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Stability and freeze-drying of cyclosporine loaded poly(D,L lactide–glycolide) carriers

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Abstract

The present paper describes the stability of poly (D,L-lactide–glycolide) nanoparticles (PLGA NP) and microspheres (MS), either alone or loaded with cyclosporine (CyA), stored at 8°C and room temperature (RT). Freeze-drying of these formulations was evaluated as an alternative method to achieve long term stability. A significant polymer rupture was detected during PLGA MS preparation by solvent evaporation, which correlated with the stirring rates used for the formation of the primary emulsion. On the other hand, the polymer remained unchanged during NP formation. After 6 months of storage, PLGA NP of a size below 80 nm aggregated when stored at RT whereas no changes of particle size were observed for the remaining formulations and experimental conditions. Drug entrapment significantly increased by about 9.5% only during PLGA NP storage at RT. The PLGA molecular weight of NP dropped at RT being these changes related to the initial particle size and amount of CyA incorporated. The same effect was observed at 8°C but only the particle size showed a significant influence. The drop of PLGA molecular weight observed during storage of MS was not dependent on the storage temperature but it was directly related to the molecular weights obtained after MS preparation. Freeze-drying studies revealed that it was not feasible to maintain the initial PLGA NP characteristics after reconstitution. On the other hand, MS lyophilized in the absence of cryoprotectants retained the drug initially entrapped; however, the presence of at least 5% cryoprotectant was essential to keep the initial particle size. Therefore, PLGA NP and MS show a significant instability when stored as suspensions. Freeze-drying offers a good alternative to stabilize polymeric MS but the preservation of the PLGA NP characteristics by freeze-drying needs for further investigations. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: PLGA; Microspheres; Nanoparticles; DSC; SEC; Cyclosporine

1. Introduction

In recent years, the application of biodegradable polymers to the preparation of pharmaceuticals has gained much importance, fundamentally due to their capacity to modulate the release of drugs or even to modify their distribution within the body (Brannon-Peppas, 1995). In this way, most of the formulations based on the incorporation of drugs to multiparticulate systems such as microspheres (MS) or nanoparticles (NP) are prepared from aliphatic polyesters such as polycaprolactone (PCL) or poly(D,L-lactide–glycolide) copolymers (PLGA), whose kinetics of degradation condition their in vivo applications. The degradation process depends on several factors including the preparation method, the intrinsic properties of the polymer (molecular weight (MW), chemical structure, hydrophobicity and crystallinity) and physical–chemical parameters as the pH, the temperature or the ionic strength of the environment (Lin et al., 1994; Lemoine et al., 1996). Despite the large number of studies dealing with the preparation of drug loaded multiparticulate carriers, the number of papers describing their stability is relatively little (Auvillain et al., 1989; Lemoine et al., 1996; Guterres et al., 1995; De Chasteigner et al., 1996). Most of the MS and NP formulations whose stability have been reported were obtained by solvent evaporation and solvent displacement, respectively (Coffin and McGinity, 1992; Spenlehauer et al., 1989; Park, 1994). Some of them contained no drug and only a few studies report on the influence that NP and MS characteristics have on their own stability (Coffin and McGinity, 1992; Molpeceres et al., 1997).

NP and MS are initially obtained as a milky suspension showing acceptable physical short term stability due to their reduced particle size. However, particle aggregation may occur after long periods of storage. Furthermore, PCL and PLGA copolymers degrade non-enzymatically in aqueous environments and drugs incorporated in such systems may be released during storage (Guterres et al.,

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1995; De Chasteigner et al., 1996). Several authors have reported that the polymer degradation rates and the release rates of drugs incorporated in polyester matrices are accelerated in the presence of basic drugs and amines (Lin et al., 1994; Maulding et al., 1986; Cha and Pitt, 1988). Since the actual tendency is to encapsulate protein and peptide drugs which may have primary amino groups in their side chains there is a potential for the interaction with the polyester matrices modifying the degradation rates and the release profiles.

Lyophilization represents a good alternative to provide the requisite of long term product stability. Nonetheless, previous studies about freeze-drying of colloidal carriers (Crowe et al., 1996; Sun et al., 1996; Fang et al., 1997; Ozaki and Hayashi, 1997; Molpeceres et al., 1997; Esquisabel et al., 1997) demonstrated that the addition of cryoprotectants is essential for the maintenance of the initial formulation characteristics.

The potent immunosuppressant cyclosporine (CyA) is a neutral fungal metabolite showing a high pharmacokinetic variability and nephrotoxicity (Fahr, 1993; Faulds et al., 1993) and therefore, based on the advantages offered by polymeric carriers, it represents a candidate drug to be incorporated in such systems.

Since we have already reported about the stability of PCL NP (Molpeceres et al., 1997), this study was focused to determine the stability of PLGA NP and MS suspensions, non-loaded and in the presence of the model peptide CyA. In addition, freeze-drying studies were also performed as an alternative method to increase the long term stability of these CyA carriers.

