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Research paper

Investigation of nanocapsules stabilization by amorphous excipients during freeze-drying and storage

Wassim Abdelwahed, Ghania Degobert *, Hatem Fessi

Université Claude Bernard Lyon 1, Villeurbanne cedex, France

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Abstract

Freeze-drying was recently applied to improve the long-term storage stability of nanoparticles. Nanocapsules have a thin polymeric envelope that may not withstand the stresses of such process. So, cryoprotectants and lyoprotectants are usually added to the formulation to protect these vectors during freezing and desiccation steps. The aim of this paper was to investigate the importance of the vitrification of cryoprotectants on the stabilization of nanocapsules during freezing, desiccation, and storage steps. Furthermore, the effect of stabilizer crystallization on the conservation of nanocapsules properties was studied. Finally, the effect of temperature storage and relative humidity on the stability of nanocapsules was tested through an accelerated stability study. Results indicate that nanocapsules stabilization during the different steps of freeze-drying requires their dispersion within a vitrified matrix of amorphous excipient to protect them against the stress of freezing and dehydration. The crystallization of this stabilizer during the freezing, the desiccation or the storage steps can destabilize these fragile particles. Electron spectroscopy for chemical analysis revealed the adsorption of nanocapsules at the interface ice/liquid during the freezing step. Such adsorption must be avoided in the case of freeze-drying of immuno-nanoparticles to preserve the native structure of proteins attached to their surface.

Keywords: Freeze-drying; Nanocapsules; Vitrification; Crystallization; Cryoprotectant; Electron spectroscopy for chemical analysis

1. Introduction

Freeze-drying is an attractive method to enhance the physico-chemical stability of colloidal vectors such as liposomes [1] and nanoparticles [2]. These particulate systems are currently well investigated to deliver the drug to a target site and thus increase the therapeutic benefit [3].

The major obstacle that limits the use of such vectors is their instability in an aqueous medium. Aggregation and fusion of particles are frequently noticed after a long period of storage of such systems [4]. So, the conservation of colloidal vectors for a sufficient period of time in dried state was well explored.

Freeze-drying is a complex drying process employed to convert solutions of labile materials into solids of sufficient stability for distribution and storage. This process removes water from a frozen sample by sublimation and desorption under vacuum [5]. Nanocapsules have a thin polymeric envelope that may not withstand the stresses of such process [4]. So, cryoprotectants and lyoprotectants are usually added to the formulation to protect the nanocapsules during freezing and desiccation steps.

In this paper, we investigate the importance of vitrification of cryoprotectants on the stabilization of nanocapsules during freezing, desiccation, and storage steps. Furthermore, the effect of stabilizer crystallization on the conservation of nanocapsules properties was studied. Finally, the effect of temperature storage and relative humidity on the stability of freeze-dried nanocapsules was tested through an accelerated stability study.

* Corresponding author. Laboratoire d'Automatique et de Génie des Procédés (LAGEP), UMR-CNRS 5007, CPE Lyon, ISPB, Université Claude Bernard Lyon 1, 43, Boulevard du 11 Novembre 1918, 69622 Villeurbanne cedex, France. Tel./fax: +33 472 431 874.
E-mail address: degobert@lagep.cpe.fr (G. Degobert).

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2. Materials and methods

2.1. Materials

Poly(epsilon-caprolactone) (PCL) (Mw: 14,000 g/mol) and hydroxypropyl β-cyclodextrin (HPβCD) were obtained from Sigma–Aldrich (France). Polyvinyl alcohol (PVA) Moviol 4-88 (88% hydrolysed, Mw 31,000 g/mol) was purchased from Clariant (France). Miglyol 829 was supplied by Condea chemie (Germany). Ethyl acetate was obtained from Carlo Erba, (France). D(+)-Sucrose was from prolabo (France). D(+)-Anhydrous glucose and D-mannitol were from Flucka biochemika (Switzerland). Polyvidon 25 (PVP) was purchased from Merck (Germany). All other reagents were of analytical grade and water was purified by Alpha-Q ultra-pure water system (Millipore, Ireland).

