



Freeze-drying of nanoparticles: Formulation, process and storage considerations[☆]

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Abstract

Freeze-drying has been considered as a good technique to improve the long-term stability of colloidal nanoparticles. The poor stability in an aqueous medium of these systems forms a real barrier against the clinical use of nanoparticles. This article reviews the state of the art of freeze-drying nanoparticles. It discusses the most important parameters that influence the success of freeze-drying of these fragile systems, and provides an overview of nanoparticles freeze-drying process and formulation strategies with a focus on the impact of formulation and process on particle stability.

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1. Introduction

In the last decade, significant effort has been done to develop nanoparticles for drug delivery [1–5]. The colloidal systems offer a suitable means for delivering as well as small molecules than macromolecules such as proteins or peptides by either localized or targeted delivery to the tissue of interest. These systems in general can be used to provide targeted (cellular/tissue) delivery of drugs, to improve oral bioavailability, to sustain drug effect in target tissue, to solubilize drugs for intravascular delivery, and to improve the stability of therapeutic agents against enzymatic degradation [6–9].

Nanoparticles are submicron sized colloidal polymeric systems. According to the process used in

preparing nanoparticles, nanospheres or nanocapsules can be obtained [1–4,10]. Nanocapsules are vesicular systems in which a drug is confined inside a cavity surrounded by a polymeric membrane, whereas nanospheres are matrix systems in which a drug is dispersed throughout the particles.

The submicron size of nanoparticles offers a numerous advantages over microparticles. Nanoparticles have in general relatively higher intracellular uptake compared to microparticles. It was demonstrated that nanoparticle size of 100 nm showed 2.5 fold greater uptake compared to 1 μm and 6 fold higher uptake compared to 10 μm microparticles in Caco-2 cell line [8]. Similar results were obtained when these formulations of nano- and microparticles were tested

in a rat in situ intestinal loop model. The efficiency of uptake of 100 nm size particles was 15–250 fold greater than larger size (1 and 10 μm) microparticles [9].

The polymers used for the preparation of nanoparticles can be biodegradable such as: polylactide, polyglycolide, and their copolymers poly(lactide-co-glycolide), polycaprolactones, polyacrylates, or non-biodegradable such as: polyacrylics, poly(vinyl chloride-co-acetate) and polystyrene. Polyesters are the most extensively used for drug delivery due to their biocompatibility and biodegradability [11–15].

Nevertheless, the major obstacle that limits the use of these nanoparticles is due to the physical instability (aggregation/particle fusion) and/or to the chemical instability (hydrolysis of polymer materials forming the nanoparticles, drug leakage of nanoparticles and chemical reactivity of medicine during the storage) which are frequently noticed when these nanoparticle aqueous suspensions are stored for an extended periods [15,16].

In order to improve the physical and chemical stability of these systems water has to be removed. The most commonly used process which allows to convert solutions or suspensions into solids of sufficient stability for distribution and storage in the pharmaceutical field is freeze-drying [17]. Freeze-drying, also known as lyophilization, is an industrial process which consists on removing water from a frozen sample by sublimation and desorption under vacuum. Nevertheless, this process generates various stresses during freezing and drying steps. So, protectants are usually added to the formulation to protect the nanoparticles from freezing and desiccation stresses. In the literature, there are a few papers on freeze-drying process of polymeric nanoparticles compared to other colloidal systems such liposomes. Furthermore most often, the investigations into nanoparticles freeze-drying have been carried out by a trial and error without studying the scientific principles of this complex process. When taking into account the physical, chemical and engineering principles, freeze-drying of colloidal systems can be controlled in order to reach a shelf-life of several years [18–20].

To improve the performances of these nanoparticles, the objectives were the following: i) an elegant lyophilizate, rapid reconstitution time of the suspension, ii) a conservation of the physico-chemical

characteristics of the freeze-dried product (a small or unmodified nanoparticle size, and the drug entrapment), iii) a weak residual humidity (<2%), iv) and also a good long-term stability of the formulation.

This article reviews the state of the art of nanoparticles freeze-drying. It discusses the most important parameters that influence the freeze-drying success of these particulate systems, and provides an overview of the nanoparticles freeze-drying process and formulation strategies with a focus on formulation and process on nanoparticles stability. A successful nanoparticles freeze-drying requires a deep investigation of the formulation and the process conditions to retain the properties of nanoparticles and to have a long shelf-life of the dried product.

2. Stability of nanoparticles

Generally, nanoparticles show a poor long-term stability due to different physical and chemical factors that may destabilize the system. In this section, the stability will be discussed to investigate the fundamental aspects of this phenomenon.

2.1. Physical stability

Many factors may affect the nanoparticles stability. Generally, the colloidal homogenous suspension does not tend to separate because submicron particles sediment so slowly that the effect is obliterated by the mixing tendencies of diffusion and convection [21]. The thermal motion of the particles in the colloidal size range is known as Brownian motion. Suspended particles continuously change direction as a result of random collisions with the molecules of the suspending medium, other particles, and the walls of the containing vessel. As a result of thermal motion, colloidal particles diffuse from a region of high concentration to a region of lower concentration until the concentration is uniform throughout. Gravitational forces, which cause particles to sediment, and Brownian motion (diffusion forces), oppose one another. Both these forces are related to the particle size. Colloids are of the size range at which the Brownian forces dominate the gravitational forces, so they tend to remain suspended [22].

In order to avoid the aggregation phenomena, a suitable stabilizer can be used in the formulation. Nevertheless, the colloidal nanoparticle suspensions may be

destabilized when other components of the formulation are added. The adsorption of active molecules on the nanoparticles may induce this phenomenon (particle agglomeration), probably by displacing at least part of the steric stabilizing surfactant layer as noticed in poly(D, L-lactide) nanospheres before and after nifedipine adsorption [21]. If a solution of a high molecular weight polymer is added to a dilute dispersion, bridging flocculation may occur. In this, it is supposed that the two ends of a polymer chain adsorb on separate particles and draw them together [23]. Charge stabilized dispersions are coagulated by the adsorption of counter-ions in the electrical double layer. Even more effective are charged particles of opposite sign. Thus heterocoagulation occurs when negatively charged and positively charged dispersions are mixed.

2.2. Chemical stability

The chemical stability of colloidal polymeric carriers depends on their storage conditions (the temperature and the pH medium) and on the exact composition of the formulation stored (the type and the molecular weight of the polymer used in preparing nanoparticles). Consequently, for each specific formulation, the corresponding stability study will have to be performed to assess the quality of the product. Polyester polymers used in preparing nanoparticles have been extensively presented and discussed in this section.

2.2.1. Effect of polymer type

It is well known that nanoparticles made of hydrolytic degradable polymers will degrade (although at a low rate if the temperature and pH are controlled) over time. Lemoine et al. [12] have found that the stability of polymeric nanoparticles depends on the type of polymer with the following increasing order of polymer stability: poly(D,L-lactide-co-glycolide) PLA25GA50 (50% D,L-lactide acid and 50% Glycolide acid) < poly(D,L-lactide-co-glycolide) PLA37.5GA25 (75% D,L-lactide acid and 25% Glycolide acid) < poly(D,L-lactide) PLA50 = poly(ϵ -caprolactone) PCL. Furthermore, for PCL nanoparticles, the initial molecular weight of the polymer did not influence the degradation profile.

Phospholipids and triglyceride are frequently used in preparing solid lipid nanoparticles [24]. Phospho-

lipids are sensitive to hydrolysis from ester bonds. When such a transformation occurred, lysophosphatidylcholine and fatty acids were formed and membrane permeability increased. The peroxidation of unsaturated acyl chains, if present, was the other way of phospholipids degradation. The degradation process produced a number of products with highly different chemical natures [25]. Triglyceride hydrolysis can also occur. It leads to mono- or di-glycerides with free fatty acids. However, due to their internal location in the studied particles, they were less susceptible to hydrolysis than external phospholipids [25].

2.2.2. Effect of pH of the aqueous dispersion

The in-vitro degradation of nanospheres made from poly(D,L-lactide) of two different molecular weight (Mw: 25 000 and 95 000) has been investigated [14]. It has been found that the aqueous dispersion pH of nanospheres has major effects on the chemical stability of the polymer. The best stability in aqueous medium was observed in a buffered solution with a pH corresponding to the physiological conditions and a temperature of 4 °C. Polymer degradation by hydrolysis was observed at extreme conditions of pH and temperature. Two mechanisms of polymer hydrolysis have been found according to the medium pH. An acidic medium led preferably to a random scission along the polymeric chain resulting in the formation of mostly insoluble oligomers and low production of free carboxylic groups. On the contrary, an alkaline medium favors a non-random cleavage of the ester bonds at the end of the polymeric chains with high production of soluble derivatives including lactic acid.

2.2.3. Chemical stability of entrapped drugs

The chemical integrity of drugs entrapped in nanoparticles is another fundamental aspect of the overall stability evaluation of these preparations. Oppenheim et al. [3] have stated that if the drug degrades in an aqueous environment, the time of contact with water will influence the amount of drug incorporated into the nanoparticles. Since most such drugs have a pH-dependent degradation profile, the pH needs to be closely controlled. The manufacturing procedure should minimize the time over which degradation may occur. A number of cytotoxic drugs are light-sensitive. Hence, during the manufacturing procedure, exposure to light should also be minimized.

However, since the final product usually consists of the drug incorporated within the bulk of a solid particle, light-induced degradation of the delivery system should be less of a problem [21].

On the other hand, when studying the stability of colloidal carriers, it is important to analyze not only the particle size and polymer molecular weight, but also the eventual leaking of the drug from the carrier during storage. For example, during the encapsulation of strong hydrophobic drugs, precipitation in the external aqueous phase causes nanocrystals that may be misinterpreted as nanoparticles. The nanocrystals, however, will become evident if the preparation is stored for several days or weeks to allow the crystallization nuclei to grow [4]. In addition, it was observed that the presence of anionic surfactants in the dispersion causes a more rapid degradation of poly(D, L-lactide) [13]. Therefore, it could be expected that drugs with strong nucleophilic groups may catalyze the degradation of the polymer.

