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A pilot study of freeze drying of poly(epsilon-caprolactone) nanocapsules stabilized by poly(vinyl alcohol): Formulation and process optimization

Wassim Abdelwahed, Ghania Degobert*, Hatem Fessi

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

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Abstract

A common limitation of using polymeric nanoparticles in aqueous suspension is due to their poor chemical and physical stability when conserved for a long time. Therefore, freeze drying of these colloidal systems is an alternative method to achieve long-term stability. Nanocapsules have thin and fragile shell structure, which may not resist to the stress of such process. The aim of this study is to investigate the formulation and process parameters in order to ensure the stability of polycaprolactone nanocapsules (PCL NC) by freeze drying.

In this paper, we studied the freeze drying of PCL NC prepared by the emulsion–diffusion method and stabilized by poly(vinyl alcohol) (PVA). Different parameters have been tested throughout the freeze–thawing study including PVA and PCL concentration, cooling rate, cryoprotectant concentrations, nature of encapsulated oil and NC purification. On the other hand, nanocapsules have been freeze dried both before and after purification. Freeze dried purified PCL NC were characterized by particle size measurement, collapse temperature, T'_g determination, scanning electron microscope observation, environmental scanning electron microscope imaging and residual humidity quantification. Finally, the effect of annealing on the NC stability and the sublimation rate has been well explored.

The results suggest that PCL NC could be freeze dried without a cryoprotectant if the concentration of PVA stabilizer is sufficient (5%), while for the purified NC the addition of 5% of cryoprotectant seems to be necessary to ensure the stability of NC. The type of cryoprotectants had practically negligible effects on the size and the rehydration of freeze dried nanocapsules. The annealing process could accelerate the sublimation with the conservation of nanocapsules size.

Keywords: Emulsification–diffusion technique; Nanocapsules; Freeze drying; Collapse; Annealing; Environmental scanning electron microscope

1. Introduction

Submicronic colloidal vectors have gained a considerable interest in the last few years because of their ability to ensure a specific drug targeting by both the oral route (Ponchel and Irache, 1998) and the parenteral route (Marty et al., 1978; Soppimath et al., 2001).

Such particulate systems have been widely investigated for gene delivery to cells and tissues (Panyam and Labhasetwar, 2003) as in the delivery of anti-sense oligonucleotides (Lambert et al., 2001) and also in cancer therapy and diagnosis (Brigger et al., 2002).

Among these vectors, liposomes and nanoparticles have special advantages with regard to the modulation of an active ingredient distribution within the human body (Soppimath et al., 2001).

Nanoparticles can be classified into nanospheres and nanocapsules. Nanocapsules are vesicular systems in which the drug is confined to an oily or aqueous cavity surrounded by a unique polymeric membrane while nanospheres are matrix systems in which the drug is physically and uniformly dispersed (Soppimath et al., 2001).

The major obstacle that limits the use of such vectors is their instability in an aqueous medium (Chacon et al., 1999). Aggregation and particle fusion are frequently noticed after a long period of storage of such systems (Auvillain et al., 1989). Furthermore, drug leakage out of the nanocapsules and non-enzymatic polymer hydrolysis can happen. Thus, the stabiliza-

* Corresponding author. Tel.: +33 472 431 874; fax: +33 472 431 874.
E-mail address: degobert@lagep.cpe.fr (G. Degobert).

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tion of colloidal vectors is deeply explored in order to reach a shelf-life of several years.

Freeze drying, also termed lyophilization, is an industrial process of drying by freezing and sublimation of ice; it is used to convert solutions of labile materials into solids of sufficient stability for distribution and storage (Franks, 1998). This technique was considered as a good method to conserve the integrity of colloidal vectors. In literature, there are many papers that investigate in detail the stabilization by freeze drying of liposomes (Crowe et al., 1986, 1994; Anchordoguy et al., 1987) and nanospheres (De Chasteigner et al., 1996; Schwarz and Mehnert, 1997; Chacon et al., 1999; Saez et al., 2000;) but few researchers, to the best of our knowledge, studied the lyophilization of nanocapsules which have a very thin and fragile envelope that may not withstand the mechanical stress of freezing (Auvillain et al., 1989; De Chasteigner et al., 1995; Schaffazick et al., 2003; Choi et al., 2002).

Auvillain et al. (1989) found that the freeze drying of polycaprolactone nanocapsules is possible but 30% trehalose as cryoprotectant was necessary to preserve the integrity of nanocapsules. In such a case, rapid freezing has been favourable and the conservation of encapsulated oil in liquid state during the freezing process improved the success of lyophilization.

De Chasteigner et al. (1995) have reported the lyophilization of polylactide nanocapsules with 10% glucose or sucrose but the produced lyophilizates showed a two-fold increase in their size after redispersion in water. The authors explained this result by a clustering of nanocapsules.

Schaffazick et al. (2003) reported the lyophilization of polycaprolactone and eudragit nanocapsules upon the addition of colloidal silicon dioxide, however such an addition makes intravenous administration of the nanocapsules impossible.

Finally, Choi et al. (2002) freeze dried nanocapsules of polycaprolactone with pluronic F68 as a stabilizer without a cryoprotectant. These authors found that the freeze process can break the nanocapsules and promote leakage of their contents. They concluded that the nanocapsules may not have been broken by water crystallization in the external phase but by the solidification of the oil (miglyol) in the internal phase.

The aim of this study is to investigate the factors which can influence the nanocapsules stability during the different steps of lyophilization. Different parameters have been tested throughout the freeze–thawing study including PVA concentration, polymer concentration, cooling rate, cryoprotectant concentration, nature of encapsulated oil and nanocapsules purification. Nanocapsules have been freeze dried both before and after purification. Freeze dried purified nanocapsules were characterized by particle size measurement, collapse temperature and T'_g determination, scanning electron microscope observation, environmental scanning electron microscope imaging and residual humidity quantification. Finally, the effect of annealing on the nanocapsules stability and the sublimation rate has been well explored. A successful nanocapsules lyophilization requires a good formulation and optimal conditions of freezing and desiccation. Such process must produce an acceptable non-collapsed cake, which can rehydrate immediately with the conservation of nanocapsules properties.