2.2. Preparation of nanocapsules

Nanocapsules (NC) studied in this work were prepared by the emulsification-diffusion method [6]. Briefly, 0.2 g PCL and 0.5 g miglyol 829 were dissolved in 20 mL ethyl acetate saturated with water. This organic phase was then emulsified with 40 mL of aqueous phase saturated with ethyl acetate and containing 2 g PVA using a high speed homogeniser (ultra-turax T 25, Ika Germany) at 8000 rpm for 10 min. Two hundred milliliters of deionised water was then added to the emulsion to induce the diffusion of ethyl acetate into the continuous phase leading to the formation of nanocapsules. The organic solvent and a part of water were evaporated under reduced pressure to get a concentrated suspension of 40 mL.

An additional purification step was applied to eliminate the free stabilizer in solution. This step was carried out by washing nanocapsules twice using deionised water after their separation via ultra-centrifugation at 50,000 rpm and 20 °C for 30 min. The purified nanocapsules were re-suspended in deionised water.

2.3. Particle size measurement

The size of nanocapsules both before and after lyophilization was determined by photon correlation spectroscopy (PCS) using Zetasizer 3000 HSa (Malvern, England) at 25 °C. Each measurement was performed in triplicate.

2.4. Freeze-drying of NC

Cryoprotectants used in this study were sucrose, glucose, hydroxypropyl β-cyclodextrin, polyvinyl pyrrolidone, and mannitol. The lyophilization of nanocapsules was performed using a pilot freeze-dryer Usifroid SMH45 (Usifroid, France). The conditions applied during our study were the same as described previously [7]: freezing for 2 h at −45 °C with a cooling profile of 1 °C/min, sublimation at a shelf-temperature −15 °C and pressure 100 μbar for 15 h and finally, secondary drying at 25 °C and 50 μbar for 6 h. The end of sublimation step was determined using an electronic moisture sensor installed inside the lyophilization chamber (Panametrics moisture sensor, USA). Under our conditions of freeze-drying, the product temperature during the sublimation was well above the collapse temperature of both sucrose and glucose. These conditions have been chosen to investigate the effect of cake collapse on the stability of nanocapsules.

0.5 mL of nanocapsules suspension and 0.5 mL of cryoprotectant solution were filled into 5 mL freeze-drying vials (Fisher Bioblock Scientific, France) to have a final cryoprotectant solution of 5% w/v [7].

2.5. Scanning electronic microscopy (SEM)

Sample preparation for the observation of porous structure of freeze-dried plugs was performed as follows: the complete freeze-dried plug was extracted from the glass vial and rapidly cut into pieces using a single-edged razor blade. Whole plugs or pieces were attached to SEM specimen mounts using silver paint. The specimens were coated with gold/palladium with a cathodic pulverizer technics Hummer II (6 V–10 mA). Imaging was realized on a FEG Hitachi S800 SEM at an accelerating voltage of 15 kV.

2.6. Environmental scanning electronic microscopy (ESEM)

ESEM imaging was performed on a Philips electron optics ESEM XL 30 at a pressure of 5.33 mbar, a temperature of 6 °C and an accelerating voltage of 15 kV. No sample preparation is needed for this technique.

2.7. Electron spectroscopy for chemical analysis (ESCA)

ESCA was used to probe the elemental composition of the powder surfaces with an analysis depth of less than 10 nm. The ESCA measurements were performed with an ESCALAB 200 R – VG Scientific USA. The instrument uses a monochromatic Al Kα X-ray source. The pressure in the vacuum chamber during analysis was less than 10^{-8} mbar. The analyzed surface was 1 mm^2. The kinetic energy of the electrons coming from the first atomic layers of the solid is measured through hemispherical analyzer with energy of analysis of 50 eV. The freeze-dried cake was gently crushed before taking samples for ESCA. Each sample was maintained by a double faced conducting adhesive.

2.8. X-ray diffraction (XRD)

Sample crystallization was studied by a X-ray diffractometer system type siemens D500 operated with Cu Kα X radiation at 40 kV and 30 mA. The scans were conducted in the 2θ range from 3° to 35°. Identification of the product was carried out by comparing the diffraction pattern of the sample with library data in the powder diffraction file (Diffrac plus software).

2.9. Thermal analysis

To measure the glass transition temperature of maximally cryo-concentrated suspensions ($T_{g'}$), a thermal analysis was performed by a differential scanning calorimeter DSC TA 125 (TA instrument USA). A heating rate of 10 °C/min was applied throughout the analysis in the (−100 to 30 °C) temperature range. The instrument was calibrated with indium for melting point and heat of fusion. The crystallization of mannitol was studied at a heating rate of 5 °C/min in the range of −100 to 30 °C.