In order to reduce this physical and chemical instability of nanoparticles in aqueous solution, freeze-drying process can be proposed. At first, freeze-drying process is briefly presented and after that the freeze-drying of nanoparticles is largely discussed.

2.2.4. Effect of storage temperature

The storage temperature has a crucial effect on the long-term stability of nanoparticles. For example, when poly(D,L-lactide) and poly(ϵ -caprolactone) nanoparticles were stored for 350 days at 5 °C, only minor changes in the molecular weight of the polymers and in nanospheres size were observed [13]. At 37 °C, there was a rapid degradation of both polymers in the dispersion. However, when PLA nanoparticles were stored at 25 °C, changes in the polymer molecular weight were detected after 4 month storage period. Similar results have been obtained by Lemoine et al. [12]. These authors have found that PCL and PLA50 nanoparticles can be kept at 4 °C and room temperature during one year, while PLA37.5GA25 and PLA25GA50 nanoparticles have to be stored at 4 °C.

3. Freeze-drying process

Freeze-drying is a widely used process for drying and improving the stability of various pharmaceutical products including: viruses, vaccines, proteins, pep-

tides, or colloidal carriers :liposomes, nanoparticles, nanoemulsions. This process is relatively slow and expensive, it is especially applies only for products having a high added value. Freeze-drying cycle can be divided into three steps: freezing (solidification), primary drying (ice sublimation) and secondary drying (desorption of unfrozen water).

3.1. Freezing step

Freezing is the first step of freeze-drying. During this step, the liquid suspension is cooled, and ice crystals of pure water forms. As the freezing process continues, more and more water contained in the liquid freezes. This results in increasing concentration of the remaining liquid. As the liquid suspension becomes more concentrated, its viscosity increases inducing inhibition of further crystallization. This highly concentrated and viscous liquid solidifies, yielding an amorphous, crystalline, or combined amorphous-crystalline phase [26]. The small percentage of water that remains in the liquid state and does not freeze is called bound water.

The understanding of freezing stress is aided by viewing the phase diagram of binary system water–sucrose (Fig. 1) [17]. This diagram shows how a dilute sucrose solution will increase in concentration during freezing until the temperature reaches T_g' value (T_g' : glass transition temperature of maximally cryo-concentrated solution). At this point, the sucrose concentration is of 80% and further cooling will not change the concentration. It should be noted that the freezing

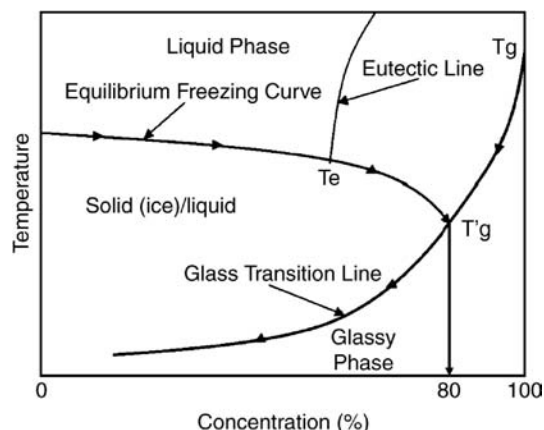


Fig. 1. Phase diagram for a binary system of sucrose–water showing T_g' .

temperature of the solution must be performed at, or below T_g' for freezing to go to completion. Sucrose does not crystallize during freezing concentration but the eutectic point is shown in Fig. 1 if crystallizing solutions (e.g. mannitol).

3.2. Primary drying step

The primary drying stage involves sublimation of ice from the frozen product. In this process, i) heat is transferred from the shelf to the frozen solution through the tray and the vial, and conducted to the sublimation front, ii) the ice sublimates and the water vapor formed passes through the dried portion of the product to the surface of the sample, iii) the water vapor is transferred from the surface of the product through the chamber to the condenser, and iv) the water vapor condenses on the condenser. At the end of sublimation step a porous plug is formed. Its pores correspond to the spaces that were occupied by ice crystals [27].

3.3. Secondary drying

Secondary drying involves the removal of absorbed water from the product. This is the water which did not separate out as ice during the freezing, and did not sublimate off [28].

A typical production scale freeze dryer consists of a drying chamber containing temperature-controlled shelves, which is connected to a condenser chamber via a large valve. The condenser chamber houses a series of plates or coils capable of being maintained at very low temperature (less than $-50\text{ }^{\circ}\text{C}$). One or more vacuum pumps in series are connected to the condenser chamber to achieve pressures in the range of 4 to 40 Pa in the entire system during operation [29].

4. Freeze-drying of nanoparticles

A freeze-dried nanoparticles should have certain desirable characteristics, including: i) the preservation of the primary physical and chemical characteristics of the product (elegant cake appearance, short reconstitution time, an acceptable suspension and low or unmodified particle size distribution of nanoparticle suspensions, unchanged activity of encapsulated drug), ii) an acceptable relative humidity, and iii) long-term stability.

One can say that for obtaining product with high quality, it is important to control the following steps: i) The formulation, ii) the freeze-drying process and iii) the storage conditions.

4.1. Importance of the formulation

The goal of the formulations scientist is to identify the right formulation conditions, the right excipients in optimal quantities, and the right dosage form to maximize stability, biological activity, safety, also and marketability of a particular product. If the formulation is intended to be freeze-dried it would be important to adapt the formulation, taking into account the thermo-physical properties of the nanoparticle suspensions.

Many components of the nanoparticles formulation have a crucial effect on the resistance of nanoparticles to the different stresses during freeze-drying, as the type and the concentration of cryoprotectant, the nature of surfactant, the chemical groups attached to the nanoparticles surface, or the polymer used to form the nanoparticles. For this reason, a wise and attentive selection of all components of the nanoparticles formulation must be performed before starting the study of freeze-drying. In this section, the different effects of the nanoparticles formulation (the protectant used, the surface of nanoparticles) on the freeze-drying will be discussed. A particular attention is brought to nanocapsules.

4.1.1. Use of cryo and lyoprotectant

Freeze-drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration. It is well known that during the freezing of a sample there is a phase separation into ice and cryo-concentrated solution. In the case of nanoparticles suspension, the cryo-concentrated phase is composed of nanoparticles and the other components of the formulation as free surfactants, buffers, and unloaded drugs [19].

This high concentration of particulate system may induce aggregation and in some cases irreversible fusion of nanoparticles. Furthermore, the crystallization of ice may exercise a mechanical stress on nanoparticles leading to their destabilization. For these reasons, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems [20]. These excipients are usually added in order to protect the

Table 1
Examples of commonly used excipients in freeze-drying of pharmaceutical products

Type	Function	Substance
Bulking agents	Provide bulk to the formulation specially when the concentration of product to freeze dry is very low.	Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine.
Buffers	Adjust pH changes during freezing.	Phosphate, tris HCl, citrate, and histidine.
Stabilizers	Protect the product during freeze-drying against the freezing and the drying stresses.	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, polyethylene glycol, dextran, and PVP.
Tonicity adjusters	Yield an isotonic solution and control osmotic pressure.	Mannitol, sucrose, glycine, glycerol, and sodium chloride.
Collapse temperature modifiers	Increase collapse temperature of the product to get higher drying temperatures.	Dextran, hydroxypropyl- β -cyclodextrin, PEG, poly(vinyl pyrrolidone).

product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage. Table 1 presents some examples of the excipients commonly used in freeze-drying process of pharmaceutical products with the presentation of their different role. The most popular cryoprotectants encountered in the literature for freeze-drying nanoparticles are sugars: trehalose, sucrose, glucose and mannitol (Table 2). These sugars are known to vitrify at a specific temperature denoted Tg' [30,31]. The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. Generally, freezing must be carried out below Tg' of a frozen amorphous sample or below Teu (eutectic crystallization temperature) which is the crystallization temperature of soluble component as a mixture with ice, if it is in a crystalline state in order to ensure the total solidification of the sample [32].

Trehalose seems to be a preferable cryoprotectant for biomolecules. It has many advantages in comparison with the other sugars as: less hygroscopicity, an absence of internal hydrogen bonds which allows more flexible formation of hydrogen bonds with nanoparticles during freeze-drying, very low chemical reactivity and finally, higher glass transition temperature Tg' [33,34].

The level of stabilization afforded by sugars generally depends on their concentrations. It has been proved that trehalose is more effective for stabilizing both comprotol (glycerol behenate) solid lipid nanoparticles and glycerol trilaurate SLN during freeze-drying at concentration 15% [35]. This concentration was also necessary to stabilize SLN made from different lipid matrixes, whereas 2% of trehalose was

not sufficient to protect the nanoparticles, as size measurements of reconstituted nanoparticles showed an increase in their average diameter and polydispersity index [36].