2. Materials and methods

2.1. Materials

Poly(epsilon-caprolactone) (PCL) (Mw: 14,000 g/mol) and hydroxypropyl beta cyclodextrin (HPβCD) were obtained from Sigma–Aldrich (France). Poly(vinyl alcohol) (PVA) Moviol 4-88 (88% hydrolyzed, Mw: 31,000 g/mol) was purchased from Clariant (France). Miglyol 810 and miglyol 829 were supplied from Condea chemie (Germany). Ethyl acetate was obtained from Carlo Erba (France). D(+)Sucrose from prolabo (France). D(+)Anhydrous glucose, D(+)trehalose and D-mannitol 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. Methods

2.2.1. Preparation of nanocapsules

Nanocapsules studied in this work were prepared by the emulsification–diffusion method (Quintanar-Guerrero et al., 1998a). Briefly, 0.2 g of PCL and 0.5 g of miglyol 810 (or 829) were dissolved in 20 mL of ethyl acetate saturated with water. This organic phase was then emulsified with 40 mL of aqueous phase, saturated with ethyl acetate, 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.

2.2.2. Purification of PCL NC

An additional purification step was applied in some cases to eliminate the free stabilizer in solution. This NC purification was carried out by washing of nanocapsules two times using deionised water after their separation via ultra-centrifugation at 50,000 rpm and 20 °C for 30 min. The purified PCL NC were resuspended in deionised water. For the purified formulations, miglyol 810 was replaced by miglyol 829 as it was denser and permitted the precipitation of NC in the tube bottom after ultra-centrifugation. Such a procedure makes the purification process easier. The amount of PVA adsorbed at the surface of PCL NC was determined by colorimetric method as described in Section 2.2.11.

2.2.3. Particle size measurement

The size of nanocapsules both before and after the freezing and lyophilization was determined by photon correlation spec-

troscopy (PCS) using Zetasizer 3000 HSa (Malvern, England) at 25 °C. Each measurement was performed in triplicate.

2.2.4. Freeze–thawing study

In order to evaluate the resistance of nanocapsules during the freezing, the first step of lyophilization, three procedures of freezing were carried out using 1 mL of nanocapsules suspension filled into 7 mL freeze drying vials (Fisher Bioblock Scientific, France): ultra-rapid freezing by immersion into liquid nitrogen (−196 °C during 1 min), placement on a pre-chilled freezer at −80 °C for 1 h and ramp shelf-temperature of freeze dryer at 0.7 or 2 °C/min to −50 °C. The frozen preparations were kept at room temperature for thawing. Different factors have been studied throughout this study, such as: PVA concentration in the external phase, polymer concentration, cryoprotectant concentration, rate of cooling and nature of encapsulated oil. The particle size was determined before freezing and after thawing, and the final to initial size ratio (S_F/S_I) was also calculated.

2.2.5. Freeze drying of nanocapsules

The lyophilization of nanocapsules was realized on a pilot freeze dryer Usifroid SMH45 (Usifroid, France). It consists mainly of three stainless steel shelf plates (3 m × 0.15 m), a coiled tube used as a condenser at −65 ± 5 °C and a vacuum pump. The operating pressure is regulated by air injection through a microvalve. The apparatus is equipped by different control instrumentations: seven thermocouples to measure the product temperature and two platinum probes to measure the temperature of shelves and condenser. The total pressure is measured by a capacitive sensor between 0 and 1000 μbar. The partial pressure of water is measured by a hygrometer installed in the lyophilization chamber to determine the end of sublimation step. The conditions applied during our study were: freezing for 2 h at −45 °C with a temperature ramp of 1 °C/min, sublimation at −15 °C and 100 μbar for 15 h and finally the secondary drying was carried out at 25 °C and 50 μbar for 6 h.

2.2.6. Thermal analysis

To measure the glass transition temperature of maximally cryoconcentrated 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.

2.2.7. 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.

2.2.8. Karl Fisher titration

The residual humidity quantification in all freeze dried preparations was carried out by a Karl Fisher titration (Metler Toledo titrator DL38, Suisse) using methanol as a sample solvent.

2.2.9. Scanning electronic microscopy

Rehydrated colloidal suspensions were deposited on a metallic probe then immersed in liquid nitrogen for 10 min then evaporated under vacuum. Samples were metallized 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.

2.2.10. Environmental Scanning electronic microscopy

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.

2.2.11. Determination of PVA adsorbed at the surface of PCL NC

The amount of PVA associated with NC was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule (Alleman et al., 1993). Nanocapsules were separated by ultra-centrifugation at 50,000 rpm and 20 °C for 30 min. One milliliter of supernatant was diluted to 50 mL by deionised water. To 0.5 mL of this solution 3 mL of 0.65 M solution of boric acid and 0.5 mL of a solution of I_2/KI (0.05 M/0.25 M) were added and the volume was increased to 10 mL by deionised water. Finally, the absorbance of the sample was measured at 690 nm after 15 min of incubation. The amount of PVA adsorbed at the NC surface was calculated from the difference between the total quantity used in the formulation and the free dosed part.

2.2.12. Effect of annealing on the nanocapsules stability and sublimation rate

Annealing is a process step in which samples are maintained at specified subfreezing temperature for a defined period of time. In general, this temperature is between the T'_g and the melting temperature (Searles et al., 2001).

To study the effect of such treatment on the sublimation rate, four lyophilization cycles were performed with 25 vials filled with 1 mL of purified nanocapsules protected by 5% of sucrose. The annealing was performed at three different temperatures: −10, −15 and −20 °C for 1 h. The fourth cycle was achieved without annealing. The freezing procedure was as follows: cooling the freeze dryer shelf to −45 °C with a rate of 1 °C/min, holding for 15 min, heating to annealing temperature with a rate of 1 °C/min, holding for 1 h, cooling to −45 °C with a rate of 1 °C/min, holding for 2 h. The sublimation conditions were the same for the four cycles. The freeze drying cycle was stopped after 5 h of sublimation and the vials were collected and reweighed. The primary drying rate was calculated using the weight

lost during the partial drying. The resistance of nanocapsules toward the annealing was estimated by nanocapsules size measurement after annealing and compared with their initial size.

3. Results

3.1. Influence of cooling rate

Nanocapsules were prepared in this study by the emulsification diffusion method which is capable of preparing nanocapsules in a simple, efficient and reproducible manner. This method was preferred to the other techniques of nanocapsules preparation such as emulsification evaporation of solvent and nanoprecipitation. Emulsification evaporation technique requires working with toxic solvents, whereas nanoprecipitation method produces low yields and poor entrapment efficacy (Quintanar-Guerrero et al., 1996).

