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Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles

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Abstract

The feasibility of producing sterile and freeze-dried polyester nanoparticles was investigated. Various poly(D,L-lactide-co-glycolide) and poly(D,L-lactide) were selected as biodegradable polymers. Using the salting-out procedure, process parameters were optimized to obtain sub-200 nm particles. After purification, the nanoparticle suspensions containing different lyoprotectants were sterilized by filtration. Freeze-drying was performed using vials covered with 0.22 μm membrane filters in order to preserve the suspensions from bacterial contamination. Sterility was assessed on the final product according to pharmacopoeial requirements using the membrane filtration method. With all polymers tested, sub-200 nm particles could be obtained. Nanoparticles with a size as low as 102 nm were prepared with good reproducibility and narrow size distribution. Upon freeze-drying, it appeared that complete redispersion of all types of polyester nanoparticles could be obtained in presence of the lyoprotectants tested such as saccharides while aggregation was observed without lyoprotectant. Sterility testing showed no microbial contamination indicating that sterile nanoparticulate formulations have been achieved.

Keywords: Nanoparticles; Biodegradable polyesters; Sterilization; Sterile filtration; Freeze-drying; Lyoprotectants

1. Introduction

Biodegradable nanoparticles based on polyester polymers such as poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA) have been widely investigated as parenteral delivery systems (Smith and Hunneyball, 1986; Scholes et al., 1993; Rodrigues et al., 1995; Allémann et al., 1998; Kawashima et al., 1998). Polyester polymers, approved by the Food and Drug Administration, have raised great interest due to their physicochemical and biological properties (Vert, 1987; Vert et al., 1998). In addition to their biocompatibility and bioresorbability properties, the possibility of modulating drug release profiles by selecting the appropriate polymer is particularly interesting for the development of parenteral drug products. If intended for parenteral administration, nanoparticulate formulations have to meet the pharmacopoeial requirements of sterility. However, sterilization of such polymeric devices by a satisfactory technique remains a challenge.

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The chemical or physical lability of the polymer matrix usually limits most conventional methods for obtaining acceptable sterile products. For example, sterilization by autoclaving induces a degradation of polyesters by hydrolysis. These polymers are also heat sensitive due to their thermoplastic nature (Athanasiou et al., 1996). With chemical sterilization by gases such as ethylene oxide, toxicological problems may be encountered due to toxic residues. Numerous studies have shown the effects of γ-irradiation on the stability and the safety of colloidal carriers based on polyesters, principally microparticles (Volland et al., 1994; Hausberger et al., 1995; Montanari et al., 1998; Mohr et al., 1999). γ-Irradiation was shown to affect drug loaded polyester microparticle properties in several ways such as radiolytic reactions, chain scission and cross-linking (Volland et al., 1994). These reactions may have consequences on the nominal drug content, the drug release pattern and the bioresorption of the system. Therefore, the selection of a suitable sterilization method for such type of formulations is crucial to ensure their physical and chemical integrity, their performance and safety in vivo. As an alternative technique, sterile filtration through 0.22 μm membrane filters may be considered as the appropriate method for chemically or thermally sensitive materials since it has no adverse effect on the polymer and the drug. Nevertheless, this sterilization method can only be used for nanoparticles with a mean size significantly below membrane cut-off and with a narrow size distribution to avoid membrane clogging. Furthermore, the ability to produce nanoparticles in the size range 100–150 nm is attractive since carriers may target sites located outside the vascular system. Some tumours, which possess a defective microvasculature, exhibit an increased vascular permeability favouring the accessibility of colloidal carriers to extravascular tumoral cells (Douglas et al., 1987).

Another problem encountered with PLGA or PLA nanoparticles is their instability in aqueous media, which leads to the chemical degradation of these polymers. Consequently, complete elimination of water is necessary to obtain anhydrous forms and therefore extend the shelf-life stability of the delivery systems. Widely used in the pharmaceutical industry, freeze-drying is one of the most efficient techniques to provide dehydrated products. However, several physicochemical phenomena such as air adsorption, modification of surface structure (Kreuter, 1983) may occur during the different steps of this complex dehydrating process and may lead to difficulties in redispersion of the nanoparticles in aqueous media (De Chasteigner et al., 1995; Allémann et al., 1998). The protection of nanoparticle formulations against these various stresses induced by freeze-drying is possible by addition of excipients such as sugars, which have been shown to be effective stabilizers (De Chasteigner et al., 1995, 1996).

The aim of this work was to produce a nanoparticle size enabling sterilization by a filtration process. Several process parameters including the concentration of the stabilizing colloid in the aqueous phase, the stirring rate, the stirring time, the percentage of polymer in the organic phase and the solvent nature were varied in order to achieve optimal conditions for the preparation of sub-200 nm nanoparticles. Sterilization of the nanoparticle suspensions and freeze-drying of the sterile products in non-aseptic areas but in protective conditions were also set up. The effectiveness of the lyoprotective additives against the adverse effects of freeze-drying on the particle redispersibility was also evaluated based on systematic particle size measurements.

2. Materials and methods

2.1. Materials

The nanoparticles were produced using three types of polyesters all with various molecular weights obtained from Boehringer Ingelheim (Ingelheim, Germany): (i) 50:50 PLGA (RG502 and RG502H (molecular weight (Mw) = 12 000) and RG504H (Mw = 48 000)); (ii) 75:25 PLGA (RG752 (Mw = 12 000), RG755 (Mw = 63 000), and RG756 (Mw = 98 000)) and finally (iii) pure PLA (R104 (Mw = 2000), R202 and R202H (Mw = 16 000) and R203 (Mw = 28 000)).