Poly(D,L-lactide-co-glycolide) and poly(ϵ -caprolactone) nanoparticles could be freeze-dried giving acceptable product upon reconstitution with no macroscopic aggregation when sucrose and glucose respectively were added at a concentration of 20% [37]. Furthermore, the weight ratio cryoprotectant: nanoparticles is important for stabilizing nanoparticles. A complete redispersion of poly(lactide acid-co-ethylene oxide) nanoparticles after freeze-drying could be obtained when trehalose was added to the nanoparticles suspension at a weight ratio trehalose: nanoparticles (1:1) [38]. Similar observations

Table 2
Some of cryoprotectants used in literature for the freeze-drying of nanoparticles

Cryoprotectant	References
Glucose	[15,18–20,37,65,69,80,82]
Sucrose	[18–20,37,59,62,65,69,74]
Trehalose	[15,16,18,41,63,71,76,86]
Lactose	[16,74,82]
Mannitol	[15,41,82]
Sorbitol	[6,7,37]
Aerosil (colloidal silicon dioxide)	[10]
Maltose	[16]
Poly(vinyl pyrrolidone)	[19,20]
Fructose	[76]
Dextran	[15,90]
Glycerol	[41]
Poly(vinyl alcohol)	[18,20,52]
Glycine	[63]
Hydroxypropyl- β -cyclodextrin	[19,20]
Gelatine	[63]

have been reported during freeze-drying of polymer-DNA complex as gene delivery system. At low sucrose concentration (about 1.25%), both the complex size and its transfection efficiency have been modified [39]. However, even high concentrations of sugars (up to 25% of glucose, fructose, mannose, maltose and trehalose) were not able to stabilize calix[4]resorcinarene-derived SLN during freeze-drying [40]. The authors explained this result by the high affinity of calix[4]resorcinarenes for carbohydrates, which does not allow the entire reconstitution of the SLN suspensions in aqueous media after the freeze-drying process, or may provoke restructuring of the colloids. On the other hand, in some cases, increasing cryoprotectant concentration to a certain level may eventually reach a limit of stabilization or even destabilize nanoparticles. For example, particle aggregation increased with higher glucose concentration during freeze-drying of cationically modified silica nanoparticles [41]. Furthermore, nanoparticles concentration has a crucial effect on the success of freeze-drying. This effect was investigated in the case of freeze-drying of poly(lactide acid)-poly(ethylene oxide) copolymer nanoparticles [42]. It has been found that regardless of the amount of lyoprotectant added (trehalose), the nanoparticles concentration in the suspension prior to freeze-drying plays a key role in the lyoprotective mechanism. The results indicated that the higher the nanoparticles concentration, the higher the lyoprotective efficiency. At 0.2% (w/w) nanoparticles concentration, the nanoparticles could not be re-dispersed, even using weight ratio of trehalose: nanoparticles (10:1), whereas at 0.8% (w/w), trehalose in a 2:1 ratio allowed total preservation of the nanoparticles size.

In general, the type of cryoprotectant must be selected and its concentration must be optimized to ensure a maximum stabilization of nanoparticles. Usually, a freeze-thawing study should be realized before freeze-drying to select the best cryoprotectant which is able to conserve the properties of nanoparticles.

The crystallization of cryoprotectant as mannitol and the formation of eutectic with ice can cause phase separation in the cryo-concentrated portion of the frozen nanoparticles suspension with no opportunity for a stabilization interaction with nanocapsules. Individual nanoparticles in the nanoparticles-rich phase can interact and form aggregates. Moreover, the growing crystals of water and mannitol may exert

mechanical forces on the nanoparticles leading to their fusion. So, any stabilization mechanism requires that at least some of the mannitol remain molecularly dispersed in the amorphous nanocapsules phase [19].

Another explanation of the mechanism of nanoparticles stabilization by cryoprotectants during the freezing step is the particle isolation hypothesis. It has been proposed that sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing above T_g' . In this case, the vitrification is not required for this effect and the spatial separation of particles within the unfrozen fraction is sufficient to prevent aggregation [43].

The dehydration steps involve the removing of ice and unfrozen water. This unfrozen water remains dissolved or adsorbed on the solid phase. Such process may destabilize unprotected nanoparticles. In general, special excipients are to be added to the nanoparticles formulation to serve as lyoprotectant.

A suggested stabilization mechanism of nanoparticles by lyoprotectants during drying steps is the water replacement hypothesis which was already explained the stabilization of liposomes and proteins [44–46]. This mechanism supposes the formation of hydrogen bonds between a lyoprotectant and the polar groups at the surface of nanoparticles at the end of the drying process. These lyoprotectants preserve the native structures of nanoparticles by serving as water substitutes. The amorphous state of nanoparticles and a lyoprotectant allows maximal H-bonding between nanoparticles and stabilizer molecules. So, the crystallization of this stabilizer can limit the formation of hydrogen bonds [19]. It has been found that disaccharides were more effective to preserve griseofulvin-lipids nanoparticles (GFNPs) size during freeze-drying than monosaccharides [47]. X-ray diffraction analysis revealed that monosaccharide-containing freeze-dried GFNPs had sugar in a crystal state. On the other hand, disaccharide-containing freeze-dried GFNPs were in an amorphous state. The authors concluded that the monosaccharides were more effective than disaccharides because of less effective interaction with the nanoparticles after their crystallization.

For some nanoparticles formulations, it was possible to freeze-dry nanoparticles without adding cryo or lyoprotectant as in the case of poly(ϵ -caprolactone) nanocapsules which have been prepared using 2.5 or 5% of PVA [20]. In this formulation, free PVA which is not adsorbed at the surface of nanocapsules plays the role of

freeze-drying stabilizer. In another formulation, poly(isobutylcyanoacrylate) and poly(isohexylcyanoacrylate) nanoparticles could be freeze-dried without any modification of their size in presence of 2% of pluronic® which was the surfactant agent to stabilize the colloidal suspension [48]. The importance of the surfactant for the freeze-drying of nanoparticles will be discussed in more details in the following section.

4.1.2. Importance of the interface composition (nanoparticle surface-dispersion medium)

To ensure the maximum stability of colloidal particles, a wise selection of stabilizing agent which should be localized at the nanoparticles surface must be done. Such stabilizers can improve the stability of the nanoparticle suspensions and prevent their aggregation. Many stabilizing agents have been used to achieve this objective as surfactants, modified polymers, and copolymers (Table 3). This table shows some examples of successful freeze-drying nanoparticles by presenting the methods used for their preparation and the nanoparticles components including: the polymers, the stabilizers, and the cryoprotectants used.

Poly(vinyl alcohol) (PVA) is one of the most frequently used stabilizer to produce stable nanoparticles, since it enhances the production of stable particles with a small size and narrow size distribution [49,50]. Many papers have mentioned that a fraction of PVA used in the formulation remains associated with the nanoparticle surface despite repeated washing [49–52].

Such polymer layer formed at the nanoparticles surface can stabilize the nanoparticles and improves their freezing resistance. Takeuchi et al. [53] 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. Many researchers [2,38,52,54] have reported a successful freeze-drying of nanospheres stabilized by PVA and purified to eliminate the free polymer. Such freeze-drying of nanoparticles has been performed without the addition of cryoprotectant.

On the other hand, nanoparticles stabilized by poloxamer were not resistant to the freeze-drying procedure [18]. The aggregation of nanoparticles could be explained by an increase of the solubility of poloxamer in the bulk solution during the freezing process. It has been found that the solubility of poloxamers is higher in cold water than in hot water, due to hydrogen-bond formation between the water molecules and the numerous ether-type oxygen bonds of the poloxamers [55]. A decrease in temperature favors the salvation of the poloxamer by increasing the hydration of shell of poly(oxyethylene) and poly(oxypropylene) blocks, thus the dynamic motion of the surface-attached chains is broken and the latter tend to remain in the bulk solution.

Another studies have reported that poloxamer used as stabilizer of nanoparticles crystallize upon freezing impairing the maintenance of nanoparticles properties in the absence of cryoprotectives. On the contrary, their presence dehydrates the surfactant in the bulk

Table 3
Examples of successful freeze-drying nanoparticles

Method of preparation	Polymer	Stabilizer	Cryo or lyoprotectant	S_f/S_i	References
Nanoprecipitation	PCL	Poloxamer	Glucose, sucrose, (10%)	1.2	[65]
Nanoprecipitation	PCL, PLGA	Poloxamer	Glucose, sucrose (20%)	1.5	[37]
Salting out	PLA, PLA-PEO	PVA	Trehalose Ratio tr/np 1/1 or 2.5/1	1	[38]
Double emulsion	MPEO-PLA	PVA	Sucrose 0.5–8% w/w	1	[59]
Microemulsion method	Emulsifying wax	Hexadecyltrimethyl ammonium	Lactose, sucrose (1–5% w/v)	1	[74]
Emulsion–evaporation	PCL–dextran	Na cholate (0.1%) Poloxamer 1% PVA 1%	Glucose 5%	1	[57]
Polymerization	Poly(methylidene malonate 2.1.2)	Dextran 1%	Dextran 1%	1.07	[90]
Melt homogenization method	Tricaprin	Tween 80 Egg Phosphatidylcholine	Sucrose 5%	1.45	[62]

S_f/S_i : ratio of nanoparticles size after and before freeze-drying.

solution forcing it to the particle surface, and thereby acting as a cryoprotective agent [37].

Insulin containing poly(ethylenimine-dextran sulphate) nanoparticles have been prepared and stabilized by zinc sulphate [56]. These nanoparticles could be freeze-dried without adding a cryoprotectant and the mean particle size remained constant before and after lyophilization. On the other hand, preparation formulated without zinc sulphate showed a mean particle size twice that before lyophilization. These results suggest that zinc sulphate may stabilize the nanoparticles through electrostatic interactions with the nanoparticles.

An interesting effect of the copolymer used to form nanoparticles on the conservation of nanoparticles size after freeze-drying has been studied. Core-shell nanoparticles have been prepared from this amphiphilic copolymers, based on dextran grafted with poly(ϵ -caprolactone) side chains (PCL-Dex) [57]. Because of their strongly amphiphilic properties, the copolymers were able to stabilize nanoparticles without the need of additional surfactants. It has been found that freeze-drying of poly(ϵ -caprolactone) nanoparticles induced an extensive and irreversible aggregation and the nanoparticles size increased to more than 1 μm even if up to 1% glucose has been added. In contrast, freeze-dried PCL-Dex nanoparticles were much easier to redisperser, 1% of glucose was enough to maintain the size of nanoparticles when PCL-Dex 33% (33% is the weight percentage content of dextran in the copolymer) was used to form nanoparticles. In the case of nanoparticles with a lower dextran content (10% to 20%) the mean size increased by about 80 nm in the presence of glucose, but no large aggregate were observed after redispersion in water. For the lowest dextran content (PCL-Dex 5%), lyophilization induced a considerable increase of the nanoparticles size even in the presence of glucose. From these results, it can be concluded that dextran as a polysaccharide, can be assumed to play an additional cryoprotectant role during freeze-drying.