2. Experimental procedures

2.1. Materials

PLGA 50:50 (iv 0.5 dl/g; MW 45 000 Da) was obtained from Boehringer Ingelheim S.A. (Germany). Cyclosporine was donated by courtesy of Sandoz Pharma (Spain). Poloxamer 188 and polyvinyl alcohol (PVA) (MW 30 000–70 000), were supplied by Fluka Chemika (Switzerland) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. Acetone, tetrahydrofurane (THF) and acetonitrile (MeCN) (Scharlau, Spain) were of HPLC quality and methylene dichloride (Panreac, Spain) was of analytical grade. The cryoprotective agents, D(+) anhydrous glucose and D(+) trehalose dihydrate (microbiology grade), and D-mannitol and D-sorbitol (microselect grade) were purchased from Fluka Chemika (Switzerland) with a purity greater than 99.5% by HPLC. Clinical grade dextran (MW 90 000) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of NP and MS

PLGA NP were prepared by the method of Fessi et al. (1989) slightly modified whereas PLGA MS were elaborated by solvent evaporation as previously described (Jeffery et al., 1991; Chacón et al., 1996). Rotatable central composite designs (RCCD) had been previously applied to the preparation of CyA-loaded PLGA NP and MS (Chacón et al., 1996). The characterization of these formulations showed that the particle sizes and encapsulation percentages covered a significant range. Therefore, these samples were further used to asses the influence of NP and MS characteristics on their stability. The orthogonal values of the RCCD and the correspondence between real and orthogonal values were reported elsewhere (Chacón et al., 1996). Secondly, NP and MS were prepared considering the physiological limitations of the oral route for further in vivo studies (mainly, the reduced gastric volume of rats). According to the previously reported RCCD results (Chacón et al., 1996), 360 and 180 mg PLGA were used to obtain NP and MS, respectively. The target particle sizes for peroral administration were 2.5 μm for MS and 100 nm for NP as deduced from the results by Eldridge et al. (1990) and Jani et al. (1990), respectively.

2.3. Stability studies

Due to the high shear stress and turbulences during preparation of MS and NP, the potential effect of the RCCD variables on polymer MW was determined by size exclusion chromatography (SEC). Experimental data was analyzed by response surface methodology. Also, the stability of PLGA NP and MS suspensions prepared under the experimental design (RCCD) protocol was assessed by comparing the initial drug encapsulation percentage, particle size and polymer MW with those obtained after storage at 25°C and 8°C in closed glass vials for 6 months.

2.4. Freeze-drying studies

Unloaded PLGA NP and MS as well as 2.5 mg/ml CyA-loaded PLGA MS were freeze-dried in the presence of 5% glucose, trehalose, mannitol and sorbitol. Unloaded PLGA NP were also freeze-dried in the presence of 10, 20 and 30% glucose, trehalose and dextran, 10% sorbitol and 10% and 18% mannitol (its maximum aqueous solubility). Samples were frozen under two different experimental conditions: −196°C during 15 min by using liquid N_2 and −70°C for 72 h. After, the samples were immediately placed into the freeze-drying chamber (Virtis Unitop 400 SL, USA). The first drying step was performed at −40°C for 24 h. Secondly, the temperature was increased to 15°C for 48 h and to 30°C for the following 24 h. The same experiments were performed in the absence of cryoprotective agents as a control. Sample reconstitution was performed by adding 2 and 4 ml of MilliQ Water to the dried cake of MS and NP, respectively. After 5 min, a mild manual shaking was applied.

The ratios between the initial particle size, standard deviation and drug entrapment percentage to those ob-

tained after freeze-drying (Sf/Si, SDf/SDi and EPf/EPi, respectively) were calculated to determine the adequacy of the processing conditions. Also, the macroscopic appearance of NP suspensions after reconstitution was evaluated according to the following criteria: (0) absence of aggregates, (1) few small sized aggregates, (2) moderate aggregation and (3) large number of big aggregates. Finally, the potential changes induced by freeze-drying on polymer structure and MW were determined by DSC and SEC using 2.5 mg/ml CyA-loaded and unloaded PLGA MS as model formulations.

2.5. Characterization of NP and MS

2.5.1. Particle size measurement and drug loading efficiency

The mean size of NP and MS was measured by light scattering (Microtrac® UPA and SRA, Leeds and Northrup, Ireland). Assuming that no changes in particle porosity occur due to different preparation conditions and considering the recovery of polymer is 100%, the total surfaces of NP and MS were calculated as follows:

Total surface (cm^2)=Average NP surface (cm^2)×NP number, Average NP surface (cm^2)=πd^2, NP number= Total amount of polymer (g)/Average NP mass (g), Average NP mass (g)=Average NP volume (cm^3)×NP density (g/cm^3), NP volume (cm^3)=$\pi d^3/6$ and NP density=1 g/cm^3.