Poly(vinyl alcohol) (PVAL) 87.7% hydrolyzed with a Mw of 26 000 (Mowiol® 4-88) and 82.6% hydrolyzed with a Mw of 18 000 (Mowiol® 3-83) (Hoechst, Frankfurt/Main, Germany) were selected as stabilizing colloid. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Fluka Biochemika, Buchs, Switzerland) was used as salting-out agent. Tetrahydrofuran (THF) (Merck, Darmstadt, Germany) and acetone (Fluka Biochemika) were used as the organic water-miscible solvents. These solvents were chosen on the basis of previous works (Allémann et al., 1992; Ibrahim et al., 1992) and pharmaceutical properties with regard to toxicity (Witschi and Doelker, 1997). D(+)-trehalose dihydrate (Sigma, St. Louis, MO, USA), lactose monohydrate (Hänseler AG, Herisau, Switzerland), D(−)-mannitol (Riedel-de Haën®, Seelze, Germany) and D(+)-glucose anhydrous (Fluka Biochemika) were used as lyoprotectants. All other chemicals were of analytical grade and used as such without further purification.

2.2. Methods

2.2.1. Nanoparticles preparation

The possibility of producing very small nanoparticles was investigated using the salting-out process. The parameters were first chosen according to previous works (Allémann et al., 1992; Ibrahim et al., 1992; Allémann et al., 1993a) and were then varied in order to investigate the influence on particle mean size. Typically, 5 g of organic solution containing variable amounts of PLGA or PLA was added under mechanical stirring to 20 g of an aqueous phase containing PVAL and 60% (w/w) of a salting-out agent ($MgCl_2 \cdot 6H_2O$). After the formation of an oil-in-water emulsion at room temperature, 60 ml of pure water was added to induce complete diffusion of the solvent into the aqueous phase, thus leading the formation of nanoparticles. Since the effectiveness of the sterile filtration process is also influenced by the microbial burden of the nanoparticle suspension to be filtered, water filtered through 0.1 μm membrane filter (Millipore®, MilliQ Academic, Switzerland) was routinely used for the preparation and the purification of the nanoparticles.

In an attempt to obtain the conditions for the preparation of sub-200 nm nanoparticles, preliminary investigations were first performed using a THF solution containing 17% (w/w) of RG755 as organic phase. The particle size was evaluated as function of: a) the stirring rate (2000–13 500 rpm) b) stirring time of the emulsification (5–50 min) c) PVAL molecular weight (18 000–26 000 Da), PVAL percentage (10–15% (w/w)). The polymer percentage (10–17% (w/w)) was also varied.

2.2.2. Purification of the nanoparticle dispersions by cross-flow filtration

Raw nanoparticles dispersions were purified by cross-flow filtration using a Sartocon® mini device fitted with ultrafiltration membrane with a molecular weight cut-off of 300 000 Da (Sartorius, Goettingen, Germany) to remove the soluble additives (THF, $MgCl_2$ and PVAL). Filtration was performed by adding volumes of water which were collected as filtrate fractions. The amount of eliminated PVAL in each filtrate fraction was determined by a colorimetric method as described under Section 2.2.5.

2.2.3. Particle size measurement

The particle size was measured using photon correlation spectroscopy using a Coulter® Nano-Sizer™ (Coulter Electronics Harpenden, Hertfordshire, UK). Polydispersity index (PI) is an indication of the size distribution with values ranging from 0 to 9. The measurements were carried out at room temperature. The accuracy of the photon correlation spectrometer was confirmed by using polystyrene uniform latex standard particles (204 ± 6 nm) (Duke Scientific Corp., Palo Alto, CA, USA). Each value is the average of three measurements. To assess the redispersibility, 2 mg of freeze-dried nanoparticles were resuspended in 1 ml of distilled water under manual shaking during 30 s and the particle size was measured.

2.2.4. Viscosity measurement

The viscosity of the various PVAL and polymer solutions was determined using a Bohlin® Controlled Stress Rheometer CVO-120 HR (Bohlin®

Rheology, GmbH, Mühlacker, Germany). The viscometry test was applied to the samples. The small sample cell (SSC 25 mm) and cone-and-plate (CP2°/60) were chosen as measuring systems for the different organic phases and PVAL solutions, respectively. The measurement was performed at constant temperature (20 °C), controlled during the test with an extended temperature option unit. The shear rate and the shear stress were measured at increasing time. The strain delay was of 10 s, the integration time of 10 s and the measurement interval of 10 s.

2.2.5. Determination of residual PVAL on nanoparticle surface

During the cross-flow filtration procedure, although most of the PVAL is removed from the nanoparticle dispersion, a certain amount of this stabilizer remains on the surface of the particles (Allémann et al., 1993a,b; Leroux et al., 1995). To determine this residual amount, 5 mg of freeze-dried nanoparticles (without lyoprotectant) were added to 5 ml of 2 N NaOH solution under magnetic stirring during 1h in order to obtain a complete degradation of the polyester polymer (whereas the main chain of PVAL remained intact). Then, this solution was neutralized with 2 N HCl solution. An aliquot of this solution was assayed according to a previously described method (Finley 1961; Allémann et al. 1993a). Briefly, 2.0 ml of this resulting solution was added to 7.5 ml of a 4% (w/w) boric acid solution and 1.5 ml of an iodine solution (1.27% (w/w) iodine and 2.5% (w/w) potassium iodide in water). The volume was adjusted to 25.0 ml with distilled water. The absorbance of this solution was measured at 644 nm using a diode array spectrometer HP8453 (Hewlett Packard, Waldbronn). Each value was the average of three assays.

