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Review on: freeze drying as a crystallization technique

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ABSTRACT

Freeze-drying has been developed as a good technique to improve the solubility, dissolution and stability of microparticle. The poor stability and dissolution in an aqueous medium of these systems forms a real barrier against the pharmaceutical use of microparticle and microparticle. This reviews paper suggest about the freeze-drying microparticle and microparticle. It discusses the most important parameters that influence the success of freeze-drying of these fragile systems, and provides an overview of microparticle freeze-drying process and formulation strategies with a focus on the impact of formulation of different particles (nanoparticles and microparticle) and crystals stability.

Introduction

Many solid dosage forms in the pharmaceutical and biotech industries are based on different form of crystals. Dry powders are inhaled as aerosols into the lung, delivered to the nose, filled into capsules, or pressed into tablets for oral applications, or even delivered transdermally. In the past, crystals were often viewed simply as carriers, usually crystallized dry material, without sophisticated attributes. The primary functions of the crystallization and drying processes were to achieve a suitable particle size and remove most of the solvent. This perspective has changed as novel drug delivery strategies were developed. More advanced therapeutic approaches have created complex requirements for dosage forms that can only be met by crystals that are designed for a range of functions such as stabilization of the active, transport and targeting of the dose, or release modulation. The particle is no longer seen as a passive carrier, but rather as an essential part of the drug delivery system. Crystals Engineering is a young discipline that combines elements of microbiology, chemistry, formulation science, colloid and interface science, heat and mass transfer, solid state physics, aerosol and powder science, and nanotechnology. It provides the theoretical framework for a rational design of structured crystals Particle engineering requires a deeper understanding of particle formation processes. Complex structured crystals are difficult to design using an empirical approach alone because of the many process and formulation variables that need to be tuned correctly to achieve the desired result. Efforts to understand and control particle formation processes were intensified in the last decade, coinciding with the development of pulmonary therapeutics that were traditionally given by injection (1–3). The pulmonary route was found to be viable for systemic delivery of proteins and peptides (4–6), in particular insulin (7–18), triggering the development of diverse administration systems and particle engineering strategies (19–28). Crystallization can be manufactured by many different processing methods like spherical agglomeration, effect of solvent,, effect of surfactants, solid deposition, solid composition, freeze drying etc. This review focuses exclusively on spray drying (29–35), with an emphasis on the literature of the last few years. Wet chemistry and phase separation processes and alternative drying processes such as spray freeze drying, or supercritical fluid technologies, have also been used widely for crystals engineering purposes and have been reviewed elsewhere (36–41).

In the last decade, significant effort has been done to develop nanoparticle and microparticles for drug delivery [42–46]. 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 [47–51]. Microparticles and microparticle are submicron sized colloidal polymeric systems. According to the process used in preparing microparticle, nanospheres or nanocapsules can be obtained [52-56]. Microparticle and 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 microparticle and microparticles offers a numerous advantages over microparticles. Microparticles have in general relatively higher intracellular uptake compared to microparticles. It was demonstrated that microparticle size of 100 nm showed 2.5 fold greater uptake compared to 1 μm and 6 fold higher uptake compared to 10 μm microparticle in Caco-2 cell line [48]. Similar results were obtained when these formulations of nano- and microparticle 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) microparticle [49]. The polymers used for the preparation of microparticles can be biodegradable such as: polylactide,