Similar interesting use of dextran to stabilize colloidal particles has been achieved by the synthesis of polymeric surfactants [58]. These polymeric surfactants could be obtained by chemical modifications of dextran via the attachment of various amounts of aromatic or aliphatic hydrocarbon groups onto the glucose units in the dextran molecule. Two different types of stable colloids could be obtained using this modified dextran, stable oil in water nanoemulsion with heavy oils having low water

solubility, and nanocapsules obtained by the polymerization of styrene following a miniemulsion process. In the two types of colloids, a permanent adsorbed dextran layer at their surface could be obtained. It has been found that the thickness of this layer increased with the increase of the weight ratio polymeric surfactant: oil. Nanoemulsion and nanocapsules freeze-dried without the addition of cryoprotectants. For nanoemulsion, results showed clearly that the presence of the modified dextran layer at the surface of nanospheres may help to prevent aggregation of oil particles during freeze-drying process. Similar results have been obtained after freeze-drying of nanocapsules. It has been found that the freeze-dried suspensions are not redispersible when the amount of polymer is too low (low weight ratio polymeric surfactant:oil). For higher amounts of polymer, limited aggregation occurs and the average size is close to that of the initial particles.

Another example of the importance of copolymer used in the formulation on the success of freeze-drying is poly(D,L-lactide acid-co-ethylene oxide)(PLA-PEO) [38]. These surface modified nanoparticles by the introduction of PEO are known to avoid the mononuclear phagocytic system to have a long circulating time in the blood. Freeze-drying of these nanoparticles induced their aggregation. No improvement of the redispersion could be achieved even using sonication, indicating a strong type of aggregation. Particle sizes in the samples with the highest PEO contents were no longer compatible with an intravenous administration. The almost linear relationship between the amount of PEO in the formulation and particle aggregation after freeze-drying ascertained the surface location of the PEO chains and therefore, highlighted the negative impact of the latter on particle redispersion. This result has been explained by the tendency of PEO to crystallize upon freezing. It can be assumed that, as a result of covalent attachment of PEO to the surface and close proximity of the particles, intra- and interparticulate bridges of crystallized PEO might have formed during freezing, resulting in aggregated particles after water removal. This effect appeared to be PEO-concentration dependent. The addition of trehalose could improve the freeze-drying of nanoparticles. By forming an amorphous matrix with water around the particles during freezing, trehalose may maintain the PEO chains in a pseudo-hydrated state through intramolecular hydrogen-bonding. When sufficient

hydrogen-bonding is formed, crystallization of the PEO chains may be prevented in the frozen samples.

The same observation has been reported for the freeze-drying of nanoparticles prepared of blends of poly(lactide acid) and monomethoxypoly (ethylene

oxide) (MPEO-PLA) [59]. The crystallization of MPEO chains during freezing without a protector could induce nanoparticles aggregation. The addition of sucrose could improve the freeze-drying by preventing MPEO crystallization. It has been found

Table 4

Encapsulated drugs and process conditions of different nanoparticles formulations freeze-drying

Polymer	Encapsulated drug	Conditions of freeze-drying process			References
		Freezing	Primary drying	Secondary drying	
Glyceryl monostearate	Clobetasol Propionate	−75 °C for 5 h	72 h		[94]
	RMEZ98	−70 °C	1.03 mbar	30 °C for 3 h	[76]
			−30 °C 7 h		
			−10 °C 2 h		
Poly(isobutylcyanoacrylate)	Ampicilline		6×10^{-2} mbar		[48]
Poly(isohexylcyanoacrylate)			for 24 h		
PCL	Itraconazole	−60 °C for 3 h	30 °C for 12 h		[59]
PCL, PLGA	Cyclosporine	−45 °C for 24 °C	4×10^{-5} atm		[37]
			for 48 h		
PLA-PLGA	Meso-tetra (4-hydroxyphenyl)porphyrin	−60 °C for 30 min	0.001 bar		[82]
Polyethylenimine, dextran sulfate	Insulin	−46 °C	1272 mTorr for 24 h		[56]
Emulsifying wax	pDNA	−20 °C overnight	For 24 h		[74]
		−80 °C			
PCL, Eudragit S90	Diclofenac	−20 °C	0.07 mbar for 24 h		[10]
Poly(isohexylcyanoacrylate)	Doxorubicine	−55 °C for 3 h			[80]
Dynasan 112	Tetracaine	−25 °C	−25 °C and 20 °C		[63]
Compritrol ATO 888	Etomidate	Liquid nitrogen	0.37 mbar for 24 h	and maximum vacuum for 24 h	
PLGA	Cyclosporine	In liquid nitrogen for 15 min	−40 °C for 24 h	15 °C for 48 °C	[15]
		At −70 °C for 72 h		30 °C for 24 h	
PLA	Indomethacin	−50 °C for 3 h	At 6 °C for 10 h	40 °C for 2 h	[65]
Trilaurine	Azidothymidine	−40 °C for 15 min	−40 °C, 0.07 mbar for 24 h		[64]
Silica	DNA	In liquid nitrogen	−40 °C and 0.05 mbar for 48 h	−20 °C and 0.01 mbar for 96 h	[41]
Tricaprin	All-trans retinoic acid	Liquid nitrogen			[63]
Polybutylcyanoacrylate	Stavudine	−80 °C for 30 min			[54]
Methylmethacrylatesulfopropylmethacrylate					
PCL	Cyclosporine	−70 °C and −196 °C			[66]
Lecithin	Paclitaxel				[86]
Stearic acid	Hydrocortisone				[93]
Glyceryl monostearate	Progesterone				

that the freeze-drying of MPEO-PLA nanoparticles depends not only on their MPEO content but also on the molecular weight of the MPEO used, because for an equal MPEO surface density on the surface of the nanoparticles, a MPEO of high molecular weight may stick out to a larger distance from the surface than a lower MPEO molecular weight, thus having a higher tendency to interact with other MPEO chains, which can perturb the nanoparticle structure.

Freeze-drying of chitosan-DNA nanoparticles was possible after the conjugation of poly(ethylene glycol) at the nanoparticles surface [60]. These nanoparticles could be lyophilized in presence of 1% mannitol without aggregation. The dried particles were easily resuspended in saline or PBS, even after storage at either 4 °C or –20 °C over one month.

The chemical modification of chitosan by the introduction of *N*-acyl groups can improve the freeze-drying of nanoparticles prepared of these modified polysaccharides [61]. The size of unmodified and modified chitosan nanoparticles was in the range of 160–200 nm before freeze-drying. After freeze-drying, unmodified chitosan nanoparticles aggregated due to the strong inter- and intramolecular hydrogen bonding and did not break down by sonication, whereas, *N*-acyl chitosan particles were readily redispersed to nano-size (about 360 nm). This result could be explained by the fact that long acyl groups lessen the inter- and intramolecular hydrogen bonding and reduce the compactness of the network.

Finally, the composition of a surfactant mixture is very important for the freeze-drying of nanoparticles. Such effect has been studied on solid lipid nanoparticles stabilized with a mixture of Egg Phosphatidyl choline and tween 80 [62]; It has been found that the best result of freeze-drying was with an egg PC:Tween 80 (54:46 weigh ratio). The size and the polydispersity index of nanoparticles after freeze-drying were increased when more than 46% of Tween 80 was used. Also, the impact of surfactant amount (30, 40 or 50 mg/g) on the conservation of nanoparticles after lyophilization was more evident. The best conservation of nanoparticles size after freeze-drying has been obtained with 50 mg/g of surfactant mixture.

4.1.3. Influence of entrapped drugs

The entrapped drug in nanoparticles may in some cases influence the freeze-drying of nanoparticles. On

the other hand, the lyophilization process may induce the drugs leakage out of nanoparticles or their degradation as in the case of proteins. In literature, there are many examples of freeze-drying of loaded nanoparticles (Table 4).

To study the interference between encapsulated drug and the freeze-drying process, Dynasan solid lipid nanoparticles have been loaded with tetracaine and etomidate [63]. These loaded nanoparticles were freeze-dried using trehalose as cryoprotectant. It has been found that both drugs, tetracaine and etomidate, impaired the quality of the reconstituted product. The number and size of large aggregates increased with increasing drug concentration in the SLN dispersion. These instabilities were mainly attributed to free drug in the dispersion medium. During the freezing process, water will crystallize at the same time the concentration of the dissolved drug in the water will increase until reaching the eutectic. Presence of electrolytes (e.g. protonated drug) in the water reduces the zeta potential with increasing concentration. The reduction in zeta potential is considered to be one cause of aggregation. Thus, it may be advantageous to remove the free drug before lyophilization.

In the other hand, solid lipid nanoparticles loaded with all-trans retinoic acid (ATRA) could be freeze-dried successfully with a minor modification of nanoparticles size using sucrose as cryoprotectant [62]. This result could be explained by the relative absence of free drug in the dispersion medium, due to the nil solubility of ATRA in the aqueous medium and the strong interaction between ATRA and phospholipids.

Freeze-drying of solid lipid nanoparticles containing azidothymidine palmitate induced the loss of encapsulated drug [64]. Drug loss was associated with changes in particle diameter, which was dependent on the amount of sugars used as cryoprotectant. Trehalose was the most effective sugar in preventing SLN diameter changes and loss of azidothymidine palmitate.

This drug loss has been explained by the phase change of phospholipids. Thus, a fully hydrated phosphatidylcholine that is in a liquid crystalline phase at room temperature will be in the gel phase when dry. When the phospholipids are rehydrated, transformation from gel to liquid crystalline phase occurs as the lipid groups head hydrate. Such phase transition may lead to temporary inhomogeneous rearrangement of phospholipids resulting in particle aggregation and loss of incorporated azidothymidine palmitate to the aqueous

medium. Based on the water replacement hypothesis, trehalose is able to hydrogen-bond to phospholipid head groups, thus supplanting water as the membrane stabilizer and reducing the gel to liquid crystalline phase transition of dry phospholipids.