2.2.6. Sterile filtration

The sterile filtration feasibility study first was initiated with a qualitative evaluation of different membrane filter systems. Three systems, including Millex®-FG_{50} (hydrophobic polytetrafluoroethylene membrane), Swinnex®47 (hydrophilic Durapore® membrane (GVWP04700)), Steriflip™ filter units (Express™ polyethersulfone membrane) (Millipore®, Volketswil, Switzerland), were tested. In order to avoid the possible clogging of the filtration device, the nanoparticle suspension containing a lyoprotectant was first pre-filtered through a membrane with a pore size of 0.45 μm before the sterilizing filtration process. Then, the pre-filtered sample was filtered through the appropriate sterilizing filter unit. Afterwards, 40 ml of sterile suspension was poured into 100 ml preweighed sterile glass vials under a laminar air-flow hood. In order to preserve the particles from contamination during the freeze-drying process, the vials were covered with sterile 0.22 μm membrane filters (Durapore® hydrophobe, type GVHP, Millipore®, Volketswil). The sterile suspensions were frozen and freeze-dried as described below. In order to determine if significant quantities of nanoparticles had been retained by the membrane, aliquots of the nanoparticle dispersions taken before and after filtration were freeze-dried. Therefore, weight field could be determined. The particle size distribution before and after filtration was also examined.

2.2.7. Freeze-drying

In order to determine the exact solid content of the nanoparticle suspension, an aliquot suspension was first freeze-dried without lyoprotectant and the amount of nanoparticles was determined by gravimetry. The amounts of lyoprotectant added to the particulate dispersions before the sterile filtration ranged from 0:1 to 2:1 (lyoprotectant:nanoparticle mass ratio). Aliquots of the filtered samples were then frozen at -60 °C for 10 min. Lyophilization was carried out in a freeze-drier Lyolab B II (Secfroid, Aclens, Switzerland) at 0.05 mBar. The accurate amount of nanoparticles in the dried powder was assessed by weighing the vials after freeze-drying. The freeze-dried samples were stored at -20 °C before analysis.

2.2.8. Validation and sterility testing

The sterility testing was performed on the freeze-dried nanoparticles, following European Pharmacopoeia guidelines (addendum 1999). The membrane filtration followed by incubation of the membrane in culture media was chosen as testing method. Typically, 50 mg of dried nanoparticles

dispersed in 100 ml of sterile water were passed through a 0.22 μm membrane filter (47 mm of diameter). Then, the membrane was aseptically removed from the holder. After rinsing with three portions of sterile peptone solution, each half of the membrane was immersed in tubes containing appropriate media. Thioglycollate resazurine broth (BioMérieux®, Marcy, France) was used as aerobic or anaerobic medium for the detection of bacteria and tripcase soy broth was used as medium for the detection of yeasts and fungi. Non-sterile membranes were used as positive controls. The tubes were incubated for 14 days at 32.5 ± 2.5 °C (thioglycollate resazurine medium) or at 22.5 ± 2.5 °C (tripcase medium). The turbidity of the media was then observed over a basic period of 14 days in comparison to positive controls. The experiment was done two times.

A validation of the sterility testing was performed using the same conditions as just described above. Several categories of microorganisms were chosen for this test: Staphylococcus aureus (ATCC 6538), Bacillus subtilis (ATCC 6633) and Pseudomonas aeruginosa (ATCC 9027) as aerobic bacteria, Clostridium sporogenes (ATCC 11437) as anaerobic bacteria and Candida albicans (ATCC 10231) and Aspergillus Niger (ATCC 19404) as yeasts and fungi, respectively. Diluted cultures of each bacteria and fungi were prepared from the strains to obtain a final concentration of microorganisms less than 100 cfu/ml. After filtration of the nanoparticle suspensions in the same conditions used for the sterility testing, the final rinse was inoculated with 1 ml of microorganism solution. The samples were incubated as described above for 7 days.

3. Results and discussion

3.1. Influence of the stirring rate on particle size

The influence of the stirring rate on particle size was evaluated using an aqueous solution containing 10% (w/w) of PVAL and 60% $MgCl_2$. As expected, it was observed that the mean size of the particles decreased when the stirring rate was increased from 2000 to 13 500 rpm (Fig. 1). Above 8000 rpm, sub-200 nm particles were obtained. From this threshold value, a further increase in the stirring rate only led to a slight decrease in the particle size. Sub-200 nm PLGA nanoparticles have been produced using the solvent displacement method (Scholes et al., 1993). Particle size reduction was achieved using high homogenous speed (13 500 rpm) combined with a sonication process (at an intensity of 60 W, during 5 min). It has been established that high-energy mixing forces including sonication and microfluidization generate large shear pressure, which may increase the temperature and induce material degradation (Krishnamurthy and Lumpkin, 1998). It has been shown that the sonication had an adverse effect on the degradation of various polyesters (Reich, 1998). At an intensity of 40 W, a significant molecular weight reduction could be observed even after short exposure time of 20 s. This phenomenon was correlated to the implosive collapse of cavitation bubbles induced by the mechanical stress. In the present study with the concern of preparing nanoparticles in gentle conditions, the evaluation of the effect of the percentage of PVAL in external phase on particle size was mainly investigated at relatively low stirring rate (2000 rpm).

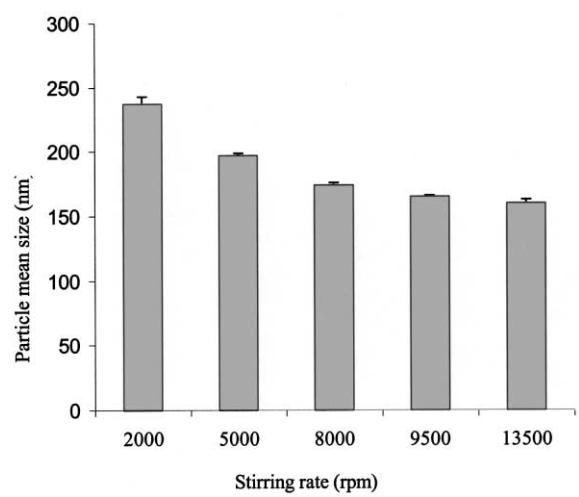

Fig. 1. Influence of the stirring rate on the mean size of RG755 nanoparticles. (Aqueous phase: 10% (w/w) of Mowiol® 4-88 and 60% (w/w) $MgCl_2$, organic phase: 17% (w/w) of polymer in THF) (mean ± SD, $n = 3$).