The mechanism of nanocapsules formation by emulsification diffusion method has been explained by a chemical instability produced by solvent transport. Thus, solvent diffusion from the oily globules into the water carries molecules of oil and polymer, forming locally supersaturated regions. It is proposed that such super saturation cannot persist, and that nanodroplets containing oil, polymer and drug are formed and rapidly stabilized by the surface active agent (Quintanar-Guerrero et al., 1998a).

The formation of nanocapsules has been confirmed by the density gradient centrifugation. The existence of a unique density band indicated high yields and a high loading efficiency of the oil phase. This density was found to be intermediate between those of nanoemulsions and nanospheres (Quintanar-Guerrero et al., 1998a).

Freezing is considered to be the most aggressive and critical step during the lyophilization. This step can cause aggregation or destruction of the nanocapsules. This preliminary study consisting on freeze–thawing PCL NC was conducted in order to determine the influence of procedure of cooling on the size of NC prepared with two concentrations of PVA (1.25 and 5%) and without cryoprotectant. Three cooling rates were applied on PCL NC: immersion in liquid nitrogen, at −80 and −50 °C with a ramp temperature of 0.7 or 2 °C/min.

Table 2
Freeze–thawing study of PCL NC prepared with different concentrations of PVA

PVA (%, w/v)	Size of nanocapsules (nm)		S_F/S_I
	Before freeze–thawing	After freeze–thawing	
0.62	401.7 ± 1.9	1610.3 ± 172.4	4
1.25	294.4 ± 6.9	442.5 ± 13.3	1.5
2.5	307 ± 1.3	342.9 ± 9.6	1.11
5	327 ± 1.5	329.8 ± 3.8	1.008

Procedure of freeze–thawing: 2 h at −50 °C with a temperature ramp 0.7 °C/min and thawing at room temperature. S_F/S_I: ratio of PCL NC size after and before freeze–thawing.

The results in Table 1 showed clearly that PCL NC prepared with 5% of PVA seem very stable during the three procedures of freezing as the ratio S_F/S_I is very near from 1 confirming a high stability. Also, the homogenous aspect of preparations after thawing and the presence of tyndal effect confirm this stability.

For PCL NC prepared with 1.25% of PVA, the cooling procedure has an influence on the size and polydispersity of NC. It was observed an increase in the size and polydispersity when a slow cooling procedure is applied, whereas a flash cooling (−198 °C) using liquid nitrogen reduces this ratio S_F/S_I to about 1.2.

3.2. Concentration of PVA stabilizer

The concentration of PVA stabilizer in the formulation was decreased in order to investigate its influence on the PCL NC freezing resistance. Four concentrations of PVA have been chosen 0.62, 1.25, 2.5 and 5% (w/v) (Table 2).

NC size decreases from 401 to 294.4 nm when the PVA concentration increases from 0.62 to 1.25% probably due to an insufficient amount of PVA to stabilize NC preparation. For a concentration of PVA greater than 1.25%, a small increase in NC size can be observed. In effect, Murakami et al. (1997) reported

Table 1
Freeze–thawing study of PCL NC and purified ones with different rates of cooling and at two concentrations of PVA stablizer

Cooling procedure	Final temperature (°C)	PVA (%, w/v)	Size PCL NC after freeze–thawing (nm)	P.I.	S_F/S_I
In liquid nitrogen[a]	−196	1.25	354.7 ± 5.3	0.15	1.20
		5	334.6 ± 1.7	0.07	1.02
		5*	447.9 ± 36	1.82	1.35
Pre-chilled freezer[b]	−80	5	322.5 ± 2.7	0.05	0.98
		5*	++	++	–
Freeze dryer shelf[c]	Ramp at 2 °C/min to −50 °C	1.25	425.1 ± 4.4	0.45	1.44
		5	321.8 ± 4.4	0.02	1.10
	Ramp at 0.7 °C/min to −50 °C	5	329.8 ± 3.8	0.10	1.08
		5*	+++	+++	–

(++) Aggregated suspension; (+++) very aggregated suspension. P.I.: polydispersity index; S_F/S_I: ratio of PCL NC size after and before freeze–thawing.
[a] Duration of freeze: 1 min.
[b] Duration of freeze: 1 h.
[c] Duration of freeze: 2 h.
* Purified PCL NC obtained using 5% of PVA stabilizer.

Table 3
Freezing–thawing study of nanocapsules prepared with different concentrations of PCL

PCL (%, w/v)	Envelope thickness (nm)	Size of PCL NC (nm)		S_F/S_I
		Before freeze–thawing	After freeze–thawing	
1	15.2	294.4 ± 6.9	442.5 ± 13.3	1.5
1.5	21.45	308.9 ± 4.6	432.61 ± 15.01	1.4
2.5	31.85	327.7 ± 1.8	436.9 ± 5.81	1.33

Procedure of freeze–thawing: 2 h at −50 °C with a temperature ramp 0.7 °C/min and thawing at room temperature. S_F/S_I: ratio of nanocapsule size after and before freeze–thawing, PCL NC prepared with 1.25% (w/v) of PVA.

an increase in the nanocapsules size when the PVA concentration increased, explaining that this phenomena occurred as a result of a viscosity increases.

After freeze–thawing (at −50 °C for 2 h with a ramp of 0.7 °C/min) of NC prepared using different concentrations of PVA, it can be noticed that with increasing the concentration of PVA stabilizer from 0.62 to 5%, the values of S_F/S_I decrease from 4 to 1 which indicate that the initial size was maintained. The concentration of 2.5% of PVA is the minimum required to stabilize the NC during the freezing (Table 2).

As the concentration of 1.25% of PVA is not sufficient to stabilize PCL NC, this PVA concentration was chosen to investigate the influence of other factors on the nanocapsules freezing resistance. The studied factors were the concentration of polymer, the concentration of cryoprotectant, the rate of cooling and the nature of encapsulated oil.

3.3. Concentration of PCL

As the concentration of 1.25% of PVA is not sufficient to stabilize PCL NC after freeze–thawing procedure (Table 2) it was chosen to investigate the influence of the envelope thickness of PCL NC. PCL concentration was varied from 1 to 2.5% (w/v) in the organic phase. It was supposed that such modification can increase the envelope thickness of nanocapsules and probably improves the nanocapsules freeze resistance; the results are presented in (Table 3). It has been found that PCL remains soluble in saturated ethyl acetate up to a concentration of 2.5%. At higher concentration, it was difficult to dissolve the polymer without a heating at 50 °C for a sufficient period of time.