Freeze-drying of itraconazole-loaded nanospheres led to the desorption of drug adsorbed at the nanoparticles surface [65]. The responsible of such effect was the crystallization of poloxamer, used as non-ionic stabilizer of nanoparticles. This stabilizer crystallization led to the destabilization of the weakly adsorbed drug at the surface of the nanospheres leading to its desorption when resuspending the lyophilizate. Replacing the poloxamer by an anionic surfactant, sodium deoxycholate, resulted in a complete stabilization of itraconazole-loaded nanospheres after freeze-drying in the presence of 10% sucrose. The ionic surfactant sodium deoxycholate, which did not crystallize, stabilized itraconazole association. However, it has been found by another group of research that freeze-drying increased significantly the cyclosporine encapsulation within poly(ϵ -caprolactone) nanoparticles stabilized by poloxamer [66]. This result was probably due to the adsorption of free cyclosporine at the nanoparticles surface as the water sublimed.

Freeze-drying of PCL and PLGA nanoparticles loaded with cyclosporine induced minor particle size increase [37]. This size modification may change the oral pharmacokinetics of loaded drug. A larger MRT (the first order moment mean residence time) and higher drug hepatic levels have been obtained after the administration of freeze-dried nanoparticles.

Finally, it has been found that freeze-drying did not induce ampicillin leakage out of nanoparticles after freeze-drying whereas freeze-drying under the same conditions induce dramatic leakage of ampicillin from liposomes [67].

4.1.4. *Specific considerations concerning nanocapsules*

Nanocapsules are more delicate structures than nanospheres. Nanocapsules have a thin polymeric envelope that encapsulates an aqueous or oily core. This envelope may not withstand the stresses of freeze-drying process. The aggregation and fusion of nanocapsules and the loose of their encapsulated drug are common results of their freeze-drying. In literature, there are few papers which have studied the freeze-drying of nanocapsules. In this section the particularity

of nanocapsules freeze-drying will be discussed in details.

Auvillan et al. [16] have studied the possibility to freeze-dry poly(lactide acid) and poly(ϵ -caprolactone) nanocapsules using trehalose as cryoprotectant agent. Three conditions were decisive for conserving nanocapsules during freeze-drying: the concentration of trehalose, the freezing rate, and the melting temperature of the encapsulated oil. 30% of trehalose was necessary to protect the nanocapsules during the process (10% was not sufficient). Rapid freezing in an alcohol bath (at $-75\text{ }^{\circ}\text{C}$) or in liquid nitrogen (at $-196\text{ }^{\circ}\text{C}$) was more suitable for preserving nanocapsules size after drying. The included oils did not affect the diameter during freezing and freeze-drying as long as the solidification temperature of the encapsulated oil was lower than the essential freezing temperature of the suspension. Oil having a solidification temperature of $+4\text{ }^{\circ}\text{C}$ was less suited than one with a temperature of $-25\text{ }^{\circ}\text{C}$. Moreover, a solidification temperature of $-65\text{ }^{\circ}\text{C}$ was generally more appropriate as it did not cause the diameter to vary. The authors presumed that the preservation of the liquid state of encapsulated oil during the solidification of surrounding medium permitted a better resistance of nanocapsules membrane against the mechanical stress of freezing.

In another study, it has been found that freeze-drying of poly(ϵ -caprolactone) nanocapsules can break them, promoting the leakage of their contents [68]. It has been proposed that the nanocapsules could have been broken not by the water crystallization in the external phase, but by the solidification of the encapsulated oil (miglyol 812) in the internal phase. A slow freezing rate was more favorable for the nanocapsules conservation. The authors suggested that slow freezing should be applied and the product temperature should be kept above the oil solidification temperature.

Freeze-drying has been used to prepare a freeze-dried oral dosage form of indomethacin-loaded nanocapsules [69]. 10% of glucose was chosen as cryoprotectant agent. Lyophilization produced an increase in particle size after redispersion in water with an average of two-fold the initial particle size. This slight increase of nanocapsules size was explained by a nanocapsule clustering, because there was not a leakage of encapsulated indomethacin from nanocapsules after redispersion.

Nanocapsules of poly(ϵ -caprolactone) or eudragit S90 could be freeze-dried without leakage of encapsulated

drug (diclofenac) or breaking the capsule wall after the addition of colloidal silicon dioxide [10]. Scanning electronic microscopy revealed that the resulting powder presented non-spherical microparticles. The surface of these microparticles was covered by nanostructures.

It has been found that poly(vinyl alcohol) (PVA) adsorbs at the surface of nanocapsules prepared from poly(ϵ -caprolactone) by emulsification-diffusion method [20]. This stabilizer attaches strongly to the nanocapsules surface and forms a stable coating layer despite repeated washing for purification. Such polymer layer formed at the nanocapsules surface can stabilize the nanocapsules and improves their freezing resistance. Furthermore, free PVA which is not adsorbed at the nanocapsules surface forms a glassy matrix around nanocapsules during freezing leading to their stabilization. Nanocapsules could be freeze-dried without the addition of cryoprotectant when the amount of PVA was sufficient (about 2.5% to 5%). However, after purification, the addition of 5% of cryoprotectant such as sugars seems to be necessary to ensure the stability of nanocapsules. The concentration of polymer (PCL) and the solidification temperature of encapsulated oil have a negligible effect on the stability of nanocapsules during freezing. Similar results have been obtained during freeze-drying of antigen loaded poly(ethylcyanoacrylate) nanocapsules [70]. These nanocapsules were prepared by interfacial polymerization of a water-in-oil microemulsion and freeze-dried in presence of different types of sugars (glucose, maltose and sucrose). In the absence of cryoprotectant, nanocapsules could not be fully redispersed after freeze-drying and sonication indicating the formation of strong aggregates. A complete redispersion of nanocapsules with the conservation of their size and polydispersity index could be achieved when sugars were added at a concentration of 5% (w/v).

In a comprehensive study, the influence of lipid nanocapsules composition on their aptness to freeze-drying has been investigated [71]. Nanocapsules were formed from an oily core surrounded by a solid shell. A mixture of two surfactants was used to prepare the shell, lecithin and solutol. It has been found that low lecithin content formulations exhibit a poor aptness to freeze-drying, while formulations with a lecithin content of 5% or more can be freeze-dried and remain stable during storage. This result is consistent with the

assumption that lecithin, the constituting surfactant of the shell with a high melting point ($T_m=83^\circ\text{C}$), should be in sufficient amount to confer the appropriate rigidity to the nanoparticulate carrier as this surfactant crystallizes and allows the shell to get hardness and aptness to freeze-drying.

Furthermore, a good interaction could be found between lecithin and trehalose, the cryoprotectant used, reinforcing the stabilizing properties of lecithin.

4.2. Importance of the freeze-drying process

In a typical nanoparticles freeze-drying process, an aqueous suspension containing the nanoparticles and various formulation aids, or excipients, is filled into glass vials, and the vials are loaded onto the temperature-controlled shelves. The temperature of the shelves is reduced to a temperature in the vicinity of -40°C , thereby converting nearly all the water into ice. Some excipients, such as buffer salts and mannitol, may partially crystallize during freezing, but most cryoprotectants remain amorphous. The nanoparticles and excipients are typically converted into an amorphous glass also containing large amounts of unfrozen water (15–30%) dissolved in the solid amorphous phase. After all water and solutes have been converted into solids, the ice-vapor is evacuated by the vacuum pumps to the desired control pressure and the shelf temperature is increased to supply energy for sublimation, and primary drying begins. The removal of ice crystals by sublimation creates an open network of pores, which allows pathways for escape of water vapor from the product [72]. The ice-vapor boundary generally moves from the top of the product toward the bottom of the vial as primary drying proceeds. Primary drying is normally the longest part of the freeze-drying process. When the judgment is made that all vials are devoid of ice, the shelf temperature is typically increased to provide the higher product temperature required for efficient removal of the unfrozen water. The final stages of secondary drying are normally carried out at shelf temperatures in the range of 25–50 $^\circ\text{C}$ for several hours.

Freeze-drying nanoparticles is not an easy process and requires a comprehensive expertise and comprehension of the process. However, one may find that most of papers published in this field studied the freeze-drying of nanoparticles by trial and error, i.e. by

trying different conditions of freeze-drying and selecting the best after the analysis of freeze-dried product. It is now well known that the various stages of lyophilization are based on very sound physical, chemical and engineering principles and can be controlled to the extent that the outcome of a given process performed on a given product can often be estimated to within fairly close tolerance, without the need for trial-and-error experimentation [17]. Even more important, stable freeze-dried nanoparticles can be designed by matching an optimum nanoparticle formulation with its associated optimum drying process cycle. Table 4 presents examples of freeze-drying nanoparticles with precisions about the process conditions of freeze-drying when they are mentioned.

In order to design an optimum nanoparticles freeze-drying process, process development scientists need to know the critical properties of the optimized formulation and how to apply this information to process design. The critical formulation properties include the glass transition temperature of the frozen sample (T_g'), the collapse temperature of the formulation (T_c), the stability of the nanoparticles and their encapsulated drug, and also the properties of the excipients used. The collapse temperature is the maximum allowable product temperature during primary drying [28]. Freeze-dried product loses macroscopic structure and collapses during freeze-drying when it is heated to above the temperature of collapse (T_c). T_c is usually about 2 °C higher than T_g' , or equals the eutectic temperature (T_{eu}).

4.2.1. Freezing step

Freezing is the first stage of freeze-drying and is the stage where most of the water is removed from nanoparticles and excipients. The system is separated into multiple phases and the interfaces between ice and nanoparticles form. Freezing often induces many destabilizing stresses for nanoparticles. These stresses include increase of nanoparticles concentration that enhances the interaction between them leading to their aggregation or fusion.

