Drug Development and Industrial Pharmacy, 32:839–846, 2006

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040600685134

informa
healthcare

Freeze-Drying of Composite Core-Shell Nanoparticles

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ABSTRACT The effects of four sugars (glucose, saccharose, maltose, trehalose) and one surfactant (Poloxamer 188), on the freeze-drying of poly(isobutylcyanoacrylate) (PIBCA), poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG), and novel core (mainly PIBCA)-shell (principally PEG) composite nanoparticles (CNP) obtained by co-precipitation were investigated. The efficiency of the additives against the adverse effect of freeze-drying on the redispersibility of the nanoparticles was evaluated, based on the visual appearance of the nanoparticle suspensions (Tyndall effect and aggregation), and on the determination of the mean diameter ratio of the nanoparticles before and after freeze-drying. The results indicated that the addition of both sugars and surfactant was essential for the good redispersion of freeze-dried nanoparticles displaying hydrophobic (PIBCA) or hydrophilic (PCL-PEG and CNP) surfaces.

KEYWORDS Freeze drying, nanoparticle composites, PIBCA, PCL-PEG

INTRODUCTION

The advantages of polymeric nanoparticles as drug carriers have been highlighted in numerous papers (Alonso, 1995; Bala, 2004; Couvreur, 1995; Mainardes, 2004). However, the industrial development of these systems may be limited due to the key issues of maintaining the integrity and the colloidal stability of the liquid nanoparticle suspensions for a prolonged period of time. Despite the addition of surface active agents in order to stabilize the suspensions, aggregation is often observed upon storage (Chacon, 1999). In addition, the chemical stability of the polymer material forming the nanoparticles remains a frequent problem (Lemoine, 1990). The freeze-drying technique appears as one of the most suitable methods to make it possible to satisfy the requisite of long-term product stability. However, several physicochemical phenomena, such as air adsorption or modification of surface structure (Kreuter, 1983), may occur during the different steps of this complex dehydrating process, which may lead to difficulties in redispersion of the nanoparticles in aqueous media (Allemann, 1998). The protection of nanoparticle dispersions against the various stresses induced by freeze-drying has been shown to feasible by addition of excipients such as sugars (Konan, 2002; Saez, 2000). Nevertheless, only few studies in the literature deal with the effect of surfactants in preserving the nanoparticule integrity after the freeze-drying process, because

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the surfactants are often already present in the original formulation, and hence no formulation can be used as an actual reference for comparison with the surfactant-added dispersions. Indeed, it should be pointed out that, despite the numerous purification steps involved in nanoparticle preparation, a significant amount of surfactant still remains associated to the nanoparticles. In the case of PLA nanoparticles, residual PVA contents up to 9% (w/w) were found, depending on the initial PVA concentration in the preparation procedure (Allemann, 1993). Moreover, it has been suggested that the surfactant used to produce the nanoparticles (PVA or Poloxamer 188) forms a hydrophilic layer at the surface, which also plays a major role in the redispersion after freeze-drying (Quintanar-Guerrero, 1998).

The surface characteristics of the nanoparticules govern their in vivo fate. It has been established that once in the blood stream, nanoparticles with hydrophobic surfaces are opsonised rapidly and end up in RES organs, mainly liver and spleen. Oppositely, nanoparticles with hydrophilic surfaces are able to circulate for hours (Gref, 2003). In particular, poly(ethyleneglycol)(PEG)-coated nanoparticles present a major interest as long-circulating devices, for site specific drug delivery (Gref, 2003). However, the presence of PEG at the surface requires specific freeze-drying conditions (De Jaeghere, 2000; Zambaux, 1999). Indeed, PEG is known to crystallize during this process (Izutsu, 1996) and this leads to dramatic nanoparticle aggregation (De Jaeghere, 1999), even if sugars such as fructose, sucrose, or palatinose could successfully inhibit PEG crystallization in solution (Izutsu, 1996). In previous studies, we have designed and produced novel composite core-shell (PCL-PEG) nanoparticles able to encapsulate busulfan, the core of these nanoparticles being made of a biodegradable polymer. Busulfan is an alkylating agent that has been available only in oral form for a long time (Layre, 2004). This drug has a wide variability in bioavailability (Hassan, 1991) and severe side effects, such as the veino-occlusive disease (Grochow, 1989). These composite nanoparticles (CNP) were prepared without the addition of any surfactant, by co-precipitation of mixtures of poly (isobutylcyanoacrylate) (PIBCA) and poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG), in different mass ratios (Layre, 2004). The busulfan-loaded composite core-shell nanoparticles combined the advantages of the PIBCA core for its good ability to encapsulate busulfan, with the steric repulsive effect of the PCL-PEG coating layer reducing complement activation (Layre, 2005).