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polyglycolide, and their copolymers poly(lactide coglycolide), polycaprolactones, polyacrylates, or nonbiodegradable 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 [42–57]. Nevertheless, the major obstacle that limits the use of these microparticles is due to the physical instability (aggregation/particle fusion) and/or to the chemical instability (hydrolysis of polymer materials forming the microparticles, drug leakage of microparticles and chemical reactivity of medicine during the storage) which are frequently noticed when these microparticle and microparticle aqueous suspensions are stored for an extended periods [57,59]. 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 [59]. 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 microparticle and microparticles from freezing and desiccation stresses. In the literature, there are a few papers on freeze-drying process of polymeric microparticle and microparticles compared to other colloidal systems such liposomes. Furthermore most often, the investigations into microparticle and microparticles 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 shelflife of several years [60-62]. To improve the performances of these microparticle and microparticles, 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 microparticle and microparticle size, and the drug entrapment), iii) a weak residual humidity b2%), iv) and also a good long-term stability of the formulation. This article reviews the state of the microparticle and microparticles freeze-drying. It discusses the most important parameters that influence the freeze-drying success of these particulate systems, and provides an view of the microparticle and microparticles freeze-drying process and formulation strategies with a focus on formulation and process on microparticles stability. A successful microparticle and microparticles freeze-drying requires a deep investigation of the formulation and the process conditions to retain the properties of both microparticle and microparticles and to have a long shelf-life of the dried product.

Stages of freeze drying

A typical freeze-drying process consists of three stages; that is, 1) freezing, 2) primary drying, and 3) secondary drying. Freezing is an efficient dessication step where most of the solvent, typically water, is separated from the solutes to form ice. As freezing progresses, the solute phase becomes highly concentrated and is termed the "freeze concentrate." By the end of freezing, the freeze concentrate usually contains only about 20% of water (w/w), or less than 1% of total water in the solution before ice formation. The freezing stage typically takes several hours to finish. Primary drying, or ice sublimation, begins whenever the chamber pressure is reduced and the shelf temperature is raised to supply the heat removed by ice sublimation. During primary drying, the chamber pressure is well below the vapor pressure of ice, and ice is transferred from the product to the condenser by sublimation and crystallization onto the cold coils/plates (<−50°C) in the condenser. Typically, the primary drying stage is the longest stage of freeze drying and optimization of this stage has a large impact on process economics. Secondary drying is the stage where water is desorbed from the freeze concentrate, usually at elevated temperature and low pressure. Some secondary drying occurs even at the very beginning of primary drying as ice is removed from a region, but the bulk of secondary drying occurs after primary drying is over and the product temperature has increased. Secondary drying normally takes only hours, and the opportunity for time reduction by process optimization is limited (16). With an optimum freeze-drying process, the freeze-drying process is optimized for all the three stages.

Freezing

Freezing is the first stage of freeze drying and is the stage where most of the water is removed from drug and excipients, the system separates into multiple phases, and the interfaces between ice and drug phase form. Freezing often induces many destabilizing stresses, particularly for protein drugs. These stresses include increase of protein concentration that enhances the protein–protein interaction leading to aggregation, pH change arising from crystallization of buffer salts, reduced hydrophobic interactions caused by the "dehydration" effect of ice formation that removes bulk water from the protein phase, formation of large ice aqueous interfaces, and an enormous increase in ionic strength (16). The introduction of the ice–aqueous interfaces and pH shifts are well-known to cause protein stability problems. The pH shift during freezing can be minimized by optimal choice of buffer salts (i.e., avoid phosphate, succinate, and tartrate) or by reducing buffer concentration to several mM (17–19). Protein degradation at the ice–aqueous interface can be minimized by increasing protein concentration (i.e., "saturate" the protein–ice interface) and/ or by using surfactants (14). For a given protein formulation, process design also plays a very important role in protein stabilization.

Cooling rate

One practical process approach to stabilization is to minimize the surface area of ice by growing large ice crystals which can be achieved by reduced super cooling. The degree of super cooling is the temperature difference between the thermodynamic or equilibrium ice formation temperature and the actual temperature at which ice begins to form, which is usually around 10 to 25°C lower but changes with cooling rate and other factors. Higher super cooling results in more/ smaller ice crystals and larger ice specific surface area. Different freezing methods, like liquid nitrogen freezing, loading vials onto precooled shelves, or ramped cooling on the shelves, give different supercooling effects with normally the highest super cooling with liquid nitrogen freezing of small volumes and the lowest supercooling for the precooled shelf method (20). It was reported that slow cooling (0.5°C/min) caused larger super cooling effects than the precooled shelf method (20). However, the precooled shelf method gave large heterogeneity in super cooling between vials, which is undesirable (20). Normally, it is not practical to manipulate the super cooling by changing the cooling rate in a freeze dryer because the cooling rates are usually limited to less than 2°C/ min, and the degree of super cooling is unlikely to change within such a small range (21). Slow freezing has the potential to increase the protein damage in