To ensure the total solidification of a frozen sample, the nanoparticles phase should be cooled to below the T_g' of the formulation if it is amorphous or below T_{eu} if it is in the crystalline state, where, T_g' is the glass transition temperature of maximally cryo-concentrated sample, and T_{eu} is the eutectic crystallization temperature of the crystalline frozen sample. This condition

requires the shelf temperature for freezing be set below T_g' or T_{eu} of the formulation. The frozen nanoparticles samples must be kept at the set temperature for a sufficient time to transform all the suspension into solid. Usually, 2 h is sufficient for this purpose if the fill depth is less than 2 cm. So, it is very important to determine T_g' or the T_{eu} of the nanoparticles formulation by thermal analysis before starting the freeze-drying study. However, few articles in literature have been determined the thermo-physical properties of nanoparticles formulation before freeze-drying [19,37,65].

In literature, different freezing methods have been used to freeze nanoparticles suspensions (Table 4), like liquid nitrogen freezing, loading vials onto precooled shelves, or ramped cooling on the shelves, these different freezing methods give different supercooling effects. Normally, the highest supercooling could be obtained with liquid nitrogen freezing of small volumes and the lowest one for the precooled shelf method. Higher supercooling results in smaller ice crystals and larger ice specific surface area [73]. A fast cooling with a higher supercooling may improve the freeze-drying of nanocapsules as mentioned in the section: freeze-drying of nanocapsules [16]. High supercooling leads to the formation of small ice crystals and may decrease the mechanical stress on nanocapsules avoiding their aggregation. On the other hand, the same authors have found that slow freezing improved the freeze-drying of nanospheres prepared from different type of polymers.

Fast freezing without any cryoprotectant resulted in less aggregation after thawing of purified mannan-coated cationic nanoparticles than slow freezing [74]. However, with cryoprotectant, the rate of freezing did not affect the resulting particle size of the thawed nanoparticles. The same results have been found for the freezing of purified poly(ϵ -caprolactone) nanocapsules without cryoprotectant [20]. On the other hand, the freezing method had an effect on the particle size of long-circulating monensin nanoparticles freeze-dried with 2.4% mannitol where rapid freezing was found to have smaller size change of nanoparticles (a 26.1% increase in size after freeze-drying), in comparison with nanoparticles freeze-dried using slow freezing in presence of the same concentration of mannitol (a 57.3% increase in size after freeze-drying) [75]. However, the freezing method had a negligible effect on the particle size of nanoparticles freeze-dried with trehalose. Such result may be explained by the ability of

mannitol to crystallize when applying slow freezing, but rapid freezing may produce more amorphous mannitol able to protect nanoparticles during freezing.

Adding of 5% of trehalose, sucrose and glucose maintained the properties of PLGA or PCL nanoparticles after freeze-thawing independently of the cooling conditions (Freezing at -70°C or in liquid nitrogen) and the polymer type [37]. On the other hand, sorbitol proved to be an effective cryoprotectant when PLGA were frozen at -70°C , also sorbitol prevented the formation of macroscopic aggregates during the freezing of PCL nanoparticles at -70°C and PLGA at -196°C .

Solid lipid nanoparticles prepared from glyceryl monostearate have been freeze-dried [76]. It has been found that quick freezing in liquid nitrogen destabilized the nanoparticles leading to a greater formation of aggregates after redispersion. Whereas slower freezing at -70°C in a deep freezer proved to be the better method for this formulation. However, fast freezing in liquid nitrogen proved to be most efficient for trehalose and glucose protected Dynasan solid lipid nanoparticles.

4.2.2. *Annealing to optimize freeze-drying process*

The freezing step can impact the texture of the frozen matrix and the final morphological characteristics of the freeze-dried cake [77]. Thus, the freezing process influences the ice crystal size and, consequently, the primary and secondary drying stages. The porosity and the specific surface of the final cake depend on these stages of the process. In general, the optimization of freeze-drying cycle is aimed at accelerating the sublimation which is the longest step of the whole process.

Annealing is a process in which samples are maintained at a specified subfreezing temperature above the glass transition temperature for a period of time [78]. 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. [78] 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 to give a more homogenous structure.

Annealing has been applied during freeze-drying of poly(ϵ -caprolactone) nanocapsules using sucrose (T_g' is about -31°C) and poly(vinyl pyrrolidone) (T_g' is about

-22°C) as cryoprotectants [79]. Annealing has been applied for 1 h at different temperatures (20°C , -15°C and -10°C). Annealing of nanocapsules suspension could accelerate the sublimation rate without any modification of nanocapsules size to about 30% and 17% in the case of sucrose and PVP respectively. Such improvement could be explained by the increase of ice crystals size after annealing and by the diminution of mass transfer resistance by the dried layer. It has been demonstrated that the influence of annealing on secondary drying is dependent on the type of cryoprotectant used. Annealing of sucrose solution slows down the secondary drying kinetic whereas no effect is observed in the case of PVP.

Annealing was also useful to optimize the freeze-drying of glyceryl monostearate solid lipid nanoparticles using fructose and trehalose as cryoprotectants [76]. Annealing was applied at 22°C for 2 h. This temperature is well above the T_g' of both trehalose (-30°C) and fructose (-42°C). Some 99% of all particles were smaller than 300 nm before and after freeze-drying and reconstitution for both cryoprotectants.

The thermal treatment by annealing is also useful to eliminate the thin skin layer formed at the top surface of freeze-dried cake [80]. This skin is formed by the migration of cryoprotectants and nanoparticles during the crystallization of ice. Such skin layer may prevent the transfer of water vapor during sublimation and slow the sublimation rate resulting in heating the product and its fusion. To cancel this effect of skin layer, a thermal treatment by annealing has been applied during freeze-drying of nanoparticles produced from monomeric isohexyl cyanoacrylate in which doxorubicin was encapsulated. The suspension contained: 1% dextran 70, 5% glucose, 10 mg of doxorubicin chlorate and 50 mg of lactose. The product (1.3 mL/vial) was frozen on shelves at -50°C for 3 h and thermally treated for 24 h at -35°C . After freeze-drying nanoparticles with doxorubicin have the same diameter after rehydration (351 ± 52 nm) as before freeze-drying (334 ± 55 nm). After freeze-drying with annealing, samples presented a homogeneous aspect with a porous surface without a shining aspect at the top of dried cake.

4.2.3. *Primary drying step*

This stage of freeze-drying involves the removing of ice by sublimation. The temperature product during primary drying must be adjusted to below the collapse

temperature of the formulation. The operating conditions (pressure and shelf temperature) must be controlled to achieve this purpose. The temperature of collapse can be determined by freeze-drying microscopy. The product temperature during lyophilization can be measured by inserting thermocouples into vials. Chamber pressure and shelf temperature have been well studied as the main factors of the freeze-drying process which can improve the sublimation rate. However, these factors must be well adjusted so as to avoid the product overheating and its collapse.

Although the formulation collapse does not modify the nanoparticles diameter as found during the freeze-drying of poly(ϵ -caprolactone) nanocapsules using glucose as lyoprotectant (T_c is about -42 °C), it produces a cake with unacceptable aspect [20]. Furthermore, high residual water and prolonged reconstitution times are common consequences of collapse in a product. The same result has been obtained after freeze-drying of different nanoparticles formulations in presence of glucose as cryoprotectant using unsuitable conditions of freeze-drying (pressure and shelf-temperature). The reconstitution of collapsed cake was very difficult with the absence of porous structure [41,81,82].

In literature, the most papers that have studied the freeze-drying of nanoparticles there is not a determination of collapse temperature before freeze-drying. Furthermore, the product temperature is not controlled in most cases. Also, the processing conditions (pressure and shelf temperature) have been chosen by trial and errors without applying the scientific principles of freeze-drying. Table 3 presents some of the process conditions applied for the freeze-drying of different formulations of nanoparticles.

For example, the collapse of solid lipid nanoparticles freeze-dried with fructose and trehalose as cryoprotectant was obtained [76]. The applied conditions during primary drying were: 1 mbar with shelf temperature -30 °C for 7 h. At a pressure of 1 mbar one would expect that product temperature will be of the order of -20 °C. This temperature is well above the collapse temperature of trehalose (-34 °C) and that of fructose (-40 °C).

4.2.4. Secondary drying step

Secondary drying involves the removal of absorbed water from the product which did not

separate out as ice during the freezing, and so did not sublimate off. The unfrozen water may be adsorbed on the surface of the crystalline product or is in the solute phase either as hydrate water in a crystalline hydrate or dissolved in an amorphous solid to form a solid solution [83]. It is usually present in enough quantities to cause rapid decomposition of the product when it is stored at room temperature as it is. Secondary drying begins locally when all ice has been removed from that region.

The residual moisture content desired for a product usually determines the length of time devoted to secondary drying. For pharmaceuticals, it appears that moisture contents of 1% and less are the most desirable [27].

However, there are very rare papers that have studied this stage of freeze-drying of nanoparticles (Table 3). In many papers there is not a quantification of the residual water after nanoparticles freeze-drying and many of them did not distinguish between the main drying and the secondary drying.

It has been found that high residual water content may destabilize nanocapsules during storage because of cryoprotectant crystallization [19]. It is well known that the crystallization of amorphous carbohydrates (sugars) is a consequence of holding the system above its T_g , and is also a time-dependent phenomenon. As water may induce a shift in the T_g of the formulation to below the temperature of storage, thus a high residual water content may start the crystallization of the formulation during storage.

4.3. Importance of storage

Long-term stability is often required after freeze-drying. It involves chemical and physical stability and includes the prevention of degradation reactions (e.g. hydrolysis). Although few studies of such investigation have been reported, our understanding is that the long-term stability of nanoparticle suspensions depends primarily on the formulation, the knowledge of the ways of degradation in solution and the conservation conditions.