2.10. Freeze-drying cryostage

The collapse temperature (T_c) was determined for the different formulations by a freeze-drying microscope (Linkam, England) equipped by a video camera and a computer to capture the collapse image. The equipment consists of a small freeze-drying chamber containing a temperature-controlled stage, a vacuum pump to ensure the evacuation and an optical window through which the drying sample can be observed by a microscope.

2.11. Accelerated stability study

The accelerated stability study was carried out according to ICH Guidelines (1993). Sealed vials of freeze-dried nanocapsules stabilized with 5% sucrose, 5% PVP, and 5% glucose were stored over a saturated solution of NaCl which produces 75 ± 5% relative humidity in closed oven at 40 °C ± 2 °C for 6 months. Every month, the size of rehydrated nanocapsules was measured by PCS to evaluate the nanocapsules stability, also the residual humidity in freeze-dried samples was measured by Karl Fischer titration.

3. Results and discussion

Polycaprolactone nanocapsules prepared by emulsion–diffusion of solvent exhibit a diameter of about 311 nm and polydispersity index of 0.06 which indicates a monodisperse distribution of colloidal suspension. Nanocapsules aggregation and the formation of macroscopic particles were noticed after the freeze-drying of nanocapsules without cryoprotectant (Table 1). The produced nanocapsules were then freeze-dried with five different types of cryoprotectants: a monosaccharide (glucose), a disaccharide (sucrose), an oligosaccharide (hydroxypropyl β-cyclodextrin), a polymer (polyvinyl pyrrolidone), and polyol (mannitol with and without NaCl).

Nanocapsules diameter measurement, polydispersity index, and the ratio of nanocapsules size after and before freeze-drying (S_F/S_I) confirmed the conservation of nanocapsules after rehydration in the case of four cryoprotectants: sucrose, HPβCD, glucose, and PVP (Table 1). These results demonstrate a successful freeze-drying of nanocapsules. On the other hand, the freeze-drying of nanocapsules with mannitol produces macroscopic particles nonmeasurable by PCS (Table 1).

Purified freeze-dried nanocapsules protected by HPβCD could be observed by ESEM after their reconstitution (Fig. 1). ESEM imaging showed spherical and monodisperse nanocapsules being well-conserved after freeze-drying.

3.1. Stabilization of NC during freezing

The thermal analysis of nanocapsules suspension by DSC with sucrose, glucose, HPβCD, and PVP shows a glass transition at different temperatures depending on the cryoprotectant used in the formulation (Table 2). Such result indicates that nanocapsules can be included within vitrified glasses of cryoprotectants during the freezing step. It is well known that during the freezing of a sample there is a phase separation into ice and cryo-concentrated solution. This cryo-concentrated solution may vitrify at a specific temperature denoted $T_{g'}$ (glass transition temperature of maximally cryo-concentrated solutions). The immobilization of nanocapsules within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. In general, freezing must be

Table 1
Characterization of nanocapsules before and after freeze-drying with four excipients

Excipient	Size ± SD (nm)		P.I. ± SD		S_F/S_I	
	Before freeze-drying*	After freeze-drying	Before freeze-drying*	After freeze-drying	After freeze-drying	After freeze-thawing
–		+++		–	–	–
Sucrose		304.1 ± 2.42		0.05 ± 0.02	0.97	0.97
HPβCD		297.3 ± 3.95		0.05 ± 0.02	0.95	0.96
Glucose	311.3 ± 2.2	307.0 ± 0.90	0.06 ± 0.01	0.05 ± 0.04	0.98	0.98
PVP		301.0 ± 3.92		0.05 ± 0.02	0.96	0.98
Mannitol		+++		–	–	–
Mannitol + 1% NaCl		1065.5 ± 100		1	3.5	1.20

P.I., polydispersity index; S_F/S_I, ratio of PCL NC size after and before freeze-thawing or freeze-thawing; SD, standard deviation; +++, macroscopic particles. PCL NC prepared with 5% (w/v) of excipient.

* All formulations have the same nanocapsules size and polydispersity index before freeze-drying.