systems prone to phase separation because phase separation is a kinetic process and provides enough time for the process to occur (22). If phase separation causes separation of protein and stabilizer, the stabilization effect will be lost. Phase separation is most common when polymers are used for stabilizers (23,24). Slow freezing also prolongs the time the protein exists in a concentrated fluid state where bimolecular degradation reactions are accelerated. We find a moderate cooling rate (about 1°C/min) is a good compromise. A cooling rate of this magnitude yields moderate super cooling with moderate ice surface area and a reasonably fast freezing rate, which is generally best for both those formulations prone to phase separation and those where phase separation is not an issue. It also (usually) produces uniform ice structure both within a given vial and from vial-to-vial.

Freezing temperature and time

After freezing, the formulation should be in solid state; that is, the drug phase should be below *T*g_ if amorphous or below *T*eu if it is in the crystalline state. This condition requires the shelf temperature for freezing be set below *T*g_ or *T*eu, and the product batch must be kept at the temperature long enough such that all solution has transformed into solid. Because of the limited thermal conductivity between vials and shelf, complete freezing requires significant time. The freezing time depends on fill volume; that is, the larger fill volume takes longer to fully freeze (1). Generally, we use a final shelf temperature of −40°C if the *T*g_ or *T*eu is higher than −38°C;

Otherwise, we use a temperature 2°C less than *T*g_ or *T*eu and allow time for complete freezing. The final temperature is held for 1 h if the fill depth is less than or equal to1 cm or 2 hr. if the fill depth is greater than 1 cm. Fill depth greater than 2 cm should be avoided when possible, but if necessary, freezing time should be increased proportionately.

Annealing processes

Anealing is simply holding the product at a temperature above the final freezing temperature for a defined period to crystallize the potentially crystalline components (usually, crystalline bulking agent) in the formulation during the freezing stage. An annealing step is frequently necessary to allow efficient crystallization of the crystalline bulking agent, such as mannitol or glycine. Failure to crystallize the bulking agent has the potential of depressing the *T*g_ and compromising storage stability by crystallizing from the solid during storage (25). If the bulking agent crystallizes during primary drying, vial breakage may result, which is common if a high fill depth of concentrated mannitol is used (26). Vial breakage can be prevented by crystallization of mannitol during freezing using slow freezing or by avoiding a temperature lower than about −25°C until the mannitol has completely crystallized. Completion of crystallization may be facilitated by annealing. The annealing temperature should be between the *T*g_ of amorphous phase and *T*eu of bulking agent to give a high crystallization rate and complete crystallization. Sufficient annealing time is needed for completion of crystallization. The optimum time depends on the ratio and properties of the bulking agent used. A high mass ratio of bulking agent to other solutes (>80% of total solute, recommended) crystallizes much faster than a lower ratio (<50% of total solute, not recommended) (Tang and Pikal, unpublished). A low annealing temperature may tend to produce high crystallinity because super saturation is higher at low temperature, but the crystallization rate may be too low because of high viscosity. The optimum annealing conditions are a compromise between crystallinity and crystallization rate. For mannitol or glycine, a temperature of −20 or −25°C and an annealing time of 2 h or longer are suggested if the fill depth is 1 cm or more. Annealing conditions can be studied using either frozen solution X-ray diffraction or DSC procedures to evaluate the development of crystallinity (27; Tchessalov et al., unpublished).Annealing often has effects beyond crystallization of solutes. Annealing above the glass transition temperature of *T*g_ causes growth of ice crystals, which decreases the product resistance to flow of water vapor and results in shorter primary drying time (28,29). Also, the product specific surface area is reduced, which decreases the water desorption rate in secondary drying and may lead to increased residual moisture content in the final product or demand longer secondary drying (30). Suggested freezing process is summarized below.