This stability study can be carried out during storage at 25 °C and residual humidity 60% for 12 months. Every month, the size, zeta potential, drug loading etc. must be evaluated to detect any instability of nanoparticles. In some cases, it is useful to realize an accelerated

testing at 40 °C at residual humidity of 75% as recommended in the International Conference on Harmonization (ICH) guidelines [84].

Indomethacin nanocapsules prepared by anionic interfacial polymerization method have been freeze-dried without a lyoprotectants [85]. The physical stability of these nanocapsules has been evaluated during storage for 12 months under different conditions (at ambient temperature, at 4 °C, and at –30 °C). The physical stability was measured by the extent to which encapsulated indomethacin was retained in the nanocapsules during storage. The authors did not mention the size of nanocapsules after freeze-drying or storage. Unexpectedly, after the rehydration of the freeze-dried indomethacin nanocapsules that were stored at ambient temperatures, losses of 8.5%, 26% and 50.5% indomethacin were found after 2, 4, 6 months respectively. But when freeze-dried nanocapsules were stored at 4 °C, the drug loss was only 9.3% at the end of 12 months.

5. Physico-chemical characterization of freeze-dried product

It is very important to characterize the freeze-dried matrix and to investigate the conservation of the nanoparticle properties. Furthermore such characterization may validate the applied conditions of the process and the optimized formulation. This section

will present the most useful methods of characterization of freeze-dried matrix and nanoparticles.

5.1. Macroscopic aspect of freeze-dried product

A critical analysis of freeze-dried products normally includes the observation of the final volume and the appearance of the cake. One of the desired characteristics of a freeze-dried pharmaceutical form includes an intact cake occupying the same volume as the original frozen mass (see vials in Fig. 2). An attentive examination of the macroscopic aspect of the freeze-dried cake must be carried out to detect any shrinkage or collapse of the formulation.

5.2. Reconstitution time

To rehydrate the freeze-dried nanoparticles one must add the same volume of water lost after lyophilization. The time of reconstitution may be recorded. In general, freeze-dried product rehydrates immediately after the addition of water, but in some cases, a long reconstitution time could be obtained as in the case of collapsed formulations. Many methods could be used to achieve the re-suspension of freeze-dried nanoparticles after the addition of water, as manual shaking, vortexing or sonication to ensure full re-suspension.

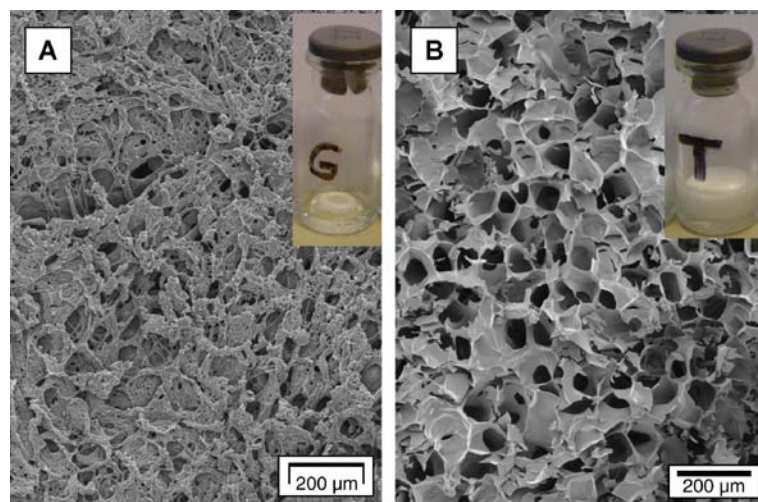


Fig. 2. Photographs of macro- (see vials) and microstructure of collapsed and non-collapsed freeze-dried (A) sucrose (Tc –30 °C) (B) PVP (Tc –22 °C). Note the partial collapse of sucrose and the apparition of holes in the structure whereas the plates of PVP are intact.

5.3. Measurement of nanoparticles size and zeta potential after freeze-drying

After reconstitution, nanoparticles size must be measured by photon correlation spectroscopy or another technique. The conservation of a nanoparticle diameter size after freeze-drying is considered as a good indication of a successful freeze-drying cycle. In general, the ratio of nanoparticles size after and before freeze-drying may be calculated. A value near from one indicates the conservation of nanoparticles size, whereas an important value of this ratio indicates the aggregation of nanoparticles. Furthermore, the index of polydispersity may be recorded after lyophilization. This index gives also an idea about the distribution of nanoparticles size and its value must be compared to the value before freeze-drying, to evaluate the conservation of nanoparticles distribution.

The measurement of zeta potential is a good method to evaluate the state of nanoparticles surface and to detect any eventual modification after freeze-drying. Furthermore, it can be used to study the interaction between the cryoprotectant molecules and the nanoparticles surface. It has been found that the addition of 10% of sucrose to itraconazole loaded poly(ϵ -caprolactone) nanospheres suspension before freeze-drying decreased the negative surface charge from -40.9 mV to -20.4 mV [65]. The authors explain this by the fact that nanosphere surface being masked as a result of hydrogen bonding between OH groups of the cryoprotectant agent and the surface of the nanospheres. After freeze-drying, the decrease in the negative surface charge is accentuated, showing a rearrangement of the surfactants (poloxamer) at the surface of the nanospheres, leading to a possible desorption of itraconazole molecules.

5.4. Microscopic observation of freeze-dried product

The microscopic visualization of freeze-dried product is a direct way on the one hand to observe the microstructure of the freeze-dried matrix, on the other hand to prove the conservation of nanoparticles integrity and to observe whether any modification has occurred on their morphology.

Many high resolution microscopic techniques could be used to observe the nanoparticle formulation after freeze-drying: transmission electron microscopy

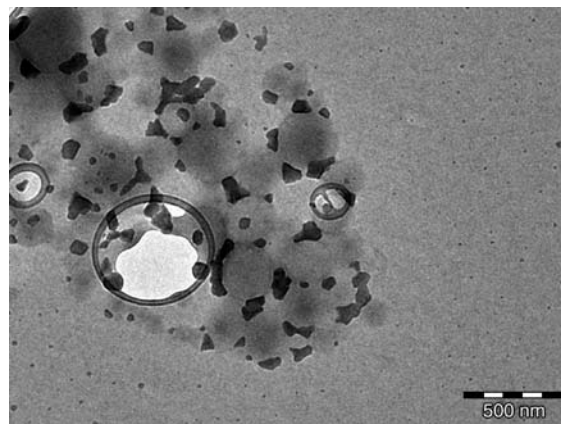


Fig. 3. TEM observation of freeze-dried nanocapsules with 5% of PVP after reconstitution. Observation was performed without negativation.

(TEM), cryogenic transmittance electron microscopy (cryo-TEM), atomic force microscopy (AFM), scanning electronic microscopy (SEM), environmental scanning electronic microscopy (ESEM).

TEM was also used to observe freeze-dried itraconazole-loaded nanospheres [65], and poly(ϵ -caprolactone) nanocapsules after reconstitution [79]. It is clear from TEM image that nanocapsules were well conserved after freeze-drying using PVP as cryoprotectant (Fig. 3). The polymeric membrane was intact around the oily cavity of nanocapsules. An amorphous matrix of PVP could be observed at the outer surface of nanocapsules.

Furthermore, freeze-dried core shell nanoparticles have been imaged by cryogenic transmittance electron microscopy to verify the formation of core/shell nanoparticles [86]. Freeze-dried cationically modified silica nanoparticles using 5% of trehalose as cryoprotectant could be observed by AFM [41]. It could be found from AFM images, that trehalose formed a matrix into which the nanoparticles were interdispersed. All particles were nicely separated by the matrix. In addition, the trehalose formed a coat that surrounded the individual particles.

SEM was used to observe the microstructure of the freeze-dried PVP and sucrose preparations. Fig. 2 demonstrates that sucrose develops holes in the structure that indicates a collapse of dried product (Fig. 2A) whereas PVP dries into intact plates with the conservation of porous structure (Fig. 2B). Purified freeze-dried nanocapsules protected by HP- β -CD have been

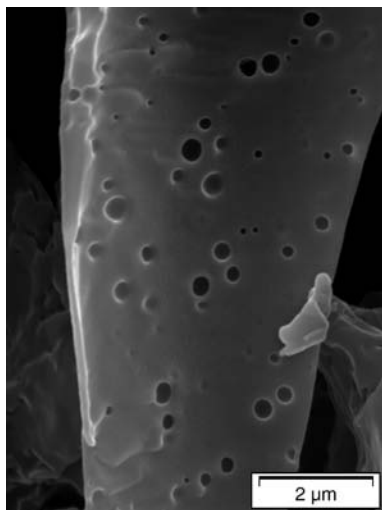


Fig. 4. SEM imaging of freeze-dried nanocapsules stabilized with 5% w/v PVA after reconstitution. Note the formation of PVA film and the inclusion of nanocapsules inside it.

observed with SEM and ESEM after their reconstitution [19]. In Fig. 4, SEM imaging shows a continuous and amorphous matrix into which PCL NC is interdispersed. All particles are well separated throughout the glassy state of HP- β -CD matrix. ESEM imaging showed spherical monodisperse nanocapsules being well conserved after freeze-drying. (Fig. 5).

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 prevents the observation of individual nanocapsules. Furthermore, this technique has the ability to image wet systems without prior sample preparation. Finally, ESEM is the best technique for observing of lyophilized nanocapsules in a hydrated state. The advantages of ESEM over SEM for observing colloidal particles with minimal perturbation are the possibility to observe hydrated samples in their native state, without need of conductive coating of the samples and no need of the preparation of the samples.

It has been demonstrated that TEM allows the observation of freeze-dried nanoparticles after dilution of the samples while SEM and AFM the observation of nanoparticles becomes more difficult when the protectant concentration is more than 5%. In this

case a continuous matrix was observed with some nanoparticles. ESEM is the most adequate technique to observe nanoparticles in a hydrated state.