The aim of the present study was to investigate the effects of four sugars (glucose, saccharose, maltose, and trehalose) and one surfactant (Poloxamer 188) on the stability of nanoparticles during freeze-drying. The nanoparticles studied displayed either a hydrophobic surface (nanoparticles of pure PIBCA), or a "pegylated" hydrophilic surface (composite nanoparticles CNP and nanoparticles of block-copolymer pure PCL-PEG). Poloxamer 188 was chosen as a surfactant, because it has been described as safe for the intravenous administration (Floyd, 1999) and is classically used for stabilization of nanoparticle dispersions. The efficiency of the additives against the adverse effect of freeze-drying on redispersibility of the particles was evaluated, based on the visual appearance of the nanoparticle suspensions (Tyndall effect and aggregation) and on the determination of the mean diameter ratio of the nanoparticles before and after freeze-drying (S_f/S_i).

MATERIALS AND METHODS

Materials

The PIBCA polymers were synthesized using an anionic polymerization process of isobutylcyanoacrylate monomers in water. Briefly, the isobutylcyanoacrylate monomers (Loctite, Ireland) (1 mL) were added in one shot to water (15 mL). The polymerization was carried on for 1h30, at 40°C under magnetic stirring (1,200 rpm). After this time, a milky suspension was obtained together with a polymer aggregate around the magnetic stirrer. This polymer was collected in two fractions: the milky suspension was freeze-dried (fraction 1), and the aggregated polymer was dissolved in acetone and dried under vacuum at room temperature (fraction 2). The polymer thus obtained in fraction 2 (representing more than 85% of the polymer synthesized) was further used in the nanoprecipitation process.

The poly(ε-caprolactone)-poly(ethylene glycol) diblock copolymer was synthesized as previously described (Gref, 2000). Briefly, given amounts of the freshly distilled monomer (ε-caprolactone) and monomethoxy polyethylene glycol (MPEG) (weight average molar mass 2000 g/mole, Sigma) were dissolved in xylene. The weight ratio ε-caprolactone:

MPEG was 9:1. Stannous octanoate (Sigma) purified by distillation was used as a catalyst in equimolar quantity with regard to MPEG. The reaction was carried on at 110°C for 6 h. Average molar masses (Mw) were determined using gel permeation chromatography equipped with a refractometric and a multiangle light scattering detector (Wyatt Dawn Model F, Milton Roy, Wyatt Technology). The diblock copolymer PCL-PEG had a PCL block with a M_w value of 10,000 g/mole and a PEG block with a M_w value of 2,000 g/mole. Poloxamer 188 (Synperonic PE/F68) and D-(+) trehalose was obtained from Fluka (France). D-(+) glucose, D-(+) saccharose, and maltose were purchased from Sigma-Aldrich (France) and acetone was purchased from Carlo-Erba (France).

Nanoparticle Preparation

Pure PIBCA nanoparticles and pure PCL-PEG nanoparticles were prepared by the nanoprecipitation process, as previously described by Fessi et al. (1992). Briefly, an organic solution of the polymer (PIBCA or PCL-PEG) (10 mg) in acetone (1 mL) was injected into water (2 mL) under magnetic stirring (1,200 rpm) at room temperature. Acetone was eliminated using a rotative evaporator (Rotavapor®) at room temperature.