As our PCL NC samples possess a very narrow distribution. The membrane thickness was calculated theoretically after assuming that PCL NC have the same mean diameter and the same value of membrane thickness. The determination of this theoretical membrane thickness is presented using the equations below (Guinebretière, 2001):

$$n = \frac{V_t}{V_{NC}} = \frac{\frac{m_{pol}}{\rho_{pol}} + \frac{m_{oil}}{\rho_{oil}}}{\frac{4}{3}\pi\left[\frac{d_{NC}}{2}\right]^3} \quad (1)$$

$$V_{oil} = \frac{\frac{m_{oil}}{\rho_{oil}}}{n} = \frac{4}{3}\pi\left[\frac{d_{oil}}{2}\right]^3 \quad (2)$$

$$\text{Membrane thickness} = \frac{d_{NC} - 2 \times \sqrt[3]{\frac{3 \times m_{oil} \times n}{4\pi \times \rho_{oil}}}}{2} \quad (3)$$

where V_t is the total volume of nanocapsules in the formulation (m^3), V_{NC} the mean of measured volume of one nanocapsule (m^3), m_{pol} the total mass of polymer in the formulation (kg), ρ_{pol} the density of polymer (kg m^{-3}), m_{oil} the total mass of oil in the formulation (kg), ρ_{oil} the density of oil (kg m^{-3}), d_{NC} the mean of measured diameter of one nanocapsule (nm), n the total number of nanocapsules in the suspension, V_{oil} the volume of oil core (m^3) and d_{oil} is the mean of diameter of oil core (nm).

The size measurement of NC supports our hypothesis, because an increase of PCL concentration from 1 to 2.5% induces an increase of NC size from 294 to 327 nm and the calculated envelope thickness is ranged from 15.2 to 32 nm. It was observed that the PCL concentration does not improve the PCL NC stability during freezing because about 1.5-fold increase of nanocapsules size was noticed after thawing with the different concentrations of PCL.

3.4. Concentration of sucrose

Sucrose was chosen as a cryoprotectant at three different concentrations 2.5, 5 and 15% (w/v) and the concentration of PVA stabilizer used for the preparation of PCL NC was of 1.25% (w/v). This disaccharide is often used for the freeze drying of colloidal vectors. According to our freeze–thawing study, a minimal concentration 2.5% of sucrose was needed to improve PCL NC stability (ratio S_F/S_I less than 1.06) during the freezing at −50 °C (Table 4).

3.5. Effect of oil type

The freeze–thawing stability of PCL NC formed with two oils (miglyol and silicon oil) were compared. These two oils possess different freezing temperatures: miglyol 810 (T_m about −3.5 °C) and silicon oil (T_m about −100 °C). During the NC freezing at −50 °C miglyol solidifies totally whereas silicon oil remains in liquid state.

Table 5 presents the results of freeze–thawing of these two formulations at −50 °C (ramp 0.7 °C/min). The conservation of encapsulated oil in liquid state did not improve the PCL NC stability during the freezing.

Table 4
Freeze–thawing study of PCL NC in presence of different concentrations of sucrose

Sucrose (%, w/v)	Size after freeze–thawing (nm)	S_F/S_I
0	442.5 ± 13.3	1.50
2.5	312.5 ± 3.22	1.06
5	304.0 ± 3.57	1.03
15	300.2 ± 1.62	1.01

Procedure of freeze–thawing: 2 h at −50 °C with a temperature ramp 0.7 °C/min and thawing at room temperature. S_F/S_I: ratio of nanocapsule size after and before freeze–thawing, PCL NC prepared with 1.25% (w/v) of PVA.

Table 5
Freeze–thawing study of PCL NC using two different encapsulated oils as inner core of NC

Encapsulated oil	Size of nanocapsules (nm)	
	Before freezing	After thawing
Miglyol 810	294.4 ± 6.9	442.5 ± 13.3
Silicon oil	297.23 ± 2.32	Two populations (263.9 and 1339.7)

PCL NC prepared with 1.25% (w/v) of PVA.

3.6. Purification of nanocapsules

PCL NC prepared with 5% of PVA were purified to eliminate the free stabilizer which is not adsorbed at the surface of PCL NC. This purification was carried out by twice washing the NC after a previous separation via ultra-centrifugation. It was found that this method of purification did not modify the size of NC and could eliminate about 88.8% of PVA used for the preparation without the formation of aggregates. In our laboratory, it has been found that the residual organic solvent remained with the nanocapsules after solvent evaporation under reduced pressure is lower than 450 ppm (Guinebretière et al., 2002). This quantity is acceptable by the European pharmacopoeia. Furthermore, such purification process may eliminate any trace of remained organic solvent which was not evaporated.

Three cooling rates were applied on purified PCL NC: immersion in liquid nitrogen, at −80 and −50 °C with a ramp temperature of 0.7 °C/min (Table 1). Macroscopic particles formed after the freezing of purified PCL NC at different cooling rates except in the case of immersion in liquid nitrogen in which the ratio S_F/S_I is about 1.35. It was concluded that the purified PCL NC could not resist the freezing stress (Table 1). The addition of 2.5% (w/v) of sucrose was sufficient to stabilize the purified NC during the freezing with three different procedures: in liquid nitrogen, at −80 and at −50 °C with a ramp temperature of 1 °C/min (data not shown).

Taking previous results into consideration, it can be concluded that the percentage of PVA stabilizer used in the formulation and the addition of a cryoprotectant are the most important factors that influence the PCL NC stability during the freezing step.

PCL NC can withstand freezing using at least 2.5% (w/v) PVA as stabilizer or with 1.25% PVA with at least 2.5% sucrose being necessary also to stabilize purified NC.