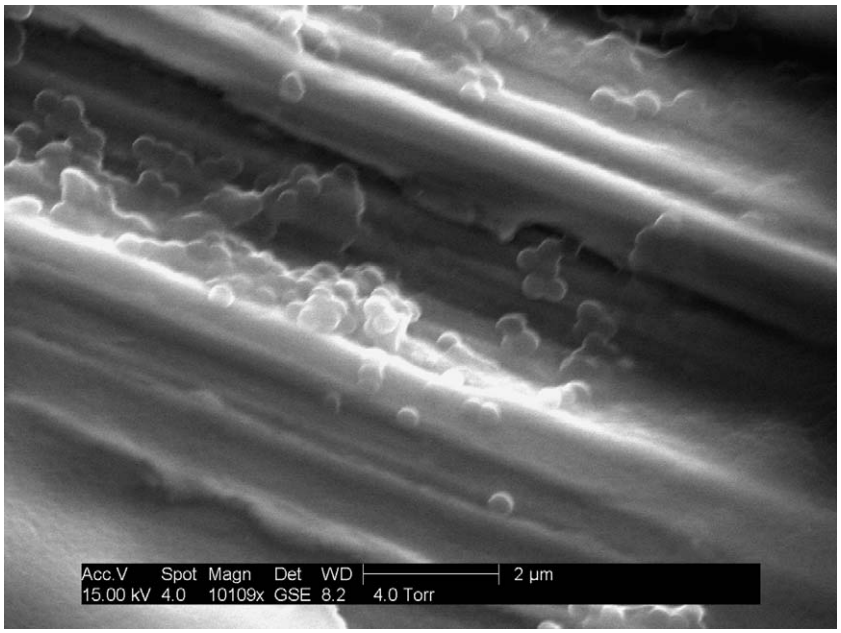

Fig. 1. ESEM imaging of freeze-dried purified PCL NC after reconstitution prepared with 5% (w/v) of HPβCD as cryoprotectant.

Table 2
Experimental determinations of collapse temperature (T_c), glass transition temperature ($T_{g'}$), and crystallization temperature (T_{cr}) of suspension of nanocapsules containing different cryoprotectants

Cryoprotectant	T collapse[a] (°C)	$T_{g'}$[b] (°C)	T_{cr}[b] (°C)
Sucrose	−30.86	−32.26	–
Glucose	−42.70	−41.36	–
HPβCD	−15.43	−14.79	–
PVP	−22.06	−21.52	–
Mannitol	–	–	−23

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

carried out below $T_{g'}$ of a frozen sample to ensure the total solidification of this sample.

The thermal analysis by DSC of mannitol solution at 10% (w/v) with and without 1% of NaCl is shown in Fig. 2. The mannitol solution shows a large exothermic peak due to the crystallization of mannitol at −23 °C, whereas the solution of mannitol solution with 1% NaCl shows a complete inhibition of mannitol crystallization which remains in amorphous state during freezing with NaCl.

Freeze-thawing study of nanocapsules using 5% of mannitol and 1% NaCl indicates a partial conservation of nanocapsules diameter size because S_F/S_I ratio is about 1.20, whereas with using mannitol alone we observe macroscopic particles (Table 1).

The aggregation of nanocapsules after freeze-thawing or freeze-drying using mannitol is probably due to the crystallization of mannitol and the formation of eutectics with ice which can cause phase separation in the cryo-concentrated portion of the frozen suspension with no opportunity for a

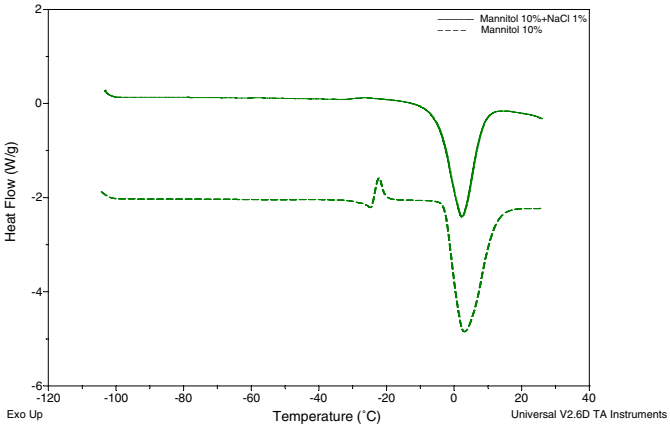

Fig. 2. DSC analysis of 10% mannitol solution and mannitol with 1% NaCl. Note the crystallization of mannitol at −23 °C and the inhibition of this crystallization in presence of NaCl.

stabilization interaction with nanocapsules. Individual nanocapsules in the nanocapsules-rich phase can interact and form aggregates (Table 1). Moreover, the growing crystals of ice and mannitol may exert mechanical forces on the nanocapsules leading to their fusion. So, any stabilization mechanism requires that at least some of the mannitol remain molecularly dispersed in the amorphous nanocapsules phase.