In order to prepare the composite nanoparticles, an acetone solution of PIBCA (10 mg/mL) and an acetone solution of PCL-PEG (10 mg/mL) were mixed in different volume ratios (80/20, 70/30, 60/40, 50/50). One milliliter of the resulting solution was then injected into water (2 mL) under magnetic stirring (1,200 rpm) at room temperature leading to spontaneous formation of composite nanoparticles. Finally, acetone was eliminated using a rotative evaporator (Rotavapor®) at room temperature (Layre, 2004). The composite nanoparticles are further named $CNP_{(PIBCA:PCL\text{-}PEG)}$. For example, $CNP_{(80:20)}$ was obtained from a PIBCA:PCL-PEG mixture in 80:20 volume ratios.

Nanoparticle Characterization

Nanoparticle Morphology

The $CNP_{50:50}$ were observed using transmission electron microscopy (TEM) after freeze-fracture. A small drop of an aqueous nanoparticle suspension was deposited into a 100 μm deep symmetric cup. Then, the sample was frozen using a high-pressure cooling device HPM 010 (Bal-Tec). Fracturing, etching, and shadowing, using Pt-C, were performed in a Bal-Tec Model BAF 400T apparatus. The replicas of the surface were then floated off by specimen submerging in successive baths of water/acetone, water, NaOH (1 M), water, and acetone. Finally, the replicas were collected onto naked 400 mesh grids that were subsequently mounted in the microscope for inspection. TEM observations were performed on a LEO 912 Omega high-resolution microscope working at 120 kV.

Nanoparticle Size Analysis

The mean particle diameter of the nanoparticles was measured by laser light scattering using a nanosizer (Coulter® N4MD, coulter Electronics, Margency, France). Each sample was properly diluted in water, in order to maintain the number of counts per second between 5.10^4 and 1.10^6. Water was filtered with a 0.22 μm filter to remove any impurity that could affect light scattering. Each sample was measured three times for at least 3 min at 20°C and at an angle of 90°

Zeta Potential Determination

The nanoparticle zeta potential measurements were carried out using a Zeta Sizer 4 (Malvern, France). Each sample was properly diluted in NaCl (1 mM), in order to maintain the number of counts per second around 600. Three measurements were carried out for each sample and the mean values and standard deviations were calculated.

Freeze-Drying

First, the effect of four sugars (glucose, saccharose, maltose, and trehalose) and one surfactant (poloxamer 188) on the stability of pure PIBCA and pure PCL-PEG nanoparticles after freeze-drying was assessed. The freeze-drying conditions used for CNP corresponded to the optimal freeze-drying conditions identified for the PIBCA and PCL-PEG nanoparticle suspensions. Practically, aliquots of nanoparticles (400 μl) at a concentration of 5 mg/mL were added to the same volume of (i) sugar solution or (ii) surfactant solution or (iii) mixture of sugar and surfactant

solution at various concentrations before freeze-drying. The weight ratio nanoparticles/cryoprotectant contained in the suspension ranged from 1/10 to 1/0.5 and the cryoprotectant concentration in the suspension ranged from 25 mg/mL to 1.25 mg/mL (Tables 1, 2, and 3). Freezing was performed in a conventional freezer (–20°C). The frozen nanoparticles were then lyophilized using a freeze-drying system (Bioblock Scientific, Christ-Alpha 1–4) over 24 hrs. Temperature cycle was –30°C: +30°C and the vacuum was 8.10^{-3} mbar.