Normal Freezing Process for Amorphous Products

1. Load vials onto the shelf and allow coming to 5°C; holding for 15 to 30 min.
2. Cool to −5°C without ice formation and hold for 15 to 30 min (this normally results in improved homogeneity of crystallization, both intra- and inter-vial).
3. Decrease the shelf temperature to a final shelf temperature of −40°C (all solutes in solid state) at about 1°C/min.
4. Hold for 1 h if fill depth is less or equal to 1 cm or 2 h if the fill depth is greater than 1 cm.

Steps of freez drying:

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 sublimes 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) thewater 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].

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 °C). One or more vacuum pumps in series are connected to them condenser chamber to achieve pressures in the range of 4 to 40 Pa in the entire system during operation [29].

Freeze-drying of microparticles

A freeze-dried microparticle 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 microparticle 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.

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 thermophysical properties of the microparticle suspensions. Many components of the microparticles formulation have a crucial effect on the resistance of microparticles 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 microparticles surface, or the polymer used to form the microparticles. For this reason, a wise and attentive selection of all components of the microparticles formulation must be performed before starting the study of freeze-drying. In this section, the different effects of the microparticle formulation (the protectant used, the surface of microparticles) on the freeze-drying will be discussed. A particular attention is brought to nanocapsules.

Use of cryo and lyoprotectant

Freeze-drying may generate many stresses that could destabilize colloidal suspension of microparticles, 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 microparticle suspension, the cryoconcentrated phase is composed of microparticles 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 microparticles. Furthermore, the crystallization of ice may exercise a mechanical stress on microparticles leading to their destabilization. For these reasons, special excipients must be added to the suspension of microparticles before freezing to protect these fragile systems [20]. These excipients are usually added in order to protect the 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 microparticles 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 microparticles 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 bimolecular. It has many advantages in comparison with the other sugars as: less hygroscopicity, an absence of internal hydrogen bounds which allows more flexible formation of hydrogen bonds with microparticles 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 microparticle 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 microparticles, as size measurements of reconstituted microparticles showedan increase in their average diameter and polydispersity index [36]. Poly(D,L-lactide-co-glycolide) and poly(ε-caprolactone) microparticle 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: microparticle is important for stabilizing microparticles. A complete redispersion of poly(lactide acid-co-ethylene oxide) microparticles after freeze-drying could be obtained when trehalose was added to the microparticles suspension at a weight ratio trehalose: microparticles (1:1) [38]. Similar observations 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 microparticles. For example, particle aggregation increased with higher glucose concentration during freeze-drying of cationically modified silica microparticle [41]. Furthermore, microparticles 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 microparticles [42]. It has been found that regardless of the amount of lyoprotectant added (trehalose), the microparticles concentration in the suspension prior to freeze-drying plays a key role in the lyoprotective mechanism. The results indicated that the higher the microparticles concentration, the higher the lyoprotective efficiency. At 0.2% (w/w) microparticles concentration, the microparticles could not be redispersed, even using weight ratio of trehalose: microparticles (10:1), whereas at 0.8% (w/w), trehalose in a 2:1 ratio allowed total preservation of the microparticles size. In general, the type of cryoprotectant must be selected and its concentration must be optimized to ensure a maximum stabilization of microparticles. Usually, a freeze-thawing study should be realized before freeze-drying to select the best cryoprotectant which is able to conserve the properties of microparticles. 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 microparticles suspension with no opportunity for a stabilization interaction with nanocapsules. Individual microparticles in the microparticles-rich phase can interact and form aggregates. Moreover, the growing crystals of water and mannitol may exert mechanical forces on the microparticles 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 microparticle 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 Tg′. In this case, the verification 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 microparticles. In general, special excipients are to be added to the microparticles formulation to serve as lyo-protectant. A suggested stabilization mechanism of microparticle by lyo-protectants during drying steps is the water replacement hypothesis which was already explained the stabilization of liposome's and proteins [44–46]. This mechanism supposes the formation of hydrogen bonds between a lyoprotectant and the polar groups at the surface of microparticles at the end of the drying process. These lyo-protectants preserve the native structures of microparticles by serving as water substitutes. The amorphous state of microparticle and a lyoprotectant allows maximal H-bonding between microparticles 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 microparticles (GFNPs) size during freeze-drying than mono-saccharides [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 themo non-saccharides were more effective than disaccharides because of less effective interaction with the microparticles after their crystallization. For some microparticles formulations, it was possible to freeze-dry microparticle 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 PVAwhich is not adsorbed at the surface of nanocapsules plays the role of freeze-drying stabilizer. In another formulation, poly (iso-butyl-cyanoacrylate) and poly (isohexylcyanoacrylate) microparticles 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 microparticles will be discussed in more details in the following section.