Table 1
Influence of the viscosity of the PVAL solutions on the particle mean size

Type of PVAL	PVAL (%)	η (Pa·s)[a]	Particle mean size (nm)[b]
Mowiol® 4-88	10	2.0 ± 0.3	275 ± 12
Mowiol® 4-88	15	29.7 ± 1.2	150 ± 8
Mowiol® 3-83	15	13.4 ± 0.8	148 ± 5

[a] Mean $\pm$ SD ($n = 22$).
[b] Mean $\pm$ SD ($n = 3$).

3.2. Influence of the stabilizing colloid concentrations

The viscosity of the aqueous phase known as important factor influencing the particle parameters can be varied by the concentration and the molecular weight of the PVAL used (Allémann et al., 1993a; Leroux et al., 1995). Accordingly, at 2000 rpm, when the percentage of PVAL was increased stepwise from 10 to 15%, the mean size of particles was reduced from 275 to 148 nm (Table 1). However, it has been noted that the nanoparticles with a mean size as low as 70 nm nanoparticles could be obtained using emulsification diffusion technique by adjusting the percentage PVAL at 28% (Leroux et al., 1995). In case of salting-out procedure, the increase of the aqueous phase viscosity has an extent limit due to the high amount of the salting-out agent (60%) in the aqueous phase, which contributes to increase the viscosity of this phase. The molecular weight of partly hydrolysed PVAL is believed to be important when this polymer is used as a dispersant and stabilizer (Bugada and Rudin, 1985). However, in our case, no evident influence of this factor on the particle size was observed since the same range of the particle size was obtained with both types of PVAL i.e. Mowiol® 3-83 and Mowiol® 4-88 (Table 1) at a percentage of 15%. Nevertheless, the results of the viscosity measurement showed that the viscosity of the Mowiol® 4-88 solution was 2.2 times higher than Mowiol® 3-83 solution (Table 1).

In subsequent investigations, aqueous solution containing 15% of PVAL (Mowiol® 3-83) was used as aqueous phase.

3.3. Influence of stirring time

The results obtained from this evaluation (Fig. 2) showed that an increase in stirring time during the emulsification step (before the dilution process) induced a decrease in particle size. First, increasing the stirring time from 5 to 15 min resulted in a reduction of particle size from 198 to 148 nm. This time of agitation was sufficient to produce stable and small emulsion droplets, which precipitated into small nanoparticles during the dilution step. However, stirring for more than 15 min did not produce a further reduction in particle size and only contributed to increasing temperature of the emulsion checked during the preparation process using a digital thermometer (TES 1310, type K, Taiwan). Fig. 3 shows how the temperature rises during the preparation process, reaching up to 50 °C after 15 min of agitation. Since this preparation process is generally intended for drug encapsulation, in the case of heat-labile drug, heat transfer to the product could be detrimental to the drug integrity. Consequently, it is crucial to maintain an acceptable temperature range as function of the drug during the emulsification step. It would be however inte-

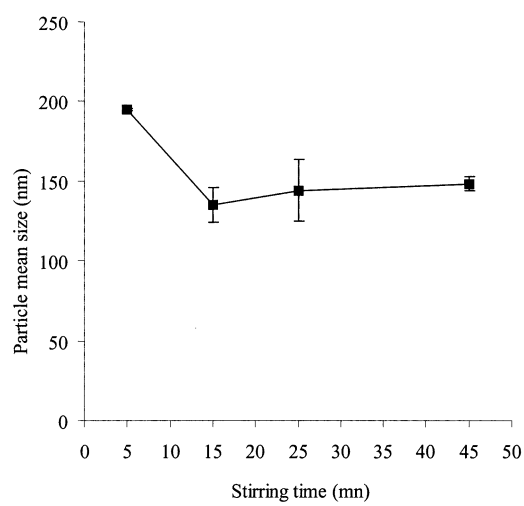

Fig. 2. Influence of stirring time on the mean size of RG755 nanoparticles. (Aqueous phase: 15% (w/w) of Mowiol® 3-83 and 60% (w/w) $MgCl_2$, organic phase: 17% (w/w) of polymer in THF) (mean $\pm$ SD, $n = 3$).

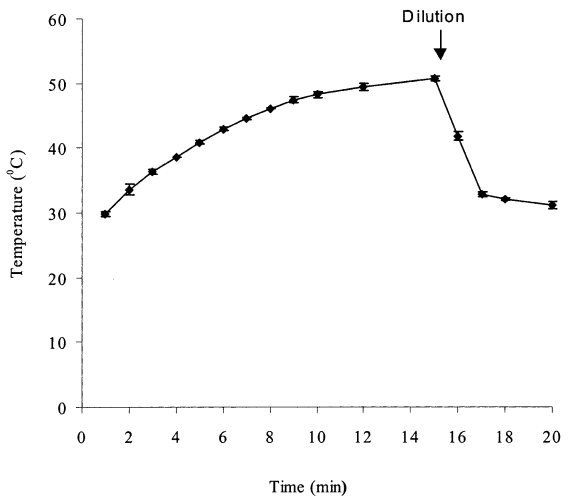

Fig. 3. Evolution of the temperature during the preparation of the RG755 nanoparticles. (Organic phase: 17% (w/w) of PLGA, aqueous phase: 15% (w/w) of Mowiol® 3-83 and 60% (w/w) $MgCl_2$, stirring rate of 2000 rpm).

resting to carry out further studies in order to establish the real impact of temperature on particle size.