RESULTS AND DISCUSSION

Nanoparticle Characterization

The mean diameter and the zeta potential of both PIBCA nanoparticles and PCL-PEG nanoparticles, as well as those of composite nanoparticles (weight ratios: from 80:20 to 50:50), are reported in Fig. 1. All the nanoparticles displayed a size lower than 200 nm, including the CNP, whatever the polymers' weight ratio. However, an increase in the amount of PCL-PEG in the PIBCA: PCL-PEG mixtures leads to a decrease of the resulting nanoparticle mean diameter. For example, the mean diameter of pure PIBCA nanoparticles was about 140 nm; it decreased to 100 nm for the $CNP_{(50:50)}$ nanoparticles. These data might be related to the amphiphilic intrinsic properties of the PCL-PEG copolymer, reducing the interfacial tension between the aqueous and the organic phase.

The CNP displayed intermediate zeta potential values between the PIBCA nanoparticles (–40 mV) and the PCL-PEG nanoparticles (–15 mV). Moreover, the higher the PCL-PEG contents in the polymer mixtures, the higher the zeta potential values of the CNP. For example, the $CNP_{(50:50)}$ nanoparticles displayed zeta potential values of –22 mV, close to the one of the PCL-PEG nanoparticles (–15 mV). These findings

TABLE 1 **Characteristics of the Freeze-Dried PIBCA Nanoparticles in the Presence of Different Cryoprotectants or Mixtures of Them. Tyndall Effect: ■ Absent or ■ Present. Aggregation Scale: ■ Absent, ■ Scarce, or ■ Significant. S_f/S_i is the Ratio Between the Final and the Initial Mean Diameters. In Same Cases (ND) Size Could Not Be Determined Because Strong Aggregation Occurred**

Cryoprotectants	Features	Nanoparticle/cryoprotectant ratio (w/w) / Cryoprotectant (mg/ml)				
		1/10 / 25	1/5 / 12.5	1/2 / 5	1/1 / 2.5	1/0.5 / 1.25
Glucose	Tyndall effect					
Saccharose						
Maltose						
Trehalose						
P188						
P188 (1/2) + Treh*						
Glucose	Aggregation scale					
Saccharose						
Maltose						
Trehalose						
P188						
P188 (1/2) + Treh*						
Glucose	S_f/S_i	ND	ND	ND	ND	ND
Saccharose		ND	ND	ND	ND	ND
Maltose		2.99	4.19	ND	ND	ND
Trehalose		3.05	3.67	3.51	ND	ND
P188		1.19	1.28	1.38	2.32	ND
P188 (1/2) + Treh*		1.14	1.17	1.16	1.22	1.21

*Mixture of Poloxamer 188 (P188) (nanoparticle/P188 weight ratio 1/2) and trehalose (Treh) (nanoparticle/Treh weight ratios from 1/10 to 1/0.5).

TABLE 2 **Characteristics of the Freeze-Dried PCL-PEG Nanoparticles in the Presence of Different Cryoprotectants or Mixtures of Them. Tyndall Effect: ■ Absent or ■ Present. Aggregation Scale: ■ Absent, ■ Scarce, or ■ Significant. In Same Cases (ND) Size Could Not Be Determined Because Strong Aggregation Occurred**

Cryoprotectants	Features	Nanoparticle/cryoprotectant ratio (w/w) / Cryoprotectant (mg/ml)		
		1/10 / 25	1/5 / 12.5	1/2 / 5
Glucose	Tyndall effect	■	■	■
Trehalose		■	■	■
Maltose		■	■	■
Saccharose		■	■	■
P188		■	■	■
P188 (1/2) + Treh*		■	■	■
P188 (1/5) + Treh**		■	■	■
P188 (1/10) + Treh***		■	■	■
Glucose	Aggregation scale	■	■	■
Trehalose		■	■	■
Maltose		■	■	■
Saccharose		■	■	■
P188		■	■	■
P188 (1/2) + Treh*		■	■	■
P188 (1/5) + Treh**		■	■	■
P188 (1/10) + Treh***		■	■	■
Glucose	S_f/S_i	ND	ND	ND
Trehalose		ND	ND	ND
Maltose		ND	ND	ND
Saccharose		ND	ND	ND
P188		1.63	2.25	>13
P188 (1/2) + Treh*		3.58	3.37	3.57
P188 (1/5) + Treh**		1.54	1.66	2.71
P188 (1/10) + Treh***		1.36	1.31	2.35