It has been found that some co-solutes can inhibit mannitol crystallization such as sodium chloride [9], sodium tetraborate [10], and sucrose [8]. Such effect has been explained by the slight melt miscibility of mannitol and NaCl which may provide cohesive force to inhibit crystallization [9]. Furthermore, water is essential for holding

mannitol and NaCl together to form an amorphous phase that resists crystallization [9]. These results confirm the importance of the amorphous state of cryoprotectant to ensure a good cryoprotective action for nanocapsules during their freezing.

The elemental composition of the powder surface of freeze-dried nanocapsules could be analyzed by electron spectroscopy for chemical analysis (ESCA). This technique is based on the emission of electrons from materials, in response to irradiation by photons of sufficient energy. These electrons are emitted at energies characteristics of the atoms from which they are emitted. ESCA has been previously used to study as well as the surface modification of nanoparticles [3], the adsorption of proteins at the air/liquid interface during spray-drying [11] and the ice crystals surface in the frozen material during freeze-drying [12].

Three samples were analyzed by ESCA, nanocapsules freeze-dried without cryoprotectant, nanocapsules freeze-dried with 5% w/v sucrose and with 5% w/v PVP. Additional analyses were performed on powder of PCL, PVA, sucrose, and PVP to be used as references.

Table 3 presents the chemical structure and the obtained results of elemental composition of powder surface for the different samples. ESCA of freeze-dried nanocapsules without cryoprotectant demonstrates that the proportions of different components are similar to that of PCL alone. The contribution of PVA (stabilizer adsorbed at the nanocapsules surface) in the obtained spectrum is small which perhaps corresponds to a very thin superficial or discontinuous layer of PVA at the nanocapsule surface.

ESCA of nanocapsules freeze-dried with PVP shows that the PCL and cryoprotectant matrix contribute to the recorded spectrum which means that some of the nanocapsules are present at the powder surface. Furthermore, the detection of N_{1s} signal is an additional signature of the matrix. A similar result was obtained with nanocapsules freeze-dried with sucrose.

The surface coverage of PVP in freeze-dried nanocapsules sample was calculated from the nitrogen content of pure PVP as measured by ESCA and the nitrogen content of the freeze-dried nanocapsules samples and it was about 38%. This result shows that the freeze-dried cake surface was enriched by nanocapsules resulting from their adsorption at the interface ice/liquid during the freezing step. Such result has a significant importance especially in the case of freeze-drying of immuno-nanoparticles which have antibodies adsorbed at their surface. The adsorption of protein at the interface ice/liquid during the freezing can loosen their native fold and result in surface induced denaturation of proteins [13]. Surfactants may drop surface tension of protein solutions and reduce the driving force of protein adsorption at the interface ice/liquid. This is perhaps the same phenomenon in the case of nanocapsule suspension. Low concentrations of non-ionic surfactants such as Tween 80 are often sufficient to serve this purpose [13].

It was not possible to calculate the surface coverage of sucrose in freeze-dried nanocapsules sample as the atomic composition of sucrose is close to that of PCL.

3.2. Stabilization of nanocapsules during the dehydration step

Nanocapsules were freeze-dried with four different excipients: sucrose, glucose, PVP, and HPβCD. The determination of collapse temperature (T_c) by freeze-drying microscope revealed that these different formulations have different collapse temperatures (Table 2). The collapse temperature is the maximum allowable product temperature during primary drying [14]. Collapse and loosening of porous structure can happen when the product is heated above the T_c during the sublimation step. The mean product temperature during the sublimation step under our conditions was about −27 °C which is above the T_c of glucose and sucrose and below that of PVP and HPβCD. These results explain the partial and complete collapse observed with sucrose and glucose, respectively. Fig. 3 shows scanning electron micrographs of freeze-dried PVP and sucrose preparations. Microscopy demonstrates that PVP dries into intact plates with the conservation of porous structure (Fig. 3A) whereas sucrose develops holes in the structure that indicates a small scale collapse of dried product (Fig. 3B).