3.7. Freeze drying of PCL NC

PCL NC prepared with 1.25% of PVA were freeze dried with 5% of different cryoprotectants whereas those prepared with 2.5 and 5% of PVA were freeze dried with 10% (w/v) of cryoprotectants including: three disaccharides (sucrose, trehalose and maltose), a monosaccharide (glucose), a cyclic oligosaccharide (HP-β-cyclodextrin), a polyol (mannitol) and polymer (PVP). Table 6 presents the characterization of different freeze dried formulations. Lyophilisates were rehydrated in 1 mL of deionised water (the same initial volume of PCL NC suspension). The rehydration time was measured after the addition of deionised water under a moderate magnetic agitation until complete resuspension was observed. The addition of such cryoprotectants was to investigate their influence on the lyophilisates properties. These concentrations of cryoprotectants have been chosen to have solid contents higher of 5% (w/v) as the solid content of the solution can impact the processing conditions (Franks, 1998).

Table 6
Characterization of freeze dried PCL NC prepared with 1.25, 2.5 or 5% (w/v) of PVA stabilizer in presence of different cryoprotectants

	PCL NC					
	5% of cryoprotectant		10% of cryoprotectant			
	1.25% PVA		2.5% PVA		5% PVA	
	S_F/S_I	Time (s)	S_F/S_I	Time (s)	S_F/S_I	Time (s)
–	1.39	90	1.06	180	0.98	180
Glucose	1.01	7	0.96	13	1.02	7
Sucrose	1.00	5	0.96	7	1.00	5
HPβCD	1.01	8	1.05	11	1.01	8
Trehalose	1.00	31	0.96	41	1.00	31
Mannitol	1.00	4.5	ND	ND	ND	ND
Maltose	0.99	17.5	ND	ND	ND	ND
PVP	1.00	32	ND	ND	ND	ND

S_F/S_I: ratio of PCL NC size after and before freeze–thawing. ND: not determined.

In general, all the presented formulations containing cryoprotectants have a good aspect without any collapse. The addition of cryoprotectant was shown to improve the rehydration of lyophilizates. The size of nanocapsules was well conserved after freeze drying. PCL NC prepared with 1.25% of PVA and without cryoprotectant presented a reconstitution time of 90 s but the ratio S_F/S_I is about 1.39 indicating an increase in the size of NC after reconstitution, where as for concentration of 2.5 and 5% of PVA, the reconstitution time is longer (180 s) (Table 6). The reconstitution time of lyophilisates and the conservation of the initial size of PCL NC are considerer as parameters of product quality and are very important for the success of nanoparticles freeze drying.

Fig. 1 shows an imaging by SEM of freeze dried PCL NC with 5% of PVA and freeze dried without cryoprotectant. A continuous film of PVA can be observed in the freeze dried nanocapsules image before reconstitution (Fig. 1A). This emulsifier film prevents the observation of individual nanocapsules. In the case of reconstituted nanocapsules (Fig. 1B), the sample preparation for SEM observation requires the drying of samples and such drying leads to the formation of film of PVA around the nanocapsules. The PVA film can be observed in the SEM image and nanocapsules can be seen within.

3.8. Freeze drying of purified PCL NC

The purification of PCL NC can reduce the undesirable effects of PVA for their in vivo administration. Moreover, the success of lyophilization becomes influenced only by the added cryoprotectant properties (T'_g, T_c and ability of cryoprotection) and not yet by that of free PVA.

Purified PCL NC were prepared with 5% of PVA and lyophilized with different cryoprotectants under the same con-

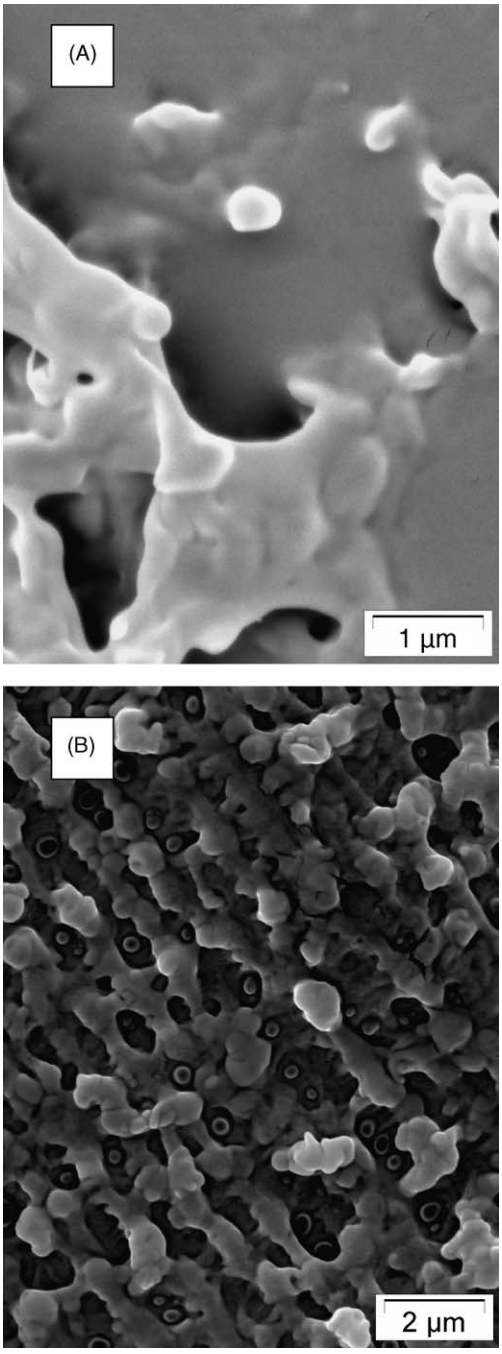

Fig. 1. SEM imaging of freeze dried nanocapsules stabilized with 5% (w/v) PVA (A) and after reconstitution (B). Note the formation of PVA film and the inclusion of nanocapsules inside it in the rehydrated nanocapsules. Whereas a continuous film of PVA can only be observed before rehydration.

ditions (freezing at −45 °C with a ramp 1 °C/min, sublimation at 100 μbar and 20 °C for 15 h and finally, secondary drying at 50 μbar and 25 °C for 6 h). All the formulations present a homogenous aspect except for the formulation freeze dried with glucose. This lyophilisate was well collapsed and had a reconstitution time about 60 s, whereas the reconstitution was immediate with the other formulations. The size measurement of lyophilized purified PCL NC after reconstitution confirms the success of lyophilization (Table 7). The conservation of PCL NC size after freeze drying using glucose as cryoprotectant affirms that such collapse during the lyophilization cycle does not modify the NC size.