*Mixture of Poloxamer 188 (P188) (nanoparticle/P188 weight ratio 1/2) and trehalose (Treh) (nanoparticle/Treh weight ratios from 1/10 to 1/2).
**Mixture of Poloxamer 188 (P188) (nanoparticle/P188 weight ratio 1/5) and trehalose (Treh) (nanoparticle/Treh weight ratios from 1/10 to 1/2).
***Mixture of Poloxamer 188 (P188) (nanoparticle/P188 weight ratio 1/10) and trehalose (Treh) (nanoparticle/Treh weight ratios from 1/10 to 1/2).

TABLE 3 **Characteristics of the Freeze-Dried CNP (PIBCA:PCL-PEG Weight Ratios from 80:20 to 50:50) in the Presence of Mixture of Poloxamer 188 (P188) (Nanoparticle/P188 Weight Ratio 1/2 or 1/10) and Trehalose (Treh) (Nanoparticle/Treh Weight Ratio from 1/10 and 1/5): Tyndall Effect (TE): ■ Present. Aggregation Scale (AS): ■ Absent. S_f/S_i is the Ratio between the Final and the Initial Mean Diameters**

Nanoparticle/P188 ratio (w/w) / P188 (mg/ml)	Features	Nanoparticle/trehalose ratio (w/w) / Trehalose (mg/ml)							
		1/10 / 25	1/5 / 12.5	1/10 / 25	1/5 / 12.5	1/10 / 25	1/5 / 12.5	1/10 / 25	1/5 / 12.5
		$CNP_{(80:20)}$		$CNP_{(70:30)}$		$CNP_{(60:40)}$		$CNP_{(50:50)}$	
1/2 / 5	TE	■	■	■	■	■	■	■	■
1/10 / 25		■	■	■	■	■	■	■	■
1/2 / 5	AS	■	■	■	■	■	■	■	■
1/10 / 25		■	■	■	■	■	■	■	■
1/2 / 5	S_f/S_i	1.68	1.52	2.29	1.37	1.62	1.49	1.90	2.34
1/10 / 25		1.15	1.22	1.27	1.19	1.17	1.30	1.16	1.14