The nanocapsules size was unchanged after freeze-drying with these four excipients which means that the collapse of lyoprotectant matrix during freeze-drying did

Table 3
ESCA of pure samples and nanocapsules freeze-dried without protectant or with 5% (w/v) of sucrose and PVP, respectively

	Chemical structure	% C atom	% O atom	% N atom	O/C	N/C	O/N
NC		75	25		0.33		
PCL[a]	$[–O(CH_2)_5CO–]_n$	73	27		0.37		
PVA[a]	$(C_2H_4O)_n$	67	33		0.50		
Sucrose[a]	$C_{12}H_{22}O_{11}$	49	51		1.03		
NC with sucrose		63	37		0.59		
PVP[a]	$(C_6H_9NO)_n$	71	16	13	0.22	0.19	1.17
NC with PVP		75	20	5	0.27	0.07	4.0

NC, nanocapsules; PCL, poly(epsilon-caprolactone); PVA, polyvinyl alcohol. % C atom, percent of carbon atom at the powder surface. O/C, ratio of oxygen atom to carbon atom.

[a] The different components forming the nanocapsules and the cryoprotectant matrix were also analyzed and used as references.

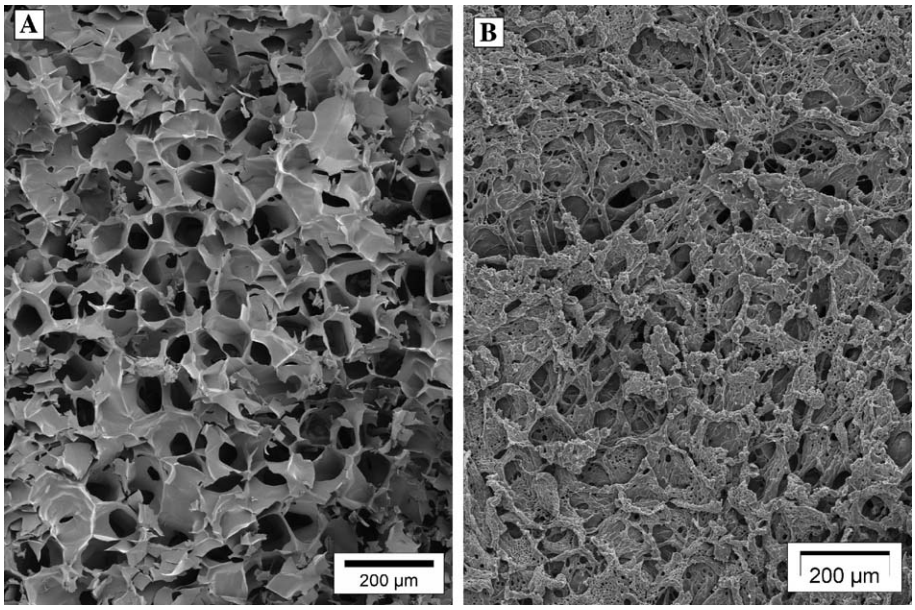

Fig. 3. Scanning electron micrographs of freeze-dried (A) PVP, (B) sucrose. Note the partial collapse of sucrose and the apparition of holes in the structure whereas the plates of PVP are intact. Scale bar: 200 μm.

not induce nanocapsules aggregation. Perhaps related is the observation that nanocapsules aggregation can be extremely slow on the timescale of processing. However, such collapse can impact the product quality, prolong the time of reconstitution, and increase the residual humidity.

During freezing step, the addition of 1% NaCl can inhibit mannitol crystallization. However, the removal of water from freeze concentrate after drying will eventually cause both NaCl and mannitol to crystallize [9] and the aggregation of nanocapsules should be expected. Indeed, the freeze-drying of nanocapsules with 5% mannitol and 1% NaCl induces their aggregation with a ratio S_F/S_I of about 3.5 and the polydispersity index shows a very polydisperse sample (Table 1). X-ray diffraction analysis of NC freeze-dried with mannitol reveals the crystallization of mannitol and the XRD patterns correspond to δ D-mannitol according to the powder diffraction files (Fig. 4B). Furthermore, XRD analysis of samples freeze-dried with mannitol in presence of NaCl showed the crystallization of both products to δ D-mannitol and halite syn-NaCl, respectively (Fig. 4B). X-ray diffraction demonstrated that pure mannitol before freeze-drying was also crystalline (Fig. 4A).