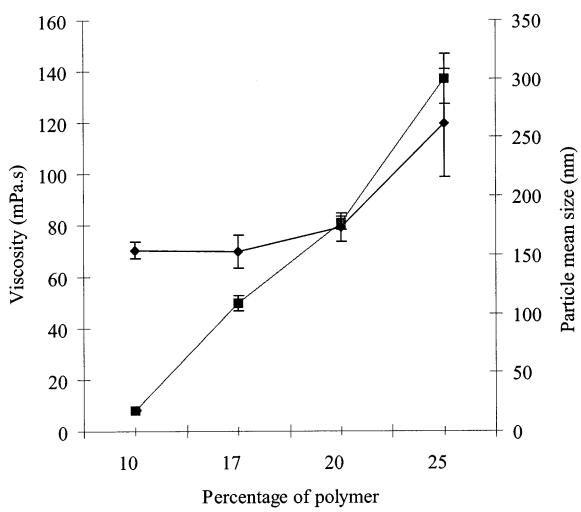

Fig. 4. Influence of the percentage of polymer on viscosity of organic phase (■) (mean ± SD, $n = 22$) and the mean size of RG755 nanoparticles (◆) (mean ± SD, $n = 3$).

3.4. Percentage of the polymer in organic phase

As shown in Fig. 4, an increase in the percentage of the polymer affected the particle size. First, from 10 to 17% (w/w), no significant effect on particle size was observed. However, increasing the percentage to 25% (w/w) resulted in a 60% increase in particle size. Indeed, at lower polymer concentrations, the viscosity of the emulsion was not modified enough to affect the size of the emulsion droplets. Increasing the concentration of the polymer resulted in an increase in the viscosity of the organic phase leading to a highly viscous emulsion. This high viscosity led to a resistance to the shear forces in the emulsion and to a less efficient stirring with a negative impact on particle size. A similar trend was described by other authors (Jeffery et al., 1991; Leroux et al., 1995; Quintanar-Guerrero 1997). However, Leroux et al., (1995) explained these results by the difference in density of internal and external phases. For the subsequent studies, the appropriate conditions for the preparation of small nanoparticles were selected, based on the results obtained in previous investigations, i.e. stirring rate 2000 rpm, duration of emulsification 15 min, concentration of PVAL 15% (w/w) in the aqueous phase and concentration of polymer 17% (w/w) in the organic phase.

3.5. Influence of the nature of the solvent in the organic phase

The nanoparticles were prepared using several types of polymers and copolymers displaying various compositions and molecular weights. The interest of choosing such polymers was the possibility of modulating drug release profile and polymer degradation since this depends on several parameters including monomer ratio, molecular weight, cristallinity and the polymer end groups (Dunn et al., 1988; Mauduit and Vert, 1993; Sah and Chien, 1995; Tracy et al., 1995; Rothen-Weinhold et al., 1997; Tracy et al., 1999). Moreover, the nature of the solvent could influence the formation of nanoparticles in terms of the viscosity of the organic phase and the particle mean size. Hence, in this study, this was assessed using THF and acetone.

Table 2
Influence of the nature of the solvent on the raw particle mean size and the viscosity of the organic phase

			THF			Acetone		
Polymer	PLA/PGA ratio	Mw	η (mPa·s)[a]	Mean size (nm)[b]	PI[c]	η (mPa·s)[a]	Mean size (nm)[b]	PI[c]
RG502H	50/50	12 000	3.6 ± 0.3	102 ± 4	2	2.5 ± 0.4	137 ± 13	3
RG502	50/50	12 000	9.7 ± 0.1	125 ± 9	4	2.7 ± 0.3	173 ± 15	2
RG504H	50/50	48 000	47.7 ± 3.4	154 ± 17	4	17.6 ± 0.5	210 ± 66	2
RG752	75/25	12 000	5.5 ± 0.3	132 ± 3	4	3.2 ± 0.5	120 ± 7	3
RG755	75/25	63 000	49.9 ± 3.1	148 ± 5	2	20.2 ± 1.1	121 ± 10	4
RG756	75/25	98 000	157.1 ± 6.3	152 ± 25	4	44.2 ± 2.5	145 ± 5	3
R104	100/0	2 000	2.4 ± 0.3	152 ± 9	3	2.2 ± 0.3	143 ± 5	3
R202	100/0	16 000	2.7 ± 0.3	138 ± 8	4	2.4 ± 0.2	183 ± 7	3
R202H	100/0	16 000	4.4 ± 0.3	166 ± 5	3	2.3 ± 0.3	174 ± 14	3
R203	100/0	28 000	7.3 ± 0.2	145 ± 4	4	3.9 ± 0.3	184 ± 5	2

Aqueous phase: PVAL 15% (w/w), organic phase PLGA or PLA 17% (w/w), stirring rate 2000 rpm, stirring time of emulsification 15 min.

[a] Mean $\pm$ SD ($n = 22$).

[b] Mean $\pm$ SD ($n = 3$).

[c] PI: polydispersibility index, 0–9.