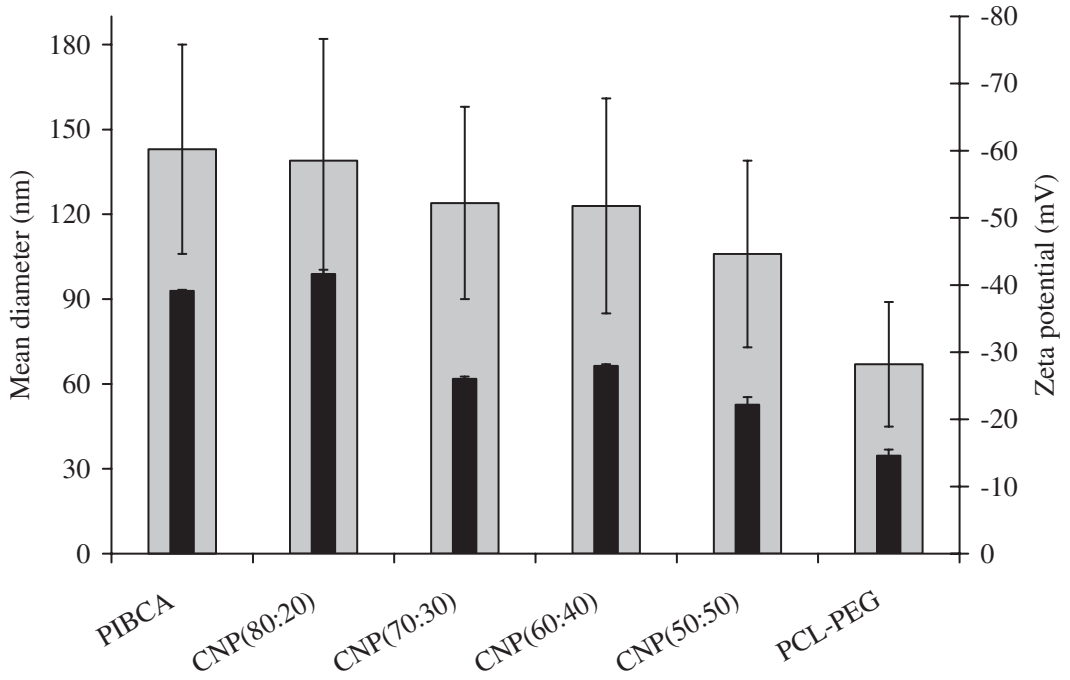

FIGURE 1 **Nanoparticle Suspensions Features: □ Mean Diameter and ■ Zeta Potential. Each Value Was the Average of Three Different Experiments ± SD.**

also suggested the preferential localization of the PCL-PEG copolymers at the surface of the CNP.

The electron microscopy observation after cryofracture (Fig. 2) showed that $CNP_{50:50}$ displayed a core-shell structure. This observation is in agreement with the zeta potential values and suggests that the amphiphilic PCL-PEG copolymer organizes itself at the surface during nanoparticle formation, forming a shell around the hydrophobic PIBCA core.

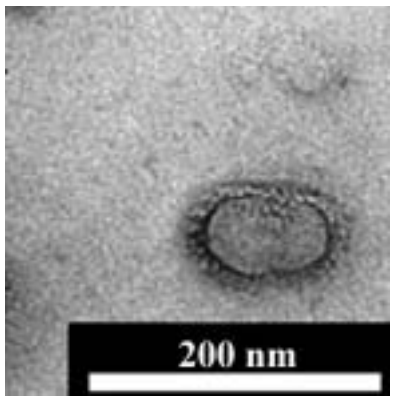

FIGURE 2 **TEM Images After Freeze-Fracture of CNP(50/50) Nanoparticles. Bar Represents 200 nm.**

Freeze-Drying of the PIBCA Nanoparticle Suspensions

The characteristics of the PIBCA nanoparticle suspensions after freeze-drying in the presence of different cryoprotectants are summarized in Table 1. In the absence of Poloxamer 188, whatever the amount of sugar (glucose, saccharose, maltose, or trehalose) added to the suspensions, the nanoparticles aggregated and could not be completely redispersed, even by prolonged vortex mixing. The strongest aggregation was obtained with glucose and saccharose. In this case, the aggregates could not be dispersed even partially, whereas when using maltose or trehalose, a partial dispersion could be achieved and the suspensions presented a Tyndall effect (Table 1). In the absence of Poloxamer 188, a significant increase of the nanoparticle mean diameter after freeze-drying was also measured ($S_f/S_i > 2$). Thus, all these sugars failed to protect the nanoparticles against aggregation during freeze-drying. Contrastingly, when the nanoparticles were freeze-dried in the presence of Poloxamer 188 (nanoparticle/Poloxamer 188 weight ratios from 1/10 to 1/1), the Tyndall effect was kept intact upon redispersion. However, only the suspensions freeze-dried with nanoparticle/Poloxamer 188 weight ratios ranging

from 1/10 to 1/2 preserved their size distribution upon reconstitution in water, with no significant aggregation observed and a S_f/S_i ratio close to 1. The optimal results were obtained with mixtures of Poloxamer 188 (nanoparticle/Poloxamer 188 weight ratio 1/2) and trehalose (nanoparticle/trehalose weight ratios from 1/10 to 1/2). When such mixtures were added to the nanoparticle suspensions, the Tyndall effect was preserved, no significant aggregation occurred, and the S_f/S_i ratio was close to 1. Thus, these findings clearly demonstrated that:

Sugars alone had limited or no cryoprotectant activity for nanoparticles of pure PIBCA

The Poloxamer 188 surfactant was needed to achieve redispersible PIBCA nanoparticle dispersions after freeze-drying

These results are in agreement with previously published data (Quintanar-Guerrero, 1998).