The amount of CyA incorporated by the carrier was determined by HPLC as previously described (Guzmán et al., 1993). CyA entrapment was calculated as the difference between the total amount of drug in the samples and that in the external aqueous phase after centrifugation of NP at 40 000 g for 1 h and MS at 11 000 g for 30 min. Drug loading efficiency was expressed as the percentage of incorporated drug relative to the total CyA in the medium.

2.5.2. Size exclusion chromatography (SEC)

Polymer MW was determined by using THF as mobile phase. The chromatographic system consisted of a pump (Waters 510) programmed at a constant flow-rate of 1 ml/min and a PL–GEL 10 μm Mixed B 30×0.75 column (Polymer Laboratories, USA) termostatized at 35°C. Eluting solutes were detected by differential refractometry (Waters 410). 50 μL samples were injected after the equipment was calibrated with polystyrene standards whose MW ranged from 550 Da to 5.48×10^6 Da (TSK Toyo Suda Manufacturing Co-Ltd, Tokyo, Japan).

SEC analysis were carried out on the pellets obtained after centrifugation of NP and MS suspensions at 40 000 g for 1 h and 11 000 g for 30 min, respectively. Freeze-dried PLGA MS were analyzed directly.

2.5.3. Differential scanning calorimetry (DSC)

The glass transition temperature (Tg) and the melting temperature (Tm) were determined with a differential scanning calorimeter Mettler TA4000 (Mettler-Toledo, Switzerland). The instrument calibration was carried out with an Indio standard. Amounts between 5 and 10 mg of dried MS were weighed and placed into an aluminum capsule. Samples were scanned from 10°C to 300°C at a rate of 10°C/min.

3. Results and discussion

3.1. Initial mean particle size and drug loading of NP and MS

PLGA formulations obtained under the RCCD conditions showed that the initial particle size ranged from 46 to 146 nm for NP and from 1.6 to 31 μm for MS, whereas the encapsulation percentage ranged from 47.9% to 84.71% for NP and 82.26% to 97.69% for MS (Table 1). The standard deviation of NP size distributions represented about 32±4% of the initial mean particle size.

On the other hand, unloaded PLGA NP and MS and CyA-loaded PLGA MS (2.5 mg/ml) presented an initial mean particle size of 103.92±50.05 nm for NP and 2.29±0.25 μm for MS, being 97.24% the initial encapsulation percentage for the latter formulation.

3.2. Polymer stability during MS and NP preparation

Response surface analysis was applied to identify those formulation variables responsible for polymer MW modifications. The statistical analysis of data generated the following polynomial equation:

$$MW = 43159.34 - 7833.57 \cdot A - 5693.94 \cdot A^2 \quad r^2 = 0.95$$

where A stands for the stirring rate.

The validity of the regression model was assessed by the application of a test described previously (Chacón et al., 1996). A determination coefficient higher than 0.9 (r^2= 0.95) was achieved with the equation terms showing statistically significant F-ratios ($P<0.05$). Therefore, the regression model resulted valid. A value of 64% of the MW variation during MS preparation is explained by the linear term of the regression fit ($F=299.69$, $P<0.001$) and the resting 31% by the quadratic term ($F=145.73$, $P<0.001$). These results suggest that the stirring rate during MS preparation must be carefully selected to avoid polymer instability. Concerning PLGA NP, no significant changes of polymer MW were detected during NP formation.

3.3. Stability of NP and MS stored as suspensions

3.3.1. PLGA NP

3.3.1.1. Particle size

After 6 months at RT, NP of an average particle size below 80 nm exhibited a significant increase of particle

Table 1
Initial size and % encapsulation values (E.P.) for PLGA NP and MS elaborated according to the experimental design (final drug concentration 100 μg/ml)

Formulation	PLGA NP		PLGA MS	
	Size (nm)	% E.P.	Size (μm)	% E.P.
1	73±12	67.73±6.66	24.74±4.49	92.51±2.62
2	83±23	84.71±2.67	1.68±0.02	82.98±5.41
3	146±21	79.69±3.32	17.24±8.57	96.85±1.45
4	72±17	69.00±2.39	1.94±0.16	85.28±0.34
5	102±20	78.32±2.72	3.31±0.62	90.91±2.73
6	97±12	78.77±2.85	2.99±0.44	90.35±1.37
7	121±25	82.84±3.77	2.94±0.47	88.86±7.18
8	50±14	71.24±1.03	4.25±1.23	90.59±2.49
9	81±1	67.81±2.36	2.77±0.70	90.09±2.27
10	119±28	82.69±2.79	1.60±0.05	82.26±4.26
11	97±10	78.61±1.24	30.67±9.09	97.69±0.78
12	99±29	76.23±3.87	2.99±0.38	90.04±4.77
13	117±16	72.66±3.89	3.51±1.35	90.07±2.79
14	46±9	83.68±2.73	2.91±0.43	90.71±3.16
15	62±4	80.54±0.65		
16	123±6	77.31±2.31		
17	120±15	83.97±1.14		
18	67±21	47.90±7.64		
19	96±20	74.65±1.41		
20	99±22	74.49±4.90		

size and standard deviation ($P<0.05$) suggesting an aggregation process. On the contrary, neither the mean particle size nor the size distribution profile of PLGA NP were affected when stored at 8°C ($P>0.05$).

