crystallization of mannitol in a metastable hydrate form during lyophilization and freezing.

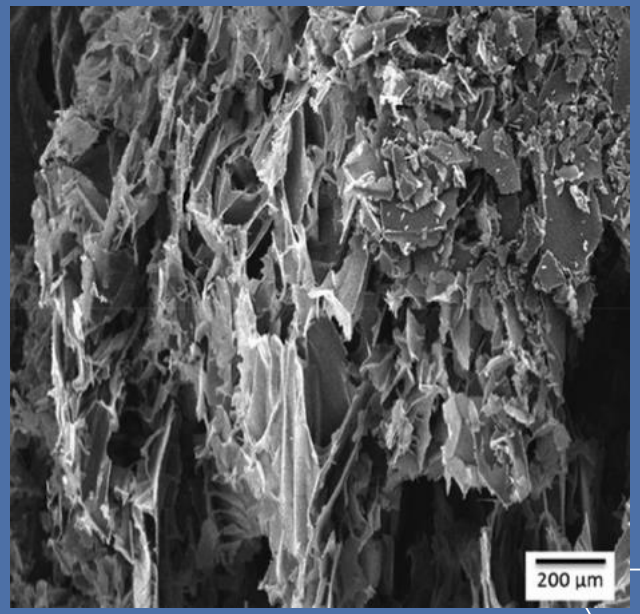
The peak area and the relative intensity of the δ-mannitol at 9.7° 2θ were used to compare de amount of δ-mannitol formed after different lyophilization cycles. For the relative intensity, the peak area of the δ-mannitol was compared to the highest peak area of the diffraction pattern, that was set as 100%.

Comparing the different annealing and secondary drying temperatures the formation of δ-mannitol was more pronounced at - 10°C annealing temperature. Compared to the effect of annealing the secondary drying temperature did not have influence on the formation of δ-mannitol when the tem- perature was increased from 20 to

Once it is essential to ensure the mannitol crystalliza- tion, the annealing process is often performed for formulations containing mannitol as a bulking agent to maximize mannitol crystallization during the freezing step. In addition, it is well known that the presence of some excipients, such as buffer components, proteins, lyoprotectants. and salts can promote mannitol crystallization.

Effect of ***annealing on porosity*** of freeze-dried product





Annealing eliminates variation in initial ice crystal size distribution induced by variable temperatures of nucleation and the resulting heterogeneity in drying rates due to the size-dependence of the phase behavior and ripening process.

X

According to Searles et al. annealing increased the ice crystals size by Ostwald repining. If the annealing temperature is above *T'g* ice will melt faster than larger ones, and the smallest ice crystals may melt completely. Ostwald ripening (recrys- tallization) is a phenomenon by which dispersed crystals smaller than a critical size decrease in size whereas those larger ones grow.

The *freeze-drying* conditions

Table 8. Pore equivalent surface area of Ova-6-*O*-CMC nanocapsules prepared at mannitol 10% (w/v) of without annealing and at four different annealing temperatures.

Process	Annealing procedure	*A* equivalent (μm)
I	without annealing	75.16 ± 26.1
II	−25.0	72.72 ± 24.0
III	−15.0	71.96 ± 25.0
IIIa	−15.0	75.29 ± 24.1
IV	−10.0	70.19 ± 25.3*
V	−5.0	79.29 ± 22.9

Data are present as the mean ± SD ($n = 3$ for pore surface area determination).
*$P < 0.05$ relative to the corresponding sample without annealing.

For annealed samples the δ-mannitol crystals randomly distributed throughout the cake block the pathways for water vapor diffusion and create a larger resistance to vapor flow, compared with the cake with large connected pores characteristic of the unannealed samples.

The rigid crystalline structure of the sugar alcohol provides an excellent matrix to support the cake structure after ice sublimation, avoiding collapse of the cake when the sample temperature exceed the glass transition temperature of the amorphous phase.

Annealing eliminates variation in initial ice crystal size distribution induced by variable temperatures of nucleation and the resulting heterogeneity in drying rates due to the size-dependence of the phase behavior and ripening process.

Annealing increased the ice crystals size by Ostwald repining. If the annealing temperature is above *T'g* ice will melt faster than larger ones, and the smallest ice crystals may melt completely. Ostwald ripening (recrys- tallization) is a phenomenon by which dispersed crystals smaller than a critical size decrease in size whereas those larger ones grow.

Stabilization of nanocapsules in dried state ***during the storage***

"Nanoparticles can be stored for a suficiente period of time with the conservation of their size and polydispersity if an amorphous excipient is present. The crystallization of this bulk lyoprotectant can destabilize the nanoparticles inducing their aggregation. However, adding special additives may delay the nucleation event which start the crystallization process."

Table 10. The size of nanocapsules, the residual humidity RH (%), and the aspects of freeze-dried Ova-6-*O*-CMC nanocapsules prepared at mannitol 10% (w/v) before and after three months of storage at 25°C.

Temperature of annealing (°C)	Before storage			After storage for 3 months at 25°C		
	Size of NCs (nm)	RH (%)	Aspect	Size of NCs (nm)	RH (%)	Aspect
−25	211.5 ± 5.3	0.76 ± 0.02	Correct	287.5 ± 6.8	1.05 ± 0.05	Correct
−15	217.4 ± 2.6	0.55 ± 0.03	Correct	279.1 ± 3.4	0.63 ± 0.03	Correct

The results of nanocapsules size and residual humidity are means of three measurements ± SD.

Table 11. The size of nanocapsules, the residual humidity RH (%), and the aspects of freeze-dried Ova-6-*O*-CMC nanocapsules prepared at mannitol 10% (w/v) before and after three months of storage at 4°C.

Temperature of annealing (°C)	Before storage			After storage for 3 months at 4°C		
	Size of NCs (nm)	RH (%)	Aspect	Size of NCs (nm)	RH (%)	Aspect
−25	211.5 ± 5.3	0.76 ± 0.02	Correct	251.9 ± 1.7	0.86 ± 0.04	Correct
−15	217.4 ± 2.6	0.55 ± 0.03	Correct	241.6 ± 5.3	0.56 ± 0.03	Correct

The results of nanocapsules size and residual humidity are means of three measurements ± SD.

Conclusions

1. Nanocapsules can be stabilized during freeze-drying process when using adequate concentrations of suitable excipients.
2. Mannitol in concentration of 10% (w/v) was well suited to preserve nanocapsules from aggregation during lyophilization and subsequent reconstitution.
3. The physical state of manitol in the freeze-dried products is affect by both formulation and processing parameters and it should be recognized when carrying out process validation studies intended to identify processing variables.
4. The the presence of mannitol hemihydrate in the final lyophilized product is undesirable because it can dehydrate during product storage
5. The addition of na annealing step shifted the *T'g* and *Tc* of manitol formulations to higher temperatures.
6. Annealing has an impact on porosity of freeze-dried cake by nearly complete crystallization of mannitol, once the crystalline matrix prevents the partial collapse and the formation of larger pores observed without annealing.

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