Importance of the interface composition (microparticle surface-dispersion medium)

To ensure the maximum stability of colloidal particles, a wise selection of stabilizing agent which should be localized at the microparticles surface must be done. Such stabilizers can improve the stability of the microparticle suspensions and prevent their aggregation. Many stabilizing agents have been used to achieve this objective as surfactants, modified polymers, and copolymers. This table shows some examples of successful freeze-drying microparticles by presenting the methods used for their preparation and the microparticle 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 microparticles, 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 microparticle surface despite repeated washing [49–52]. Such polymer layer formed at the microparticles surface can stabilize the microparticles 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 freezedrying of microparticle has been performed without the addition of cryoprotectant. On the other hand, microparticle stabilized by poloxamer was not resistant to the freeze-drying procedure [18]. The aggregation of microparticles 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 microparticles crystallize upon freezing impairing the maintenance of microparticles properties in the absence of cryoprotectives. On the contrary, their presence dehydrates the surfactant in the bulk solution forcing it to the particle surface, and thereby acting as a cryoprotective agent [37]. Insulin containing poly (ethylenimine-dextran sulphate) microparticle have been prepared and stabilized by zinc sulphate [56]. These microparticles 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 microparticles through electrostatic interactions with the microparticles. An interesting effect of the copolymer used to form microparticles on the conservation of microparticles size after freeze-drying has been studied. Core-shell microparticles 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 microparticles without the need of additional surfactants. It has been found that freeze drying of poly(ε-caprolactone) microparticles induced an extensive and irreversible aggregation and the microparticles size increased to more than 1 μm even if up to 1% glucose has been added. In contrast, freeze-dried PCLDex microparticle were much easier to redisperser, 1% of glucose was enough to maintain the size of microparticle when PCL-Dex 33% (33% is the weight percentage content of dextran in the copolymer) was used to form microparticles. In the case of microparticles with 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 microparticles 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 increasedwith 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 microparticles 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 microparticles induced their aggregation. No improvement of the redispersion could be achieved even using sonication, indicating a strong type of aggregation. Particle size 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 interparticular 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 microparticles. By forming an amorphous matrix with water around the particles during freezing, trehalose may maintain the PEO chains in a pseudo-hydrated state through intermolecular 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 microparticles 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 microparticles aggregation. The addition of sucrose could improve the freeze-drying by preventing MPEO crystallization. It has been found that the freeze-drying of MPEO-PLA microparticles 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 microparticles, 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 microparticle structure. Freeze-drying of chitosan-DNA microparticle was possible after the conjugation of poly(ethylene glycol) at the microparticles surface [60]. These microparticles 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 microparticles prepared of these modified polysaccharides [61]. The size of unmodified and modified chitosan microparticle was in the range of 160–200 nm before freeze-drying. After freeze drying, unmodified chitosan microparticle aggregated due to the strong inter- and intermolecular 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 intermolecular hydrogen bonding and reduce the compactness of the network. Finally, the composition of a surfactant mixture is very important for the freeze-drying of microparticles. Such effect has been studied on solid lipid microparticles stabilized with a mixture of Egg Phosphatidyl choline and tween 80 [62]; It has been found that the best result weigh ratio). The size and the polydispersity index of microparticle 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 microparticle after lyophilization was more evident. The best conservation of microparticles 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 microparticles may in some cases influence the freeze-drying of microparticles. On the other hand, the lyophilization process may induce the drugs leakage out of microparticles or their degradation as in the case of proteins. In literature, there are many examples of freeze-drying of loaded microparticle. To study the interference between encapsulated drug and the freeze-drying process, Dynasan solid lipid microparticles have been loaded with tetracaine and etomidate [63]. These loaded microparticles 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 microparticles loaded with all-trans retinoic acid (ATRA) could be freeze-dried successfully with a minor modification of microparticles size using sucrose as cryoprotectant [62]. This result could be explained by the relative absence of free drug in the dispersionmedium, due to the nil solubility ofATRA in the aqueous medium and the strong interaction between ATRA and phospholipids. Freeze-drying of solid lipid microparticles 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 SLNdiameter 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 microparticles surface [65]. The responsible of such effect was the crystallization of poloxamer, used as non-ionic stabilizer of microparticles. 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 freezedrying increased significantly the cyclosporine encapsulation within poly (ε-caprolactone) microparticles stabilized by poloxamer [66]. This result was probably due to the adsorption of free cyclosporine at the microparticles surface as the water sublimed. Freeze-drying of PCL and PLGA microparticles 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 microparticles. Finally, it has been found that freeze-drying did not induce ampicillin leakage out of microparticles after freeze-drying whereas freeze-drying under the same conditions induce dramatic leakage of ampicillin from liposomes [67].