3.3.1.2. Drug encapsulation

All samples stored at RT, except sample 14, exhibited a statistically significant increase ($P<0.05$) of drug entrapment (average of 9.46%±3.85%). Sample 14 corresponded to NP with the smallest size within the NP group (46±9 nm) and it presented the highest total surface area (130 434.78 cm^2) of all RCCD formulations. This is consistent with one of the highest drug loadings (83.7%) obtained with PLGA NP considering that CyA shows a great tendency of adsorption. On the other hand, sample 18 also presented a small particle size (67±21 nm) although it was elaborated with the lowest amount of polymer (20 mg) and therefore, its total surface only amounted to 17 910.45 cm^2. As a consequence it showed the lowest encapsulation percentage (47.9%) (Fig. 1a). When the increase of drug encapsulation percentage after sample storage was plotted against the total surface of NP an inverse relationship (Fig. 1b) indicated that the higher the initial amount of drug in the supernatant the higher the increment of drug incorporation during storage. When NP were stored at 8°C the increase in drug entrapment followed the same pattern but the effect was less pronounced. This finding may be the result of the CyA solubility behaviour (less soluble at higher temperatures) (Molpeceres et al., 1996; Ismailos et al., 1991). Thus, variations of the total surface of NP are counteracted by the higher solubility of the drug in the external aqueous medium at 8°C as compared to 25°C, and

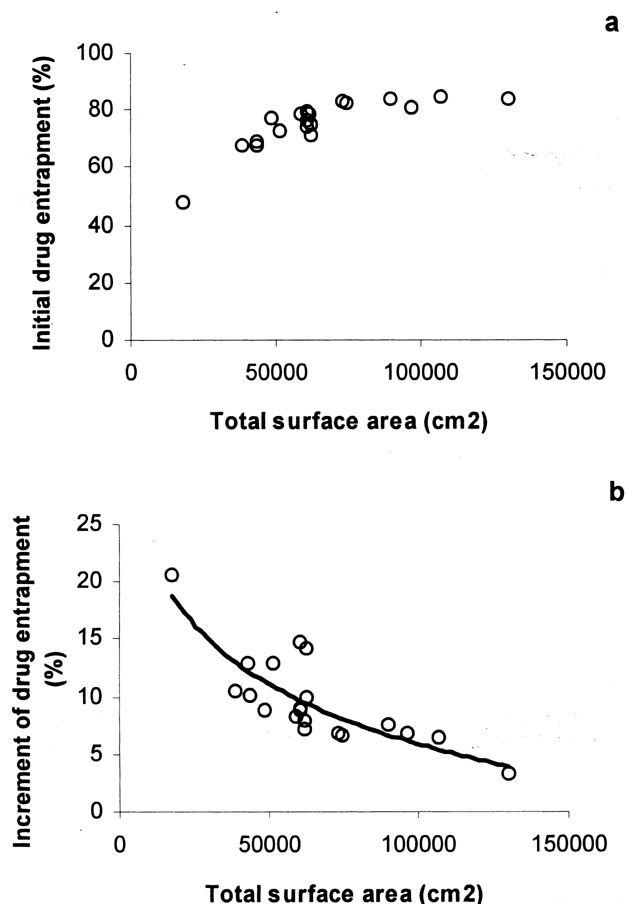

Fig. 1. Initial CyA encapsulation percentage in PLGA NP (a) and its increment (b) upon storage for 6 months at room temperature as a function of total surface area.

then the changes in encapsulation percentage did not achieve statistical significance.

3.3.1.3. Polymer degradation

After 6 months of PLGA NP storage, the polymer MW decreased more significantly at RT (MW dropped on average by 16 682±3060 Da) than at 8°C (MW dropped on average by 11 200±3200 Da). The analysis of MW variations by stepwise multiple linear regression when sample storage at RT indicated that particle size and drug entrapment explained 82% of the variability found in MW. When the same statistical analysis was applied to the samples stored at 8°C only the particle size showed a significant effect ($P=0.01$) but it only accounted for the variation of MW in 31% of the cases. The resulting mathematical relationships were the following:

At RT % MW variation = 30.42 + 0.83 (drug loading) − 0.34 (size)
At 8°C % MW variation = 64.84 − 0.21 (size)

