Formulation and Lyoprotection of Poly(Lactic Acid-*Co*-Ethylene Oxide) Nanoparticles: Influence on Physical Stability and *In Vitro* Cell Uptake

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Purpose. To investigate the feasibility of producing freeze-dried poly(ethylene oxide) (PEO)-surface modified nanoparticles and to study their ability to avoid the mononuclear phagocytic system (MPS), as a function of the PEO chain length and surface density.

Methods. The nanoparticles were produced by the salting-out method using blends of poly(D,L-lactic acid) (PLA) and poly(D,L-lactic acid-*co*-ethylene oxide) (PLA-PEO) copolymers. The nanoparticles were purified by cross-flow filtration and freeze-dried as such or with variable amounts of trehalose as a lyoprotectant. The redispersibility of the particles was determined immediately after freeze-drying and after 12 months of storage at −25° C. The uptake of the nanoparticles by human monocytes was studied *in vitro* by flow cytometry.

Results. PLA-PEO nanoparticles could be produced from all the polymeric blends used. Particle aggregation after freeze-drying was shown to be directly related to the presence of PEO. Whereas this problem could be circumvented by use of trehalose, subsequent aggregation was shown to occur during storage. These phenomena were possibly related to the specific thermal behaviours of PEO and trehalose. In cell studies, a clear relationship between the PEO content and the decrease of uptake was demonstrated.

Conclusions. The rational design of freeze-dried PEO-surface modified nanoparticles with potential MPS avoidance ability is feasible by using the polymer blends approach combined with appropriate lyoprotection and optimal storage conditions.

KEY WORDS: nanoparticles; poly(lactic acid); poly(lactic acid-*co*-ethylene oxide); freeze-drying; stability; flow cytometry.

INTRODUCTION

The main incentive to administering nanoparticles into the vascular compartment is the targeting of active compounds to desired organs or cells, allowing a site-specific action of the compound together with a minimization of side effects.

At the present time, this objective has still not been fully achieved. Conventional nanoparticulate systems are rapidly removed from the blood circulation by the action of the cells of the mononuclear phagocytic system (MPS). Mediated by the serum opsonins, this clearance process has been shown to be correlated with the particle surface properties (1). Therefore, surface modification of nanoparticles dominates current strategies to extend their circulation half-lives *in vivo*, mainly by coating them with hydrophilic and non-ionic polymers (1,2). So far, poly(ethylene oxide) (PEO) has been the most successful synthetic material used to this aim. The protective nature of PEO is attributed to its unique solution properties and molecular conformations in aqueous solutions (3,4). In contact with an aqueous environment, highly hydrated and flexible PEO chains would form dense "conformational clouds" over the particle surface, impairing interactions with approaching opsonins as well as phagocytic cells (2).

In the first attempts, nanoparticles were modified by physical adsorption of amphiphilic PEO-based surfactants such as the series of poloxamers and poloxamines (5), and more recently, by physical adsorption of amphiphilic block copolymers with a R-PEO structure (where R is a synthetic polymer similar to the polymer forming the particle core) (6,7). In both cases, the adsorption proceeds via hydrophobic interactions between the hydrophobic moieties and the surface, while the hydrophilic PEO portions form a "hair-like" coat around the particle. However, in these systems, the stability of the adsorbed steric barrier is, by nature, unreliable because rapid desorption by biological compounds with higher affinity may always be possible *in vivo*. To address this limitation, nanoparticles with a covalently attached PEO steric barrier were developed. These so-called "PEO-grafted nanoparticles" could be directly produced from R-PEO block copolymers using emulsification-solvent evaporation (8,9) or direct precipitation-solvent diffusion (10) methods. PEO-grafted nanoparticles may also be prepared by direct grafting of PEO moieties on functionalized preformed particles (11) or by *in situ* polymerization in the presence of PEO moieties (12).

From the wide range of manufacturing procedures and polymeric materials which are now available, it is presently possible to design PEO-stabilized nanoparticles displaying improved features of size, biodegradability, drug entrapment capacity and MPS avoidance ability. Beside those primary features, the ability to be freeze-dried, stored and reconstituted in solution without aggregation are other important requirements for these formulations, especially regarding the up-scaling of the production and potential clinical use. So far, these critical pharmaceutical aspects have been rarely addressed, especially in the field of PEO surface-modified nanoparticle design.

In the present work, the feasibility of producing PEO-grafted biodegradable nanoparticles with different PEO chain lengths and surface densities has been investigated. For this purpose, various blends of poly(D,L-lactic acid) (PLA) and poly(D,L-lactic acid-*co*-ethylene oxide) (PLA-PEO) diblock copolymers were used. The influence of the purification and freeze-drying processes on the redispersibility of these systems was investigated, as well as the influence of the storage conditions. In parallel, the ability of the systems produced to avoid phagocytosis was studied *in vitro* by flow cytometry using human monocytes as phagocytic cell models.