The measurement of collapse temperature T_c by freeze drying microscope and T'_g by DSC can explain these results. The T_c is the maximum allowable product temperature during primary drying (Pikal and Shah, 1990). Collapse can happen when the product is heated to above the T_c during the sublimation step. The microcollapse is the onset of collapse which is characterized by the formation of small holes in the dried portion of frozen matrix. Such small-scale product collapse can decrease the resistance to water vapour flow through dried layer in primary drying (Overcashier et al., 1999).

Fig. 2 presents the observation by freeze drying cryostage of a sample of 5% (w/v) of sucrose. Upon evacuation of the chamber, ice sublimation was observed to take place from the edge toward the centre of the sample. In Fig. 2A, the drying is carried out at −50 °C with the advancement of sublimation front inside the frozen matrix. In Fig. 2B, the sample is heated to −33.6 °C and the appearance of small holes in the dried portion marks the microcollapse. In Fig. 2C, complete collapse occurred upon heating to −32 °C.

The collapse temperatures T_c, microcollapse temperatures μT_c and phase transition temperatures T'_g for the cryoprotectant in aqueous solutions and when added to the purified PCL NC suspensions are presented in Table 7. In general, the addition of purified NC to the cryoprotectants solutions does not significantly modify T_c, μT_c and T'_g. Therefore, the success of freeze drying cycle depends only on the cryoprotectant thermal properties. The highest T_c corresponds to the solution of HP-β-cyclodextrin (−14.7 °C) whereas the solution of glucose has the lowest one (−42.15 °C). The T_c of PCL NC freeze dried with glucose is about −42.7 °C which can explain the collapse observed with this formulation under our condition of freeze drying. The phase transition temperatures T'_g of the different formulations with and without purified NC as determined by DSC are different by 1–2 °C from the T_c data and show the same formulation dependence.

Karl Fisher titration of purified freeze dried PCL NC with different cryoprotectants showed that all the formulations have a residual humidity <2% except for collapsed formulation with glucose which has a residual humidity of 3.67% (Table 7). Heating the product during primary drying above T_c causes the loss of pore structures in the dried region. High residual water and prolonged reconstitution times are common consequences of collapse in a product (Pikal and Shah, 1990).

In the SEM images of purified lyophilized nanocapsules after reconstitution (Fig. 3), we can observe large aggregates which are formed from the PCL NC containing PVP as cryoprotectant. It was necessary to dry the samples for the SEM imaging which makes the observation of individual NC very difficult in the presence of a high percentage of cryoprotectant which covers them. Another microscopic method is needed for the observation of lyophilized nanoparticles. ESEM offers the possibility to control the dehydration of sample by gradual reduction of pressure and temperature in the sample chamber. Such samples can be observed in a hydrated state without a complete drying which

Table 7
Experimental determinations of residual humidity (RH), microcollapse temperature (μT_c), collapse temperature (T_c), glass transition temperature of maximally concentrated cryoprotectants (T'_g) in aqueous solutions and when added to the suspension of purified PCL NC

Cryoprotectant[a]	Aqueous solutions				Suspension of purified PCL NC					
	μT_c (°C)	T_c (°C)	T'_g (°C)	RH (%)	μT_c (°C)	T_c (°C)	T'_g (°C)	RH (%)	S_F/S_I	Time (s)
Glucose	−44.75	−42.15	−41.92	3.6	−43.33	−42.7	−41.36	3.67	0.98	60
Sucrose	−33.6	−32	−30.78	1.7	−31.6	−30.86	−32.26	1.76	0.97	5
PVP	−23.1	−21.1	−21.67	0.72	−23.7	−22.06	−21.52	0.81	0.96	5
HPβCD	−15.9	−14.7	−15.55	0.51	−16.46	−15.43	−14.79	0.44	0.95	5

In the case of purified PCL NC the ratio (S_F/S_I) and the reconstitution times were presented.
[a] Concentration of cryoprotectant 5% (w/v).

prevents the observation of individual NC. Furthermore, this technique has the ability to image wet systems without prior sample preparation. Finally, ESEM allows the observation of dehydration and rehydration phenomena.

Purified freeze dried PCL NC containing HPβCD could be observed by ESEM after their reconstitution (Fig. 4). ESEM imaging showed spherical and monodisperse NC being well conserved after freeze drying. The average NC size obtained by images analysis was about 341 nm. In the images, we notice that the cryoprotectant starts to dry and form a matrix around the nanocapsules. ESEM is the best technique for the observation of lyophilized nanocapsules in a hydrated state. It confirms the success of nanocapsules freeze drying by the conservation of their structure.

3.9. Annealing of NC suspension

Purified PCL NC could resist the annealing procedure very well without any size modification. The ratio S_F/S_I is about 1 after annealing at three different temperatures by using 5% of sucrose as cryoprotectant (Fig. 5).

Annealing has dramatic effects on the particle size distribution of ice crystals. Such thermal treatment can lead to the growth of ice crystals. Searles et al. found that an increase in ice crystals size caused by annealing should accelerate primary drying by increasing pores diameter in the plug structure which were occupied by ice crystals. Such thermal treatment can also reduce the drying rate heterogeneity between samples.

We found that annealed purified PCL NC protected by 5% sucrose for 1 h at −10 °C can accelerate 1.4-fold the sublimation rate whereas annealing at −20 °C has a less obvious effect (Fig. 5).

4. Discussion

PCL NC prepared by emulsion–diffusion method produces NC with a mean size of 294–401 nm in a reproducible and an efficient way. In this present study, PVA was used as stabilizer for