Coffin and McGinity (1992) studied the effect of temperature on the stability of poly-lactic (PLA) pseudo-latexes, reporting that the rate of decrease in the MW of PLA was much faster at room temperature or 37°C than at 5°C. Furthermore, the acceleration of polyester hydrolysis in the presence of basic drugs or peptides containing primary amino groups has been reported (Lin et al., 1994). Then, it may be possible that CyA which contains secondary and tertiary nitrogen atoms as well as a hydroxilic group in aminoacid 1 catalyze the polymer breakdown. In fact, when the amount of CyA associated to the carrier increased the drop of MW was more marked. On the contrary, an increase of particle size inversely correlated with the variation of polymer MW, probably due to the smaller surface area exposed to the aqueous environment. These results are not in accordance to previous papers reporting that the degradation of polyesters (empty NP) is bulk mediated as opposed to surface mediated (Coffin and McGinity, 1992). The explanation of such discrepancy is likely related to the additive effects of particle size and drug loading. Unpublished results evidenced that most of the CyA associated to the carrier is adsorbed onto the particle surface. Thus, the larger the surface area, the higher the CyA amount associated to NP (Fig. 1a) and consequently, the more favored the surface mediated degradation. Fig. 2 shows the plot of percentage MW variations against the regression function for the 20 PLGA NP formulations.

3.3.2. PLGA MS

PLGA MS tended to sediment after a certain time but globally they seemed to be more stable. In fact, drug encapsulation and particle size distribution remained constant over time regardless the storage temperature. Moreover, no difficulties were found to redisperse the particles

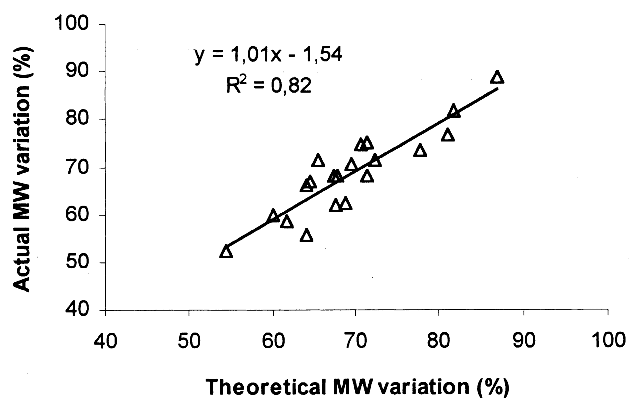

Fig. 2. Correlation between the molecular weight variation of PLGA NP stored for 6 months at 25°C and the theoretical model generated by multiple linear regression.

by applying a gentle manual shaking. With respect to MW, its variation was not related to the storage temperature, however, it showed a direct relationship with the MW obtained after MS preparation (Fig. 3). As shown by the regression analysis of data, the lower the initial MW the lower the MW variation after storage. All samples were considered as a pool because no differences were found between both storage temperatures. Similar findings have been recently reported for the degradation of D,L PLA MS (Delgado et al., 1996). These authors suggest a different breakage mechanism for high and low MW chains. Then, larger chains probably undergo scission at predetermined sites since they are subjected to higher tensions than shorter ones which probably suffer a random scission. Furthermore, the potential production of soluble polymeric fractions during degradation was not evaluated and therefore, the MW variations may have been underestimated.

3.4. Freeze-drying studies

3.4.1. PLGA MS

Table 2 summarizes the changes of particle size and drug encapsulation percentage when the MS were freeze-dried in the presence of 5% glucose, sorbitol, trehalose and mannitol. The formulations were assayed in triplicate except for the samples whose macroscopic cake appearance indicated a possible solute migration to the surface. These samples showing a brittle cake surface were assayed once. Concerning drug leakage, all MS formulations retained the amount of drug initially incorporated as shown by the ratio EP_f/EP_i. However, the presence of 5% mannitol induced a very slight decrease of drug entrapment. In most cases, the ratios S_f/S_i and SD_f/SD_i indicated an adequate reconstitution of unloaded MS even in the absence of cryoprotectants. However, a marked increase of the mean particle size and standard deviation was observed when they were frozen at −196°C in the presence of 5% mannitol. The lack of cryoprotectants and freezing of CyA-loaded MS at −196°C induced particle