A suggested stabilization mechanism of liposomes and proteins is the water replacement hypothesis [13]. This mechanism supposes the formation of hydrogen bonds between a protein and a lyoprotectant at the end of the drying process to satisfy the hydrogen bonding requirement of polar groups on the protein surface. These lyoprotectants preserve the native structures of proteins by serving as water substitutes. Such hydrogen bonds may exist between a lyoprotectant and the groups OH of PVA adsorbed at the surface of nanocapsules which can keep the nanocapsules in pseudo-hydrated state. The amorphous state of NC and a lyoprotectant allows maximal H-bonding between NC and stabilizer molecules. So, the crystallization of this stabilizer can limit the formation of hydrogen bonds.

3.3. Stabilization of nanocapsules in dried state during the storage

After six months of storage at accelerated condition NC freeze-dried with 5% PVP seem very stable without any collapse or shrinkage of the dried cake. The measurement of NC size demonstrates the conservation of nanocapsules properties during the stress testing because S_F/S_I remained very near from 1 (Table 4). Karl Fischer titration shows that the residual humidity increased from 0.8% before storage to about 8.39% after storage for 6 months. Glass transition of PVP at this water content could be estimated from Fox equation [15]

$$1/T_g = W_1/T_{g_1} + W_2/T_{g_2},$$

where W_1, W_2 are the weight fractions of components 1 and 2, respectively, T_{g_1}, T_{g_2} are the glass transition temperatures of pure components 1 and 2. T_g was about 110 °C. This means that NC freeze-dried with PVP remained in glassy state during the six months of storage at 40 °C. X-ray diffraction analysis confirms the amorphous state of PVP (Fig. 4C). The same amorphous state was found for pure PVP before freeze-drying (Fig. 4A) and after freeze drying with nanocapsules (Fig. 4B). The physical separation of dry nanocapsules by bulky lyoprotectant glass could be an important factor for preventing nanocapsules aggregation in the glassy state. The glassy state is