The particle size obtained using THF as organic solvent is reported in Table 2 together with the respective polymer properties. Small particles in a range of 102–166 nm were produced with all the polyesters tested. For PLGA 75:25 copolymers, the viscosity increased with an increase in the PLGA molecular weight and the effect of this parameter on particle size was observed (Fig. 5). However, with PLGA 50:50 copolymers, the viscosity of the organic phase was dependent on the molecular weight as well as on the structure of the end groups of the polyester. In fact, the same trend between the viscosity and the molecular weight was observed (Fig. 6). Nevertheless, with the same molecular weight, the viscosity was influenced by the presence of the free carboxylic end groups. The RG502 solution was more viscous (9.7 ± 0.1 mPa·s) than the RG502H solution with free carboxylic end groups (3.6 ± 0.3 mPa·s). Consequently, particle size was decreased in the same trend (Fig. 6). The inverse trend was observed for PLA polymers such as R202 and R202H, which are more hydrophobic. This may be explained by the affinity of these polymers towards the solvent. However, for PLA polymers, no evident effect of the polymer molecular weights and viscosities on the particle mean size could be demonstrated from the results (Table 2).

Since acetone is commonly used in the salting-out technique, it has been chosen to evaluate the influence of the solvent nature on particle size

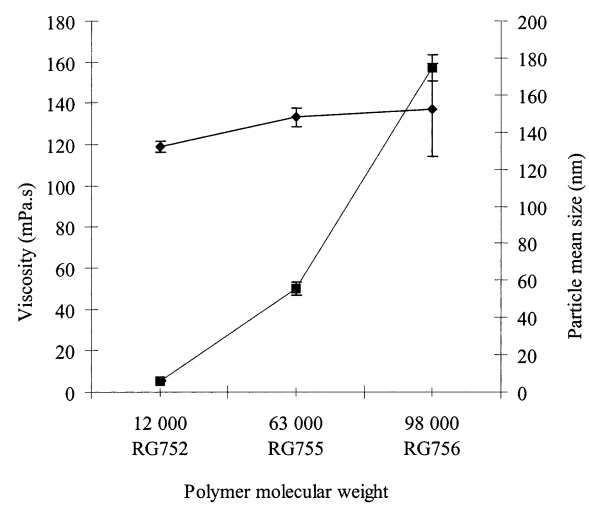

Fig. 5. Correlation between the molecular weight of PLGA 75:25 copolymers, the viscosity of organic solution (■) (mean $\pm$ SD, $n = 22$) and the particle mean size (◆) (mean $\pm$ SD, $n = 3$).

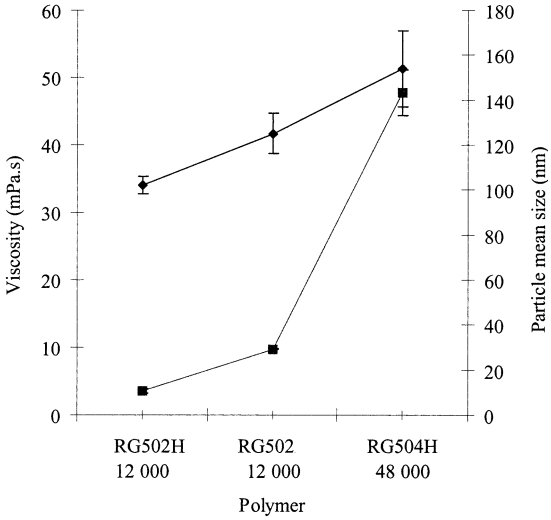

Fig. 6. Influence of the nature and the molecular weight of PLGA 50:50 copolymer on the viscosity of organic solution (■) (mean ± SD, $n = 22$) and the particle mean size (◆) (mean ± SD, $n = 3$).

using the same process parameters as described with THF. The determination of the organic phase viscosity showed values lower than those obtained with THF (Table 2). A correlation between the polymer molecular weight, the viscosity and the particle size cannot really be defined. Sub-200 nm nanoparticles were also produced with all the polyesters tested. These results indicate that sub-200 nm nanoparticles can be obtained with a large range of polymers using identical process parameters. This represents a great advantage as it is possible, for a given particle size, to select different polymer degradation and drug release rates by choosing the appropriate polymer.

3.6. Nanoparticle purification

Cross-flow filtration was used as a suitable purification process to remove the solvent, the salting-out agent and the stabilizing colloid (Allémann et al., 1993a, 1998; Quintanar-Guerrero et al., 1996). In order to check the elimination profile of the colloid stabilizing, a quantification of PVAL was performed during the purification. As shown in Fig. 7, 75.2% of the PVAL was eliminated after collecting 4 l of filtrate. A total of 10 l was used in order to achieve complete elimination of the free stabilizing colloid in the nanoparticle suspension. The amount of the residual PVAL remaining on the nanoparticles was generally around 14% (w/w) of the total weight of the freeze-dried nanoparticles (Table 3). In previous studies (Allémann et al., 1993b; Lee et al., 1999), a linear relationship between the amount of the residual PVAL adsorbed on the nanoparticle surface and the specific surface area of particle was demonstrated. This hydrophilic layer on the surface of the nanoparticles was found to favour the redispersion of the freeze-dried nanoparticles after rehydration (Allémann et al., 1993b; De Chasteigner et al., 1996). For small nanoparticles, this hydrophilic layer is not sufficient to preserve the particle size during the freeze-drying process since particle aggregation was observed irrespective of the polymer nature (Table 3). Thus, the drying process which may be considered as a simple dehydration process appeared to be problematic for small nanoparticles due to their tendency to aggregate. Indeed, this procedure seems to generate a variety of freezing and drying stresses which induce particle surface modifications resulting in the formation of aggregates.