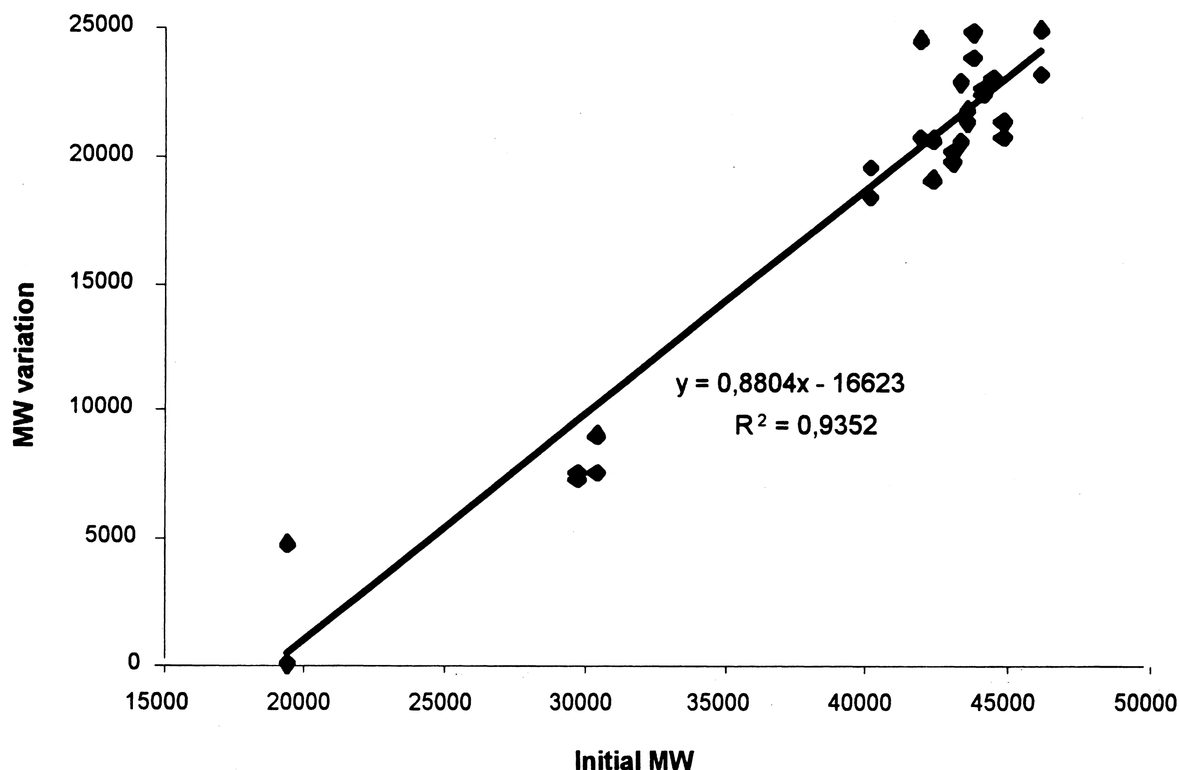

Fig. 3. Molecular weight variation of PLGA MS after 6 months of storage at both temperatures (25°C and 8°C) vs. the initial molecular weight for each formulation.

aggregation as well (Table 2). Fig. 4 shows the DSC thermograms corresponding to the different formulations frozen at −70°C and freeze-dried in the presence of each cryoprotectant. The thermograms obtained for the cryoprotectants as raw material are included for comparison purposes. Fig. 4A shows the DSC scans of glucose (1) and empty (2) or CyA-loaded PLGA MS (3) lyophilized with 5% glucose. Glucose showed three endothermic peaks, two of which corresponded to the hydrated form of α-D glucose (82°C) and the anhydrous β-D glucose (155°C) (Wade and Weller, 1994). The origin of the third peak at 221°C is unknown at the moment but it is probably related to product decomposition. The thermograms corresponding to freeze-dried unloaded MS basically showed the same thermal events, however, the peak of hydrated α-D glucose was less pronounced and another peak at 145°C evidenced the presence of anhydrous α-D glucose (Wade and Weller, 1994). In addition, the glass transition of PLGA was detected at about 50°C. When CyA was incorporated into MS no anhydrous forms of glucose were detected and the peak for hydrated α-D glucose broadened suggesting a strong interaction between the cryoprotectant and the

Table 2
Values of EP_f/EP_i, S_f/S_i and SD_f/SD_i for PLGA MS unloaded and loaded with CyA after freeze-drying without and with 5% cryoprotective agents ($n=3$)

Cryoprotector	EP_f/EP_i [1]	S_f/S_i [2]		SD_f/SD_i [3]	
		Unloaded MS	Loaded MS	Unloaded MS	Loaded MS
Glucose-70	1.008±0.002	1.027±0.016	1.007±0.031	1.028±0.103	1.125±0.177
Glucose-196	1.010	1.063±0.075	6.459	1.130±0.207	20.875
Sorbltol-70	1.004±0.002	1.012±0.066	0.988±0.017	1.010±0.197	1.000±0.059
Sorbitol-196	1.002	1.021±0.080	40.102	1.045±0.214	737.250
Trehalose-70	0.993±0.002	1.069±0.043	1.002±0.010	1.226±0.188	1.083±0.059
Trehalose-196	0.991	1.152±0.206	36.527	1.389±0.576	690.625
Mannitol-70	0.956±0.008	1.103±0.138	1.027±0.058	1.235±0.384	1.125±0.176
Mannitol-196	0.974	27.960±23.197	1.908	319.50±314.50	8.125
Without CP-70	0.995	1.051±0.040	2.275	1.126±0.159	9.917
Without CP-196	1.003	1.096±0.042	8.184	1.445±0.084	16.792

[1] EP_f/EP_i: ratio between final and initial CyA encapsulation percentage.
[2] S_f/S_i: ratio between final and initial size.
[3] SD_f/SD_i: ratio between final and initial standard deviation of the size population.