Specific considerations concerning microspheres and nanocapsules

Nanocapsules are more delicate structures than microspheres. 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 °C) or in liquid nitrogen (at −196 °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 °C was less suited than one with a temperature of −25 °C. Moreover, a solidification temperature of −65 °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 glucosewas 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 PVAwas 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 nancapsules 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 (Tm=83 °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 foundb between lecithin and trehalose, the cryoprotectant used, reinforcing the stabilizing properties of lecithin.

Importance of the freeze-drying process

In a typical microparticles freeze-drying process, an aqueous suspension containing the microparticles 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 microparticle 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 °C for several hours. Freeze-drying microparticles 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 microparticles 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 microparticles can be designed by matching an optimum microparticle formulation with its associated optimum drying process cycle. Freeze drying microparticle with precisions about the process conditions of freeze-drying when they are mentioned. In order to design an optimum micro particles 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 (Tg′), the collapse temperature of the formulation (Tc), the stability of the microparticles 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 (Tc). Tc is usually about 2 °C higher than Tg′, or equals the eutectic temperature (Teu).

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 microparticle 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 microparticles. 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.

Physico-chemical characterization of freeze-dried product

It is very important to characterize the freeze-dried matrix and to investigate the conservation of the microparticle 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 microparticles.

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. 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.

Reconstitution time

To rehydrate the freeze-dried microparticles 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 microparticle after the addition of water, as manual shaking, vortexing or sonication to ensure full re-suspension.

Measurement of microparticles size and zeta potential after freeze-drying

After reconstitution, microparticles size must be measured by photon correlation spectroscopy or another technique. The conservation of a microparticle diameter size after freeze-drying

is considered as a good indication of a successful freeze-drying cycle. In general, the ratio of microparticles size after and before freeze-drying may be calculated. A value near from one indicates the conservation of microparticles size, whereas an important value of this ratio indicates the aggregation of microparticles. Furthermore, the index of polydispersity may be recorded after lyophilization. This index gives also an idea about the distribution of microparticles size and its value must be compared to the value before freeze drying, to evaluate the conservation of microparticles distribution. The measurement of zeta potential is a good method to evaluate the state of microparticles 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 microparticles 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.