MATERIALS AND METHODS

Polymers and Chemicals

D,L-PLA (Medisorb® Technologies International L.P., Cincinnati, USA) with an average molecular weight (Mw) of

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130,000 Da was used to prepare surface-unmodified nanoparticles. Methoxypoly(ethylene oxide) with Mw of 2,000 (MeO-PEO2000) (Fluka) and 5,000 Da (MeO-PEO5000) (Sigma) were washed twice with an excess of dioxane, and dried over night in the melt (70°C, 0.06 mbar). D,L-lactide was prepared by co-crystallizing D-lactide and L-lactide (Purac Biochem b.v., The Netherlands) from dry toluene solution. The crystals were dried overnight in vacuo and sublimed prior to use (105°C, 0.06 mbar). Poly(vinyl alcohol) (PVAL) with a Mw of 26,000 Da (Mowiol® 4-88, Hoechst, Frankfurt-am-Main, Germany) and Nile Red (Sigma) were used as stabilizer and fluorescent dye, respectively. D(+)-trehalose dihydrate (Fluka) was used as lyoprotectant. The solvents were of analytical grade and all other chemicals were commercially available.

PLA-PEO Copolymers Synthesis and Purification

The PLA-PEO diblock copolymers were synthesized by ring-opening polymerization of D,L-lactide in the presence of the corresponding MeO-PEO homopolymer and stannous octoate as a catalyst, as described previously (13).

A mixture of D,L-lactide (20 g), stannous octoate (0.08 g) and an appropriate amount of MeO-PEO (0.8 g of MeO-PEO2000 or 2 g of MeO-PEO5000) was melted at 150°C for one hour. The reaction was continued for sixteen hours at 130°C. Samples from the melt showed a conversion of 97% in both cases. The resulting polymer was dissolved in dichloromethane, precipitated in a ten-fold excess of methanol and dried in vacuo overnight. The resulting PLA-PEO2000 and PLA-PEO5000 copolymers had average Mw of 33,000 and 31,000 Da as determined by gel permeation chromatography, and PEO contents of 3.8 and 7.2% (w/w) as determined by ^{1}H-NMR spectroscopy, respectively.

Nanoparticle Preparation and Labeling

Fluorescently labeled PLA and PLA-PEO nanoparticles were produced by the salting-out process, as described elsewhere (14). An aqueous phase (24 g) containing 60% (w/w) of magnesium chloride hexahydrate and 10% (w/w) of PVAL was added under mechanical stirring (1600–1800 rpm) to an acetone solution (9.5 g) containing 0.02% (w/w) of Nile Red and 18% (w/w) of polymer, namely PLA alone, PLA-PEO alone or blends of PLA and PLA-PEO in various ratios (Table I). In this system, the miscibility of both phases was prevented by the high salt concentration in the aqueous phase. Addition of the aqueous phase was continued until a phase inversion occurred and an oil-in-water emulsion was formed. Then, a sufficient amount of pure water (30 g) was added to allow complete diffusion of acetone into the aqueous external phase, with the result of forming spherical nanoparticles.

Nanoparticle Purification and Freeze-Drying

The raw nanoparticle suspensions were purified by cross-flow filtration using a Sartocon® Mini device (Sartorius, Göttingen, Germany) equipped with a polyolefin cartridge microfilter with 0.1 μm pore size (15). A total volume of 8 L of pure water was used to purify the particles. The resulting concentrated suspensions were then divided into aliquots, some being freeze-dried as such, others after addition of a determined amount of trehalose as lyoprotectant (trehalose/nanoparticle weight ratio of 1/1 or 2.5/1). Each fraction was frozen at −55°C for 10 min and freeze-dried for 24 h at 0.03 mBar in a Lyolab BII (Secfroid, Aclens, Switzerland).

Nanoparticle Characterization

The nanoparticle mean size and polydispersity index (PI) (scale from 0 to 9) were determined by photon correlation spectroscopy using a Coulter Nano-Sizer® (Coulter Electronics, Harpenden, UK) with an upper size detection limit of 3000 nm. Size measurements were performed after the different steps of production, purification and freeze-drying. Freeze-dried nanoparticles were redispersed by vortexing (2 min) in distilled water. Each size and PI value were calculated as the mean of three measurements. The redispersibility index (RI) was evaluated as the ratio of mean size of the rehydrated nanoparticles to that of the original nanoparticles.