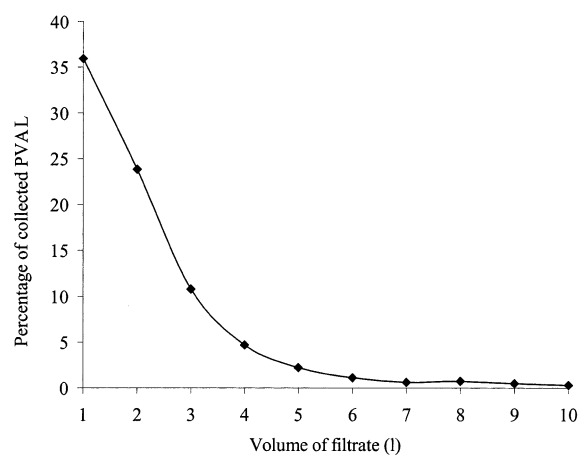

Fig. 7. PVAL elimination in the filtrate during the purification procedure of RG755 nanoparticles.

Table 3
Nanoparticle surface characterization and redispersibility after freeze-drying without lyoprotectants (mean ± SD, $n = 3$)

Polymer	Particle mean size before freeze-drying (nm)	Mean size of the rehydrated nanoparticles (nm)	Residual PVAL (% w/w)
RG752	143 ± 3.1	354 ± 16	14.8 ± 2.5
RG755	145 ± 5.0	233 ± 10	14.5 ± 0.5
RG756	151 ± 1.5	251 ± 1.5	14.1 ± 3.2
R202	150 ± 8.5	213 ± 25	14.8 ± 1.1
R203	155 ± 1.4	311 ± 11	15.2 ± 0.8

3.7. Nanoparticle sterilization by membrane filtration

The parenteral route requires sterile and apyrogenic products. The sterile filtration technique has been widely used for the sterilization of liposomes (Goldbach et al., 1995) and emulsions (Lidgate et al., 1992). Surprisingly, although numerous studies have reported the manufacture of small nanoparticles (Leroux et al., 1995; Rodrigues et al., 1995; Quintanar-Guerrero 1997), very few have addressed the issue of sterile filtration applied to these systems. In addition, many attempts at sterile filtration failed due to membrane clogging problems. This clogging phenomenon is observed when the particle size is too close to the membrane pore size. It can be noted that sub-300 nm liposomes have been filtered through 0.2 μm cellulose acetate membranes without any significant change in the vesicle size and with no breakage of the vesicle membrane or leakage of the entrapped solute (Goldbach et al., 1995). However, in the case of non-flexible polymeric nanoparticles, the size of the filtered particles should be much below the membrane cut-off to avoid clogging problems.

The initial feasibility study showed significant differences in efficiency between different membranes. In most cases, the filters clogged quickly and little filtrate could be collected. Only Steriflip™ filter units with a 0.22 μm Millipore Express™ membrane (modified surface polyethersulfone), provided satisfactory results in terms of flow rate and high filtering capacity under vaccum. After the sterile filtration, the measurements of particle size showed no significant change in particle size and size distribution. Upon filtration, the loss of material was also assessed by freeze-drying aliquots of nanoparticle dispersions taken before and after filtration. From the results obtained after weighing of the dried powder or cake, it could be shown that no significant loss of material by adsorption on the membrane surface had occurred (data not shown). These results are also supported by Zheng and Bosch, (1997). They showed that a 0.2 μm polyethersulfone membrane disposable capsule filter was suitable for sterilizing sub-200 nm Nanocrystal™ drug dispersions without measurable drug loss.

3.8. Lyoprotective effect during the freeze-drying process

Numerous studies have shown the lyoprotective effect of excipients such as sugars to prevent particle aggregation during the freeze-drying process (Auvillain et al., 1989; De Chasteigner et al., 1995, 1996). In order to choose the appropriate lyoprotectant, which effectively prevents particle aggregation, a screening of sugars at variable concentrations was performed. A range of weight ratios of sugar to nanoparticles ranging from 0.15:1 to 2:1 was tested during this screening. As shown in Fig. 8, regardless of the nature of the sugar tested, redispersion of the freeze-dried nanoparticles could be obtained with all the lyoprotectants while important aggregation was always observed without lyoprotectant. A 30 s manual shaking was sufficient to ensure complete redispersion of the freeze-dried particles. At sugar:nanoparticle mass ratio lower than 0.5:1, an increase in particle size was observed after redispersion in water with an average of at least 1.5 times the initial particle size (Fig. 8). A weight

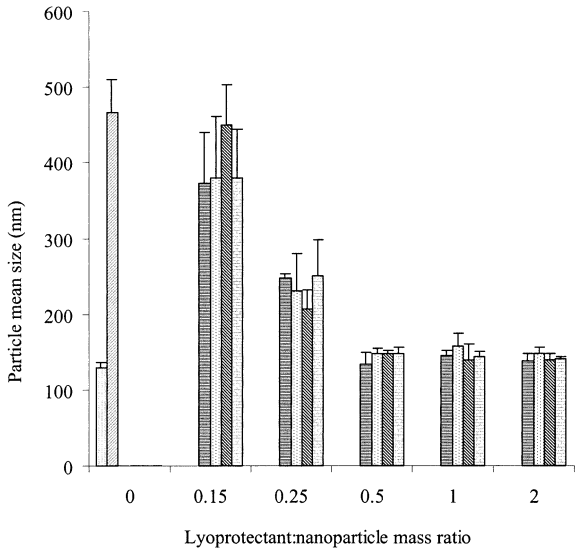

Fig. 8. Effect of different lyoprotectants on RG755 nanoparticle size (range of weight ratio of sugar to nanoparticles from 0:1 to 2:1). Before freeze-drying ((▥),), after freeze-drying without lyoprotectant ((▨),), after freeze-drying with trehalose ((▤),), lactose ((▦),), glucose ((▧),), mannitol ((▩).) (mean ± SD, $n = 3$).

ratio of sugars to nanoparticles of at least 0.5:1 is required for good redispersion of the cake. The lyoprotective effect was attributed to the ability of the sugar additive to form a glassy amorphous matrix around the particles, preventing the particles from sticking together during the removal of water (Ford and Dawson, 1993; Carpenter et al., 1997; Randolph 1997).