5.5. Thermal analysis by differential scanning calorimetry (DSC)

During storage, freeze-dried nanoparticles included within a vitrified matrix of lyoprotectant must be stored at a temperature below the temperature of glass transition (T_g) of the dried formulation to prevent any shrinkage of the freeze-dried cake or any destabilization of included nanoparticles as a result of lyoprotectant crystallization. The temperature of glass transition may be determined by differential scanning calorimetry. Furthermore, this technique is very useful to study the interaction between the lyoprotectant and the nanoparticles. For example, in the case of solid lipid nanocapsules freeze-dried with trehalose, DSC study points out a complexation between lecithin (forming the shell of nanocapsules) and trehalose, reinforcing the stabilizing properties of lecithin [71].

5.6. Drug content determination

The drug content in nanoparticles must be determined by an adequate analysis method as High performance liquid chromatography (HPLC) and its value must be compared to that before freeze-drying to detect any leakage of drug from nanoparticles during freeze-drying.

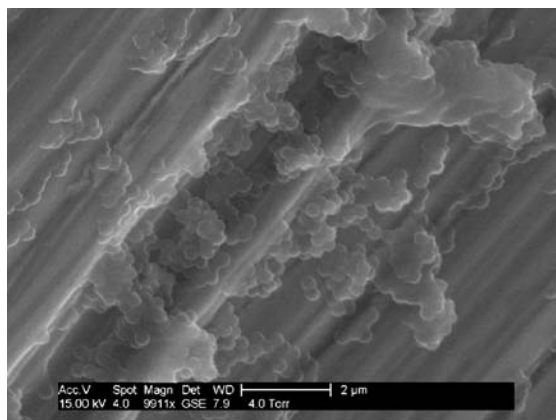


Fig. 5. ESEM imaging of freeze-dried purified PCL NC after reconstitution prepared with HP- β -CD as cryoprotectant.

5.7. Powder surface analysis

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 the surface modification of nanoparticles [11], and the adsorption of proteins at the air/liquid interface during spray-drying [87] and to the ice crystals surface in the frozen material during freeze-drying [88].

ESCA analysis of nanocapsules freeze-dried with poly(vinyl pyrrolidone) shows that the poly (ϵ -caprolactone) and cryoprotectant matrix contribute to the recorded spectrum which means that some of the nanocapsules are present at the powder surface. A similar result was obtained with nanocapsules freeze-dried with sucrose [19].

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 results in surface induced denaturation of proteins [89]. 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 nonionic surfactants such as tween 80 are often sufficient to serve this purpose [89].

5.8. Study of water sorption and determination of residual moisture

The thermal and the structure properties of freeze-dried nanoparticles are influenced by residual moisture

in the product. Residual moisture is determined by the water desorption process during secondary drying.

Sorption isotherm of water study is realized in order to determine on the one hand the degree of hygroscopicity of the product and on the other hand to assess the ease in secondary drying. In general, the easier the water adsorption, the easier water desorption. The content of residual moisture in freeze-dried nanoparticles can be determined by Karl Fischer titration or by other methods as the gravimetric method or the thermal gravimetric analysis.

6. Application of freeze-drying in the domain of nanoparticles

The main use of freeze-drying in the field of colloidal nanoparticles is to improve their long-term stability. However, freeze-drying has been applied for other objectives, as the improvement of drug association to nanoparticles, the preparation of core/shell nanoparticles, the production solid dosage form and for the analytical characterization of colloidal systems.

6.1. To improve the stability of nanoparticles

Freeze-drying as a drying method has many applications for nanoparticles technology. The literature contains many examples of such applications. The main use of freeze-drying is essential for improving long term nanoparticles stability. The transformation of colloidal suspension into solid form has the advantage of preventing particles aggregation, also the degradation of polymer forming nanoparticles and the leakage of encapsulated drug out of nanoparticles. Furthermore, freeze-drying could be transformed into another solid dosage form intended for different administration routes (parenteral, oral, nasal, or pulmonary.).

It has been found that freeze-drying could stabilize fragile poly(ϵ -caprolactone) nanocapsules for six months during storage under extreme conditions of temperature and humidity. Such result has been obtained when a suitable lyoprotectant as PVP and optimized conditions of freeze-drying have been applied [19].

The stability of freeze-dried poly(methylidene malonate 2.1.2) (PMM 212) nanoparticles was

evaluated after storage for 12 month under various storage conditions of temperature and illumination [90]. The results revealed that nanoparticles maintained at 40 °C underwent significant alterations revealed by the suspension pH decrease, the progressive modification of the HPLC chromatogram of encapsulated components and the decrease in vitro cytotoxicity. Furthermore, the degradation of the polymer side chains and generation of carboxyl moieties could be observed. On the other hand, lyophilized PMM 212 colloidal nanoparticles conserved at room temperature or below, either in darkness or in daylight may be assumed to have a satisfactory shelf-life.

The size of freeze-dried solid lipid nanocapsules (SLN) remained stable after three months of storage under two storage temperatures: 5 °C and 40 °C at 75% relative humidity. The stored nanocapsules did not exhibit any oil leakage after 3 months storage [71].

Dehydroemetine nanoparticles for treating visceral leishmaniasis have been freeze-dried using glucose 5% as cryoprotectant. These freeze-dried nanoparticles could be stored at –20 °C for 24 months without any modification of their size or the level of drug binding [91].

Finally, freeze-drying with trehalose was a good alternative to stabilize solid lipid nanoparticles loaded with azidothymidine (AZT) without any modification of their size or their drug content, because the storage of these nanoparticles at both 37 °C or 4 °C induces an increase in particle size and loss of AZT [64].

6.2. To improve the drug association to nanoparticles

Freeze-drying has been also used to improve the association of polar drugs as Amikacin sulfate to the surface of hydrophobic carriers as poly(alkylcyanoacrylate) nanoparticles [92]. Nanoparticles were prepared by the emulsion polymerization method from butylcyanoacrylate monomer and stabilized by dextran 70. The drug was dissolved in the polymerization medium at several concentrations; once polymerization was over the suspension was neutralized and freeze-dried in order to adsorb the free drug not incorporated in the polymer matrix more efficiently. Drug loading was determined by polarization fluoro-

immunoanalysis and found to be about 66 µg/mg. Whereas, drug loading was about 5.95 µg/mg for the standard procedure of loading nanoparticles without freeze-drying. The large difference in drug-polymer association rate when the polymer was freeze-dried shows that freeze-drying facilitates the drug-polymer interaction.

6.3. To produce solid dosage forms intended for various administration routes

Freeze-drying has the advantage of producing stable solid dosage forms for various administration routes. The pharmaceutical applications of such nanoparticles orale lyophilizate as a freeze-dried oral dosage form of indomethacin-loaded nanocapsules [69]. Poly(lactide acid) nanocapsules containing indomethacin has been prepared by nanoprecipitation method. Then, a large amount of lactose as inert additive was added to build up a paste solid lyophilizate. The second step was to include a colloidal additive as Arabic gum in order to avoid settling of the suspension before freezing. Arabic gum was used as an aqueous solution and added to the suspension in order to obtain 2.5 to 10% of dry Arabic gum in the formula. These texture additives were incorporated into 10 mL of a nanocapsules suspension containing 10% glucose as cryoprotectant. Finally, the freeze-drying was applied to obtain the oral dosage form.

6.4. To prepare core/shell nanoparticles

Another interesting application of freeze-drying is to prepare core/shell nanoparticles [86]. These nanoparticles are formed of a drug-loaded lipid core composed of lecithin and polymeric shell composed of pluronics (Pluronic® or poloxamer) (poly(ethylene oxide)poly(propylene oxide)-poly(ethylene oxide) triblock copolymer. After the preparation of drug-loaded lipid core, it was freeze-dried with a solution of pluronics in presence of trehalose to induce the formation of a polymeric shell on the surface of the lipid core. Freeze-drying may enhance the adsorption of pluronics at the surface of lipid core to form core/shell nanoparticles. The formation of these core/shell nanoparticles was confirmed by cryogenic transmittance electron microscopy, differential scanning calorimetry, and a particle size analyzer.

6.5. To obtain dry product suitable for analytical characterization

Freeze-drying has been used to obtain dry nanoparticles used for the analytical determination of the drugs and the thermal analysis. Solid lipid nanoparticles (SLN) containing hydrocortisone and progesterone complexes with β -cyclodextrins were freeze-dried without the addition of cryoprotectants [93]. A thermal analysis by differential scanning calorimetry was performed on these freeze-dried nanoparticles. Furthermore, the amount of hydrocortisone or progesterone incorporated in SLN was determined on the freeze-dried SLN by HPLC analysis.

Hu et al. [94] have used freeze-drying to calculate the recovery of glyceryl monostearate solid lipid nanoparticles loaded with clobetasol propionate after their preparation. The recovery of SLN was defined as the weight ratio of the freeze-dried SLN to the initial loading of monostearin and drug and calculated from the following equation

$$\text{Recovery} = \frac{\text{analyzed weight of SLN}}{\text{theoretical weight of SLN}} \times 100$$

However, this equation does not take into account the residual humidity in the final freeze-dried cake which, must be determined to have a correct estimation of SLN weight.

7. Conclusion

Freeze-drying of nanoparticles is a very complex process that requires a major investigation of the formulation and the process conditions. Many parameters of the formulation may decide the success of freeze-drying as the nanoparticles composition (type of polymer, type and concentration of surfactant, type and concentration of cryo and lyoprotectants, interaction between cryoprotectants and nanoparticles, surface modification of nanoparticles). Furthermore, the applied conditions of freeze-drying can impact the stabilization of nanoparticles during and after freeze-drying, especially the velocity of freezing with or without annealing, the pressure and shelf temperature, and the duration of each stage of the process. Many methods are available for assessing final freeze-dried product to ensure the conservation of nanoparticles properties and the required qualities of freeze-dried cake.

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