Freeze-Drying of the PCL-PEG Nanoparticle Suspensions

The features of the PCL-PEG nanoparticle suspensions after freeze-drying in the presence of cryoprotectants are summarized in Table 2. Whatever the amount of sugar used (glucose, saccharose, trehalose, and maltose), PCL-PEG nanoparticles strongly aggregated and could not be redispersed after freeze-drying. In the presence of Poloxamer 188, the Tyndall effect was preserved upon redispersion, but the S_f/S_i ratio was dependent of the amount of Poloxamer used. Indeed, the value of the S_f/S_i ratio was of 1.63 when the nanoparticle/Poloxamer 188 ratio was 1/10, and it was higher than 13 when the nanoparticles/Poloxamer 188 ratio was only 1/2. When adding trehalose to Poloxamer 188, the S_f/S_i ratio of PCL-PEG nanoparticles strongly decreased and was found to be dependent of the amount of Poloxamer used (Table 2). The best result was obtained with a mixture of Poloxamer (nanoparticle/Poloxamer 188 weight ratio 1/10) and trehalose (nanoparticle/trehalose weight ratios from 1/5 to 1/10). Therefore, our findings suggested that there were two additively contributing protecting effects, arising both from the surfactant and from the sugar, needed to avoid the aggregation of the "pegylated" nanoparticles during the freeze-drying process. It is noteworthy that previously published studies (De Jaeghere, 2000; Zambaux, 1999) did not report the protecting effect of a surfactant, such as PVA or Poloxamer, against the aggregation of the "pegylated" nanoparticles after free-drying.

Freeze-Drying of the CNP Nanoparticle Suspensions

The optimal freeze-drying conditions for PIBCA and PCL-PEG nanoparticle suspensions (Table 1 and Table 2) were used for freeze-drying CNP suspensions. Results are summarized in Table 3. The S_f/S_i ratio after freeze-drying CNP in the presence of a mixture of Poloxamer 188 (nanoparticles/Poloxamer 188 weight ratio 1/2) and trehalose (nanoparticles/trehalose weight ratios 1/10 and 1/5) increased when the PCL-PEG polymer proportion in the CNP formulation increased. However, the use of a mixture of Poloxamer 188 (nanoparticle/Poloxamer 188 weight ratio 1/10) and trehalose (nanoparticle/trehalose weight ratios: 1/10 and 1/5), whatever the CNP suspensions, resulted in a high redispersibility and a low aggregation behavior, with a S_f/S_i ratio close to one. In other words, the best freeze-drying conditions for CNP suspensions were found to be the same as those obtained for the PCL-PEG nanoparticle suspensions. Thus, it was concluded that the PCL-PEG polymer in the PIBCA:PCL-PEG mixture governed the behavior of the CNP in the freeze-drying process. These findings indirectly support our hypothesis, based on zeta potential experiments and electron microscopy observations, that the PCL-PEG copolymer was preferentially localized at the surface of the composite nanoparticles (CNP).

CONCLUSION

This work shows that freeze-drying can be successfully used to dry polymer colloidal suspensions displaying either a hydrophobic (PIBCA) or a hydrophilic (PCL-PEG and CNP) surface, provided that the simultaneous addition of two appropriate cryoprotectant agents (a sugar and a surfactant) is achieved prior to freezing. None of these cryoprotectant agents used alone had enough effect to preserve the nanoparticle suspension integrity and to allow for redispersion with preservation of the initial mean diameter of the nanoparticles. In particular, the use of a surfactant (Poloxamer 188) was found to be the key for the production of

freeze-dried nanoparticles with high redispersibility and low aggregation behavior.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the French National Research Center (CNRS) and Ethypharm for their financial support, G. Frebourg and J. P. Lechaire from the Service Microscopie Electronique de l' IFR de Biologie Interactive (PARIS V) for the freeze-fracture observations.

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