In Vitro Cell Interaction Experiments

Fresh blood was collected from healthy volunteers in tubes containing ethylenediamintetraacetic acid disodium salt (EDTA). Mononuclear leukocytes (mixture of lymphocytes and monocytes) were isolated using the density gradient separation procedure Accuspin™ System-Histopaque®-1077 (Sigma). Using this isolation technique, the cell viability was always

Table I. Nanoparticle Formulation and Characterization

Batch #	Composition	PLA % (w/w)	PLA-PEO % (w/w)	PEO content (μmol/g)	PEO density (μmol/m^2)	Mean size ± SD[a] (nm)	PI[b]
1	PLA	100	0	0	0	279 ± 10	1
2	PLA-PEO2000	81	19	3.6	0.22	245 ± 4	1
3		62	38	7.2	0.49	275 ± 11	3
4		43	57	10.8	0.68	257 ± 9	4
5		24	76	14.4	1.01	282 ± 6	3
6		0	100	19.0	1.14	241 ± 15	4
7	PLA-PEO5000	75	25	3.6	0.25	281 ± 13	3
8		50	50	7.2	0.50	282 ± 7	3
9		25	75	10.8	0.69	253 ± 16	3
10		0	100	14.4	1.00	280 ± 3	3

[a] SD: standard deviation (n = 3).
[b] PI: polydispersity index of the size distribution after rehydration (expressed using a 0–9 scale).

above 90%, as demonstrated by the trypan blue exclusion test. The cells were washed by centrifugation (400 G) and were redispersed either in phosphate buffer saline (PBS) or human EDTA-plasma (12×10^6 cell/ml). Aliquots of 100 µl were withdrawn and added to 10 µl of a 0.01 to 1% (m/v) suspension of nanoparticles (40 to 4000 nanoparticles/cell). The incubation wells were gently stirred at 37°C in a thermostatically controlled chamber. After 30 min, the incubation medium and the unbound particles were removed by centrifugation and the cells were washed twice in fresh PBS. The cells were finally redispersed in 1 ml of PBS and analyzed using a FACScan® flow cytometer (Becton Dickinson, San Jose, CA, USA) (14,16). Only the fluorescence associated with the monocyte population was analyzed, these cells being known as "professional phagocytic cells" (17), as opposed to the lymphocytes which were used as negative control. Each given value results from a triplicate determination and is normalized as a percentage of maximal fluorescence emission recorded during the experiment. Each experiment was done two or three times.

RESULTS AND DISCUSSION

Nanoparticle Preparation and Purification

As discussed elsewhere (9,10), we assumed that upon the emulsification step of the salting-out process, PEO blocks of the copolymer migrated to the water interface, whereas the PLA blocks, and a certain proportion of pure PLA, remained within the organic droplets. After organic solvent removal, nanoparticles with a PLA central core and a grafted PEO hydrophilic shell were formed. The different batches were formulated in such a way that equivalent amounts of PEO2000 and PEO5000 might be present at the particle surfaces (Table I).

The mean particle size values that are expressed in Table I relate to freshly produced nanoparticles, sampled from the raw suspensions just before the purification step. It appeared that the salting-out process enabled the production of PLA-PEO nanoparticles from all polymeric blends, and this, in the very reproducible size range of 250–300 nm. As illustrated by the PI values (Table I), all the systems exhibited rather narrow size distributions. Size equivalence of the different batches was set as an initial prerequisite in view of the subsequently planned *in vitro* cells experiments. To this end, the stirring rate during the emulsification had to be adapted for the different formulations, varying from 1600 to 1800 rpm as the PLA-PEO:PLA proportion in the blend increased.

The cross-flow filtration method was used to remove acetone, free PVAL and electrolytes from the raw nanoparticle suspensions (15). No alteration of the particle size was observed after this procedure (data not shown). This is in accordance with a previous study (15) which demonstrated the advantage of the cross-flow filtration procedure over the commonly used centrifugation procedure with respect to particle size preservation.

Redispersibility of the Particles After Freeze-Drying

Freeze-Drying Without a Lyoprotectant

As shown in Fig. 1 and Table II, when the PLA-PEO nanoparticles were freeze-dried without a lyoprotectant, full redispersion of the particles could not been achieved after rehydration, as opposed to the control batch (pure PLA nanoparticles). No improvement of the redispersion could be achieved even using sonication (data not shown), indicating a strong type of aggregation. Particle sizes in the samples with the highest PEO contents were no longer compatible with an intravenous administration. The almost linear relationship between the amount of PEO in the formulation and particle aggregation after freeze-drying ascertained, in an indirect way, the surface location of the PEO chains and therefore, highlighted the negative impact of the latters on particle redispersion. The question arising was: by which mechanism did the POE chains impair the redispersion of the freeze-dried particles?