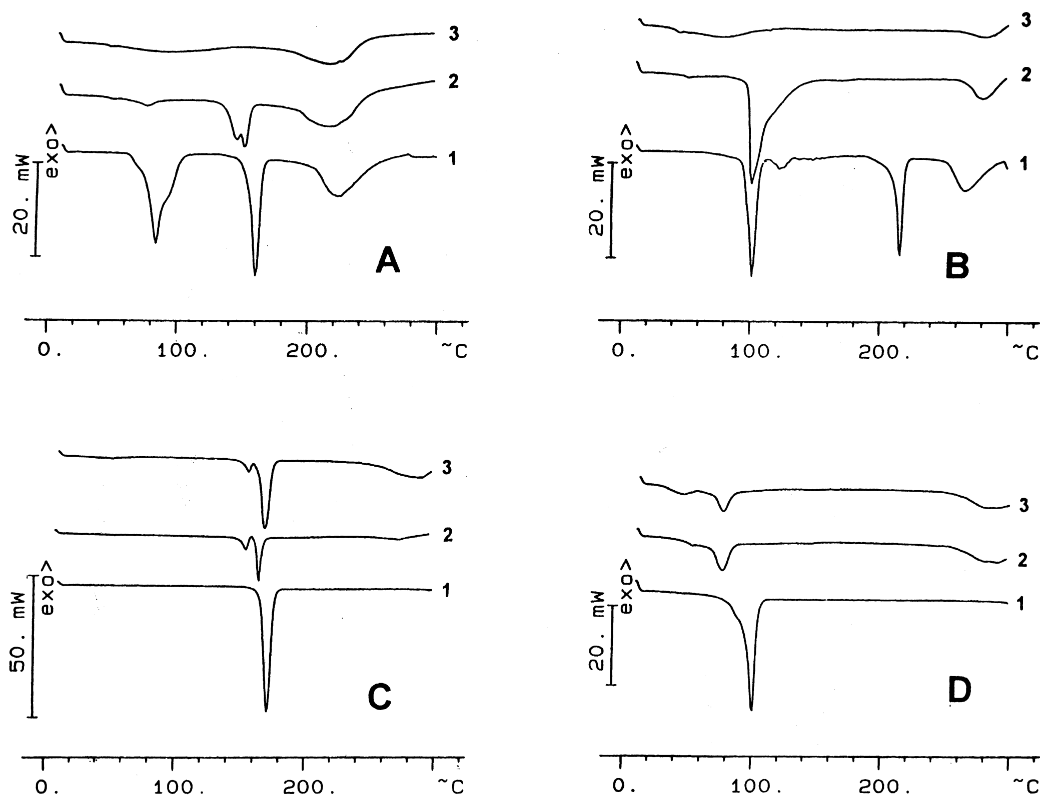

Fig. 4. DSC thermograms of PLGA MS freeze-dried in the presence of 5% of the cryoprotectives glucose (A), trehalose (B), mannitol (C) and sorbitol (D). Each group contains the thermograms of the raw cryoprotectant (1), unloaded PLGA MS (2) and CyA-loaded PLGA MS (3).

carrier induced by the presence of CyA. The thermogram corresponding to trehalose dihydrate and trehalose containing formulations are shown in Fig. 4B. The thermal events between 90°C and 130°C represent the dehydration of the dihydrate crystal (Taylor and York, 1998) while the anhydrous compound melted at 215°C. The freeze-drying of unloaded MS with 5% trehalose caused the fusion of the two peaks at 97°C and 120°C, however, no anhydrous compound was detected. In the presence of CyA, as occurred with glucose, the dramatic change of the thermal profile of MS suggested the existence of strong interactions between the different compounds in the formulation. Furthermore, it is not possible to clearly identify whether the Tg of the polymer has suffered significant changes. The thermograms of mannitol containing formulations (Fig. 4C) showed a sharp endotherm at 166°C for the melting of the cryopreservative. However, after freeze-drying of either unloaded or CyA-loaded MS another endothermic peak at 155°C appeared which is probably related with the mannitol polymorphism and its strong tendency to crystallize (Yu et al., 1998). Sorbitol is an stereoisomer of mannitol with a marked tendency to adsorb moisture. The anhydrous form melts at 110–112°C whereas the monohydrate and its metastable form melt at 98°C and 93°C, respectively. These events are well represented by the thermogram corresponding to the raw material (Fig. 4D). Upon freeze-drying of MS, the characteristic endotherm of sorbitol shifted towards lower melting temperatures (76°C) and the Tg of PLGA may have been masked by the peak observed at 40°C when CyA was present. It has been pointed out that the addition of liquid PEG to sorbitol solutions produces a decrease in the melting point of the cryoprotectant, hence similarly, the shift of the endotherm may be related to interactions of the OH groups of sorbitol with those of the polymer (Wade and Weller, 1994).