A critical analysis of freeze-dried products normally includes the observation of the final volume and appearance of the cake. One of the desired characteristics of a freeze-dried pharmaceutical form includes an intact cake occupying the same shape and size as the original frozen mass (Snowman, 1991). In our case, the structure of the final cake was dependent on the nature of the lyoprotectant added. All the sugars tested, except glucose, provided a fluffy, shelf stable cake, whereas a shrinkage was observed with glucose. Indeed, the volume of the final freeze-dried cake containing glucose as lyoprotectant was smaller than the initial suspension. In addition, since these freeze-dried powders were very hygroscopic, suitable storage conditions have to be taken (DeLuca and Boylan, 1984) to avoid moisture contamination.

3.9. Sterile nanoparticles freeze-drying set-up

In the pharmaceutical environment, freeze-drying of sterile products in aseptic conditions remains a challenge since this procedure is labour-intensive and requires expensive equipment (Snowman, 1991). In this study, freeze-drying of the sterile nanoparticle suspensions in a non-aseptic area was set up. Indeed, by covering the vials with sterile 0.22 μm membrane bacterial-retentive filters, bacterial contamination of the product was prevented and it did not impede the freeze-drying process (Fig. 9). However, the dia-meter of the vial neck and the depth of vial filling were critical. For 40 ml of suspension, if the vial neck (100 ml) was lower than 40 mm, elimination of the subli-

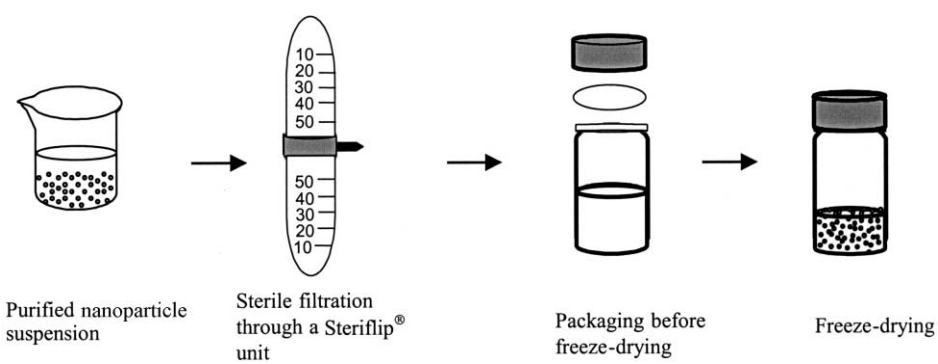

Fig. 9. Schematic procedure for sterilization and package of the sterile nanoparticle suspensions for the freeze-drying in non-aseptic environment.

mated water was impeded. Due to incomplete sublimation into the vial, the temperature within the frozen product increased, leading to either shrinking or thawing out of the product. Meltback a collapsed cake was also observed due to the change from the solid to liquid state. However, with a larger flask fitted with at least 40 mm of sterile membrane filter diameter, the nanoparticle suspensions remained frozen during the drying step and the final product was of a good quality. In this case, the presence of a membrane on top of the vials was not an obstacle to the escape of water vapour. Freeze-dried in these conditions, the nanoparticle suspensions containing a lyoprotectant could be successfully reconstituted. In addition, this packaging system has the advantage of facilitating the handling and the transport of vials to the freeze-drier and of preserving the product from potential contamination. However, the vials must be sealed immediately after removal from the freeze-drier, in order to minimize the risk of moisture contamination. All these conditions were fulfilled and the sterilization of the formulations was possible in a relatively simple and non-costly way.

3.10. Validation and sterility testing

Before performing the sterility testing, it was essential to validate the method used. Such a validation requires a demonstration that the product itself does not inhibit the growth of common contaminating organisms. In practical terms, the microorganism proliferation must be produced to the point where the liquid medium becomes visibly cloudy. For all organisms, proliferation was observed in every media containing the tested nanoparticulate formulations. These results demonstrated that neither PLGA nor PLA nanoparticles have a bacteriostatic and fungistatic activity.

In order to ensure the sterility of the final freeze-dried powder (i.e. free of all forms of viable microorganisms), the sterility testing was performed according pharmacopoeial requirements. Sterility was assessed by the observation of the media during the incubation period. All batches of nanoparticles tested showed no detectable visible growth of microorganisms, contrarily to positive controls for which a substantial increase of turbidity was systematically observed (data not shown). These observations suggested that sterile final nanoparticulate formulations had been achieved.

4. Conclusion

In recent years, biodegradable nanoparticles based on polyesters were the focus of increased attention as drug delivery systems. However, the sterilization of these formulations by a suitable method is often problematic. This study demonstrates that using the salting-out technique, various types of PLGA and PLA nanoparticles with a mean size below 200 nm can be produced using a large range of polymers. Subsequently, these particles can be sterilized by filtration through 0.22-μm filters. Provided the filtering membrane is correctly selected, this technique allows the preparation of nanoparticulate suspensions free from unwanted organisms, in a very rapid and simple way. The lyophilization of the sterile suspensions in a non-aseptic environment was made possible by adjustment of an appropriate packaging technique. Indeed, contamination was successfully prevented by covering the vials with sterile bacterial membrane filters. It appeared that the diameter of the vial neck was a key parameter insuring the correct course of the freeze-drying process. The freeze-dried nanoparticles could be easily redispersed in water by simple manual shaking, when freeze-dried in the presence of a lyoprotectant. It appears that large ranges of lyoprotectants are suitable as additives for these types of formulations. Considering possible clinical issues, the various procedures established in this study seem to be appropriate for the successful development of drug loaded nanoparticulate formulations intended for parenteral administration.

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