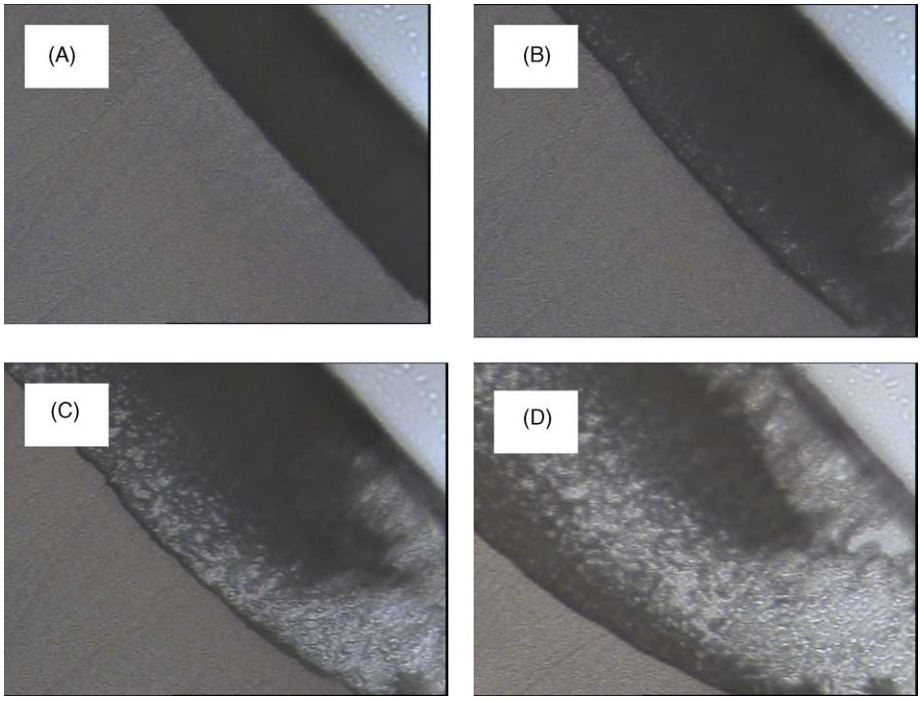

Fig. 2. Determination of collapse temperature of 5% (w/v) of sucrose by freeze drying microscope. Solution of sucrose at: (A) −50 °C, (B) −33.5 °C, (C) −32 °C and (D) −30 °C.

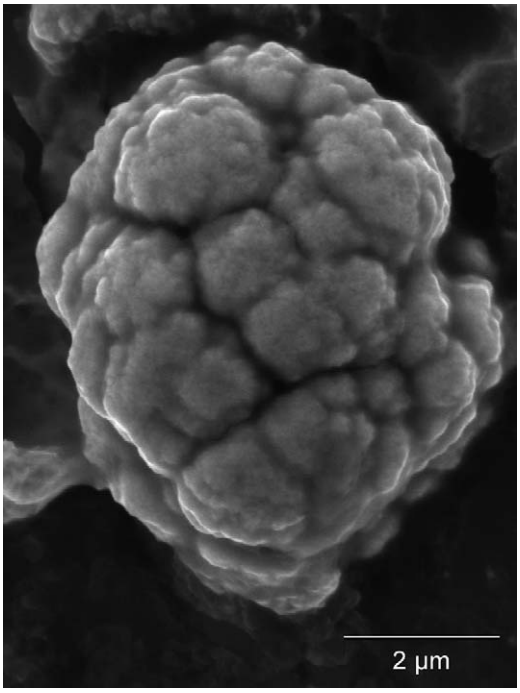

Fig. 3. SEM image of lyophilized purified PCL NC after reconstitution lyophilisate containing PVP.

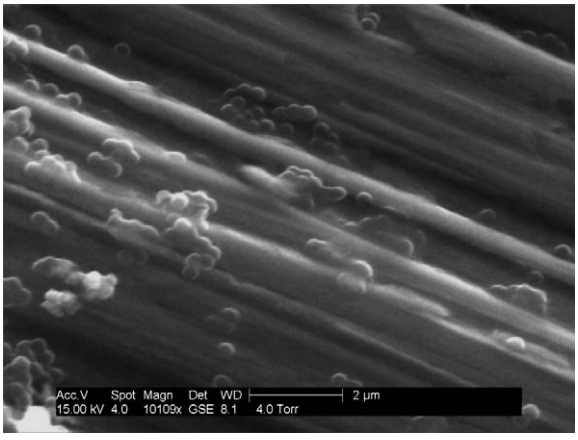

Fig. 4. ESEM imaging of freeze dried purified PCL NC after reconstitution prepared with HPβCD as cryoprotectant.

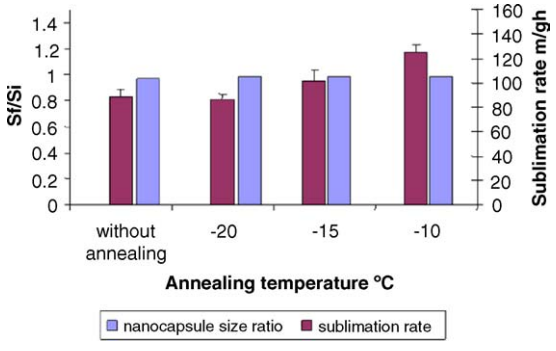

Fig. 5. Effect of annealing procedure on the PCL NC stability and the rate of sublimation.

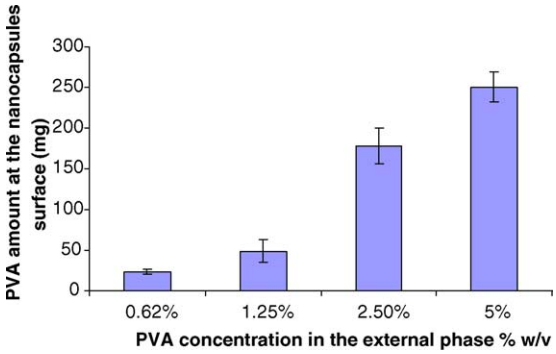

Fig. 6. Determination of PVA adsorbed at the surface of PCL NC with different concentrations of PVA.

preparing PCL NC. This polymer is one of the most frequently used stabilizers to produce stable nanoparticles, since it enhances the production of stable particles with a small size and narrow size distribution (Zambaux et al., 1998; Sahoo et al., 2002). Many papers have mentioned that a fraction of PVA used in the formulation remains associated with the nanoparticles surface despite repeated washing (Zambaux et al., 1998; Murakami et al., 1999; Sahoo et al., 2002).

A comprehensive rheological study demonstrated that PVA interacts strongly with poly lactic acid in the interfacial region during the preparation of microparticles by oil in water emulsion-based process (Boury et al., 1995). These authors concluded that the interfacial region is the location of irreversible adsorption of PVA and that makes its elimination from the surface of microparticles very difficult. Also, Murakami et al., 1999 observed a strong adsorption of PVA on the surface of poly(DL-lactide-co-glycolide) nanoparticles. They explained this by the fixation of hydroxyl groups of PVA molecules to the acetyl groups of PLGA via hydrophobic bonding.