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 microparticles integrity and to observe whether any modification has occurred on their morphology. Many high resolution microscopic techniques could be used to observe the microparticle formulation after freeze-drying: transmission electron microscopy (TEM), cryogenic transmittance electron microscopy (cryo-TEM), and atomic force microscopy (AFM), and 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. 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 microparticles have been imaged by cryogenic transmittance electron microscopy to verify the formation of core/shell microparticles [86]. Freeze-dried cationically modified silica microparticles 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 microparticles were inter dispersed. 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. Demonstrates that sucrose develops holes in the structure that indicates a collapse of dried product whereas PVP dries into intact plates with the conservation of porous structure. Purified freeze-dried nanocapsules protected by HP-β-CD have been observed with SEM and ESEM after their reconstitution [19]. In SEM imaging shows a continuous and amorphous matrix into which PCL NC is inter dispersed. All particles are well separated throughout the glassy state of HP-β-CD matrix. ESEM imaging showed spherical mono disperse nanocapsules being well conserved after freeze-drying. 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 microparticles after dilution of the samples while SEM and AFM the observation of microparticles becomes more difficult when the protectant concentration is more than 5%. In this case a continuous matrix was observed with some microparticles. ESEM is the most adequate technique to observe microparticles in a hydrated state.

Thermal analysis by differential scanning calorimetry (DSC)

During storage, freeze-dried microparticles included within a vitrified matrix of lyoprotectant must be stored at a temperature below the temperature of glass transition (Tg) of the dried formulation to prevent any shrinkage of the freeze-dried cake or any destabilization of included microparticles 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 microparticle. 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].

Drug content determination

The drug content in microparticles 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 microparticles during freeze-drying. 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 microparticles [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. 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-microparticles 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].

Study of water sorption and determination of residual moisture

The thermal and the structure properties of freeze dried microparticle 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 adsorption, the easier water desorption. The content of residual moisture in freeze-dried microparticles can be determined by Karl Fischer titration or by other methods as the gravimetric method or the thermal gravimetric analysis.

Application of freeze-drying in the domain of microparticles

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

To improve the stability of microparticles

Freeze-drying as a drying method has many applications for microparticles technology. The literature contains many examples of such applications. The main use of freeze-drying is essential for improving long term microparticles stability. The transformation of colloidal suspension into solid form has the advantage of preventing particles aggregation, also the degradation of polymer forming microparticles and the leakage of encapsulated drug out of microparticles. 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) microparticles was evaluated after storage for 12 month under various storage conditions of temperature and illumination [90]. The results revealed that microparticles 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 microparticles 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 microparticle for treating visceral leishmaniasis have been freeze-dried using glucose 5% as cryoprotectant. These freeze-dried microparticles 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 microparticle loaded with azidothymidine (AZT) without any modification of their size or their drug content, because the storage of these microparticles at both 37 °C or 4 °C induces an increase in particle size and loss of AZT [64].

To improve the drug association to microparticles

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) microparticle [92]. Microparticles 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 absorb the free drug not incorporated in the polymer matrix more efficiently. Drug loading was determined by polarization fluro immuno analysis and found to be about 66 µg/mg. whereas, drug loading was about 5.95 µg/mg for the standard procedure of loading microparticles 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.

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 microparticle orale lyophilizate as a freeze-dried oral dosage form of indomethacin-loaded nanocapsules [69]. Poly(lactide acid) nanocapsules containing indomethacin has been prepared by nano precipitation method. Then, a large amount of lactose as inert additive was added to build up a paste solid lyophilizate. The second stepwas 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 in corporated into 10 mL of a nanocapsules suspension containing 10% glucose as cryoprotectant. Finally, the freeze-drying was applied to obtain the oral dosage form.

To prepare core/shell microparticles

Another interesting application of freeze-drying is to prepare core/shell microparticles [86]. These microparticles 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 microparticles. The formation of these core/shell microparticles 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 microparticles used for the analytical determination of the drugs and the thermal analysis. Solid lipid microparticles (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 microparticles. 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 microparticles 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 Recovery ¼ analyzed weight of SLN _ 100=theoretical weight of SLN: 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.

Conclusion

Freeze-drying of microparticles 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 microparticles composition (type of polymer, type and concentration of surfactant, type and concentration of cryo and lyoprotectants, interaction between cryoprotectants and microparticle, surface modification of microparticles). Furthermore, the applied conditions of freeze drying can impact the stabilization of microparticles 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 microparticles properties and the required qualities of freeze-dried cake.

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

Type	Function	Substance
Bulking agents	Provide bulk to the formulation especially 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).

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]