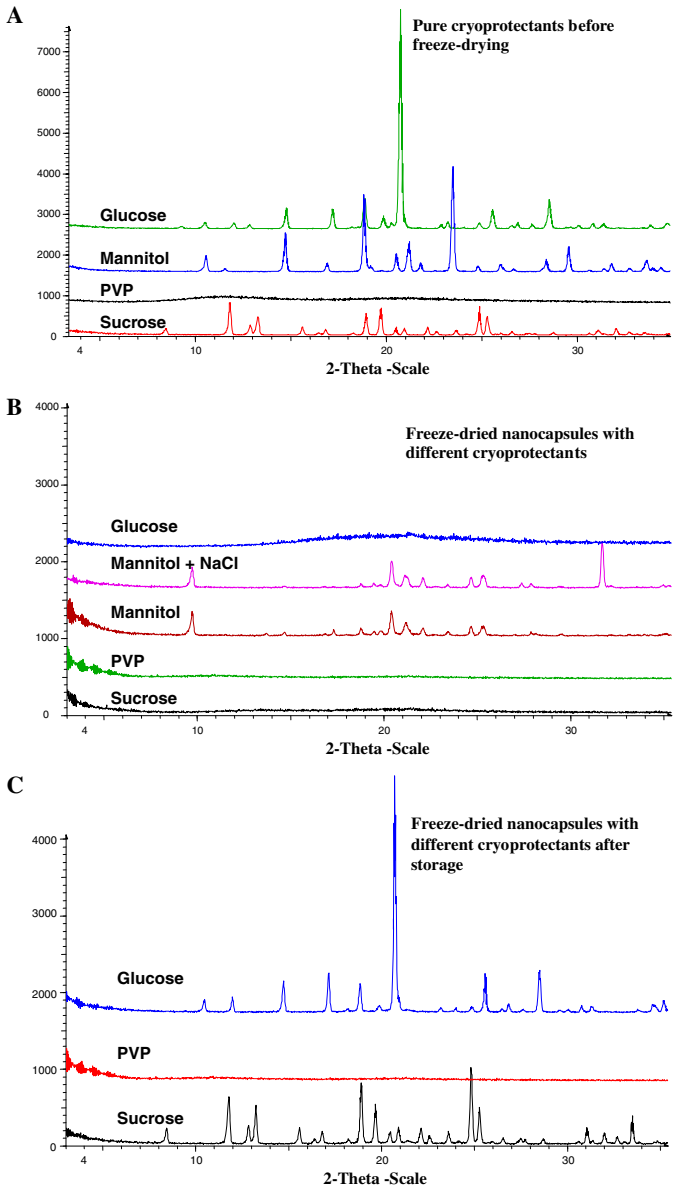

Fig. 4. X-ray diffraction analysis of: (A) pure cryoprotectants before freeze-drying. (B) freeze-dried nanocapsules with different cryoprotectants. (C) freeze-dried nanocapsules with different cryoprotectants after storage for six months at temperature 40 °C and relative humidity 75%.

characterized by a high viscosity which lowers the mobility of molecules and thus prevents the aggregation of nanocapsules.

On the other hand, the lyophilisates of NC obtained using sucrose and glucose were well collapsed with an important shrinkage of their volume after one month of storage under these stress conditions. The size of NC could not be measured because of the formation of macroscopic particles after rehydration. X-ray diffraction analysis revealed an amorphous state of both sucrose and glucose after freeze-drying (Fig. 4B). Conversely, after 6 months of storage under high relative humidity and at 40 °C, the same analysis showed the crystallization of both products which can explain the instability of nanocapsules included within (Fig. 4C). The same crystalline structure was found with the pure sugars before freeze-drying (Fig. 4A). These results are in accordance with the work of Cartensen et al. [16]. They studied the transformation of freeze-dried amorphous sucrose (metastable) into crystalline one when exposed to moist atmospheres. These lyophilisates can absorb moisture to a constant weight. The amount of moisture addition is a function of relative humidity of the atmosphere and temperature, so the lyophilisates collapse to form a denser amorphous phase denoted hydrated amorphous form. After a lag time which varies with relative humidity of the atmosphere and temperature, the hydrated amorphate loses moisture and forms crystalline sucrose. According to our results, nanocapsules can be stored for a sufficient period of time with the conservation of their size if an amorphous excipient is present. The crystallization of this adjuvant can destabilize the nanocapsules inducing their aggregation. Such crystallization should be avoided by keeping freeze-dried nanocapsules away from humidity and elevated temperature. Moreover, adding special additives may delay the nucleation event which starts the crystallization process [17].

4. Conclusion

A successful freeze-drying of fragile nanocapsules requires their dispersion within a vitrified matrix of amorphous excipient such as sucrose, glucose, and PVP during the different steps of the process to protect them against the stress of freezing and dehydration. In the case of mannitol, the crystallization of this excipient during the freezing or the desiccation step can destabilize these fragile particles. During storage, nanocapsules freeze-dried with sucrose and glucose could be aggregated after exposition

Table 4
The size of nanocapsules, the residual humidity (RH %), and the aspects of freeze-dried nanocapsules before and after six months of storage at accelerated conditions (40 ± 2 °C and 75 ± 5% RH)

Cryoprotectant	Before storage			After storage for 6 months		
	Size of NC (nm)	RH (%)	Aspect	Size of NC (nm)	RH (%)	Aspect
PVP	301 ± 3.92	0.81 ± 0.32	Correct	309.3 ± 1.2	8.39 ± 0.49	Unchanged
Sucrose	304.1 ± 2.42	1.76 ± 0.06	Partially collapsed	+++	2.73 + 0.55	Collapsed
Glucose	307 ± 0.90	3.67 ± 0.34	Collapsed	+++	ND	Collapsed

+++, macroscopic particles; ND, not determined.
The results of nanocapsules size and residual humidity are means of three measurements ± standard deviation.

to high temperature and relative humidity. Such conditions could start the crystallization of amorphous lyoprotectants causing nanocapsules aggregation. So, freeze-dried nanocapsules should be stored at a temperature below the glass transition temperature of the formulation to maintain the glassy state of lyoprotectant and to prevent any aggregation of nanocapsules. This was the case of freeze-dried nanocapsules with PVP which remained stable after six months of storage under stress conditions. ESCA revealed the adsorption of nanocapsules at the interface ice/liquid during the freezing step. Such adsorption must be avoided in the case of freeze-drying of immuno-nanoparticles to preserve the native structure of proteins attached to their surface.

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