The mechanism of PVA binding to the surface of PCL NC can be due to the interpenetration of PVA and PCL molecules during nanoparticles preparation. The hydrophobic vinyl acetate segment of partially hydrolyzed PVA interpenetrates with PCL molecule when ethyl acetate diffuses toward the aqueous phase during the dilution step.

It was found that the amount of PVA adsorbed at the NC surface increases when the PVA concentration in the aqueous phase increases (Fig. 6). Some authors have proposed that PVA can adsorb at the surface of nanoparticles in multilayers (Quintanar-Guerrero et al., 1998b).

Such polymer layer formed at the NC surface can stabilize the nanoparticles and improves their freezing resistance. Takeuchi et al., 1998 found that the coating of liposomes by a modified PVA which forms a thick layer on their surface can enhance the liposomes stability during freeze drying.

After the elimination of free PVA, purified NC cannot resist the freezing stress. These results suggest that free PVA contributes to the protection of nanocapsules in addition to the surface-adsorbed PVA. About 2% (w/v) of free PVA is at least needed to stabilize nanocapsules.

Many researchers (De Jaeghere et al., 1999; Murakami et al., 1999) have reported a successful freeze drying of nanospheres

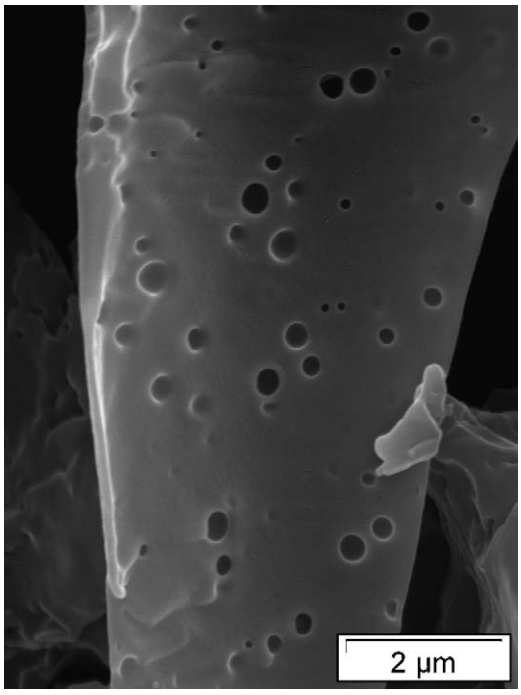

Fig. 7. SEM image of powdered NC freeze dried with HPβCD as cyoprotectant.

stabilized by PVA and purified to eliminate the free polymer. However, nanospheres have a matrix structure and are more rigid than nanocapsules. The fragile structure of nanocapsules requires further stabilization by free PVA.

During the freezing step there are dramatic changes of frozen NC suspension. The ice crystallization leads to a phase separation and cryoconcentration of nanocapsules. Such modification can damage labile bioproducts and probably induce aggregation or fusion of nanocapsules. Suitable cryoprotectant can prevent such damage. It has been suggested that stabilization of liposomes and nanoparticles requires only that they be maintained in a vitrified state (Crowe et al., 1994; De Chasteigner et al., 1996). Carbohydrates are widely used for the protection of nanoparticles and liposomes during freeze drying. Such excipients can be easily vitrified during freezing and form a protective amorphous matrix around nanoparticles.

PVA forms a glassy state at low temperature, and hydrogen bonds form between the polymer and water molecules (Takeuchi et al., 1998). Furthermore, it has been found that small concentrations of PVA inhibited the formation of ice in vitrification solutions during cooling and rewarming (Wowk et al., 2000). It has been suggested that the polymer inhibits heterogeneous nucleation of ice by preferential binding to heterogeneous nucleators in a manner similar to that of natural anti-freeze proteins. Also, this water-soluble synthetic polymer enhances the vitrification of cryoprotectant solution. Perhaps, inhibition of ice nucleation induced by PVA can improve the protection of nanocapsules during freezing.

Replacing Free PVA by a cryoprotectant can give satisfying results. In Fig. 7, we can observe the SEM imaging of powdered PCL NC freeze dried with HPβCD. This cryoprotectant forms an amorphous matrix into which the PCL NC are interdispersed. All particles are well separated throughout the matrix. The glassy state of HPβCD was confirmed by thermal analysis and the T'_g measured by DSC was about −15 °C. Crowe et al. (1994) suggested the formation of hydrogen bonding between the sugar and the polar head group of liposomes phospholipides. Such bonding can exist between the sugar and hydroxyl groups of PVA adsorbed at the surface of nanocapsules.

The annealing of purified PCL NC freeze dried with 5% sucrose could accelerate the sublimation rate. The best result was obtained with the annealing at −10 °C. Such result could be probably explained by regarding the T'_g of this formula which is about −32 °C. If the annealing temperature is above T'_g, ice will melt and smaller ice crystals will melt faster than larger one. The ice crystals size distribution during the annealing is governed by Ostwald ripening which is a phenomenon by which dispersed crystals smaller than a critical size decrease in size as those larger than the critical size grow (Searles et al., 2001). It has been found that above T'_g molecular relaxation times decrease exponentially with $(T - T'_g)$. In Ostwald ripening, the cubed average crystal diameter increases linearly with time, and it has been found that the WLF equation can describe this temperature dependence (Sutton et al., 1996).

$$\ln\left(\frac{k}{k_0}\right) = K\frac{T - T_0}{T - T_\infty} \tag{4}$$

where k and k_0 are the recrystallization rates at T and T_0, respectively, K the constant and T_∞ is a reference temperature at which $k = 0$. According to this equation, it can be found that molecular relaxation times and related viscosity and molecular mobility phenomena require a temperature-dependent activation energy $> T'_g$.

5. Conclusion

The results of this study demonstrate that polycaprolactone nanocapsules stabilized by PVA and prepared using the emulsion–diffusion method can be freeze dried without a cryoprotectant when the concentration of PVA is sufficient to prevent the NC from aggregation during freezing. However, for purified NC, the addition of cryoprotectant at a concentration of 5% seems necessary. The type of cryoprotectants had practically negligible effects on the size and the rehydration of freeze dried nanocapsules at the studied concentration (5%, w/v).

The environmental microscopy revealed NC with spherical and monodisperse size after freeze drying. Annealing process could accelerate the sublimation with the conservation of nanocapsules size. Further studies will be necessary to determine the long-term stability of the freeze dried PCL NC.

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