SEC studies revealed that the polymer MW of unloaded MS decreased by 21.8±2.4% and 13.2±1.7% when lyophilized in the absence of cryoprotectant or in the presence of 5% mannitol, respectively. Taken together, DSC and SEC data suggest that 5% mannitol did not exert a cryoprotectant effect when MS were frozen at −70°C. The rest of cryoprotectants, particularly glucose and trehalose, exhibited the strongest interactions with the MS preventing MW changes due to overconcentration and excessive growing of ice crystals during sample freezing. In addition, CyA also prevented the polymer rupture in mannitol containing formulations pointing towards a protective role of the drug. This finding may be associated with an increase of the particle hydrophobicity making the MS more stable from a chemical point of view.

The fact that MS frozen at −196°C were not adequately reconstituted in the presence of CyA and the good results obtained for empty MS indicate that MS freezing at −196°C and CyA together exerted a negative effect. The

underlying mechanism may be associated to the large number of small sized ice crystals formed during MS freezing and the increased particle hydrophobicity induced by CyA that might contribute to modify sample reconstitution by altering the particle surface wetting.

3.4.2. PLGA NP

Table 3 shows the macroscopic characteristics (Tyndall effect, aggregation and number of size populations) as well as the S_f/S_i and SD_f/SD_i of reconstituted unloaded NP. None of the samples retained their initial characteristics upon reconstitution, but the best results were obtained when 5% glucose and trehalose were used as shown by their respective S_f/S_i and SD_f/SD_i ratios. The Tyndall effect was maintained only in these samples, however, more than one size population was obtained. As no satisfactory results were obtained, NP were freeze-dried in the presence of higher cryoprotectant concentrations (10, 20 and 30% glucose, trehalose and dextran, 10% sorbitol and 10 and 18% mannitol), but the results were similar to those described above, and no changes were detected because of the cryoprotector percentage increase. The successful lyophilization of MS made from 180 mg of PLGA suggested that particle concentration might play a significant role. Thus, a second lyophilization study evaluated the effect of diluting the original PLGA NP suspensions until the polymer concentration became equal to that found in MS preparations (18 mg/ml). Values of 10 and 20% glucose, trehalose, dextran, mannitol and sorbitol were used as cryoprotectants. Similar to preceding studies, the Tyndall effect was evident only in those samples with glucose and trehalose (whatever concentration), but the dilution did not provide any advantage.

Finally, changes of the freeze-drying conditions were assayed. Unloaded PLGA NP plus 5% glucose, trehalose, dextran, mannitol or sorbitol were freeze-dried at −20°C for 20 h followed by a second 4 h period at 20°C. Under these conditions, the Tyndall effect was achieved only with glucose and trehalose and after reconstitution a small number of aggregates were found. On the contrary, too many aggregates and the absence of Tyndall effect characterized the remaining samples. Particle size measurements were carried out on the samples with an apparent good reconstitution (i.e.- NP freeze-dried with 5% glucose at −70°C). The initial particle size was 103.92±50.05 nm and two populations of 128.2±62.2 nm and 106.79±64.84 μm were obtained after reconstitution. Since freeze-dried NP could not be satisfactorily reconstituted, further studies are in progress to optimize this procedure.

4. Conclusion

PLGA exhibits significant instability during MS preparation by solvent evaporation. Therefore, resources other than increasing stirring rates should be used to reduce particle size. PLGA NP and MS were unstable when stored as suspensions for 6 months. CyA accelerate the chemical degradation of PLGA NP when adsorbed to the particle surface, particularly at RT. Otherwise, the MW changes of PLGA MS were directly related to the initial MW obtained after MS preparation. Freeze-drying of NP under the experimental conditions described herein failed to obtain a product of the same particle size. On the contrary, unloaded MS were successfully lyophilized even in the absence of additives. Likewise, CyA-loaded MS were stable under lyophilized form if frozen at −70°C with cryoprotectants.

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Table 3
Tyndall effect, aggregation characteristics, number of size populations, S_f/S_i ratio and SD_f/SD_i ratio after reconstitution of unloaded freeze-dried PLGA NP without and with 5% cryoprotector

Cryoprotector	Tyndall effect	Aggregation[1]	Number of Size populations	S_f/S_i	SD_f/SD_i
Glucose-70	Yes	2	2	1.028±0.682	0.624±0.284
Glucose-196	Yes	2	2	1.349±1.415	0.969±0.967
Sorbitol-70	No	3	2	10.933±14.946	4.287±5.776
Sorbitol-196	No	3	2	19.804±7.493	12.069±10.886
Trehalose-70	Yes	3	2	8.736±0.455	19.837±2.749
Trehalose-196	Yes	3	3	1.639±0.856	1.207±0.677
Mannitol-70	No	3	2	8.808±0.886	12.679±7.620
Mannitol-196	No	3	2	18.930±9.603	10.698±10.524
Without CP-70	No	3	2	10.010±0.684	7.663±5.903
Without CP-196	No	3	3	7.367±4.083	10.392±12.828

[1] Aggregation after reconstitution (1) few small sized aggregates (2) moderate aggregation and (3) large number of big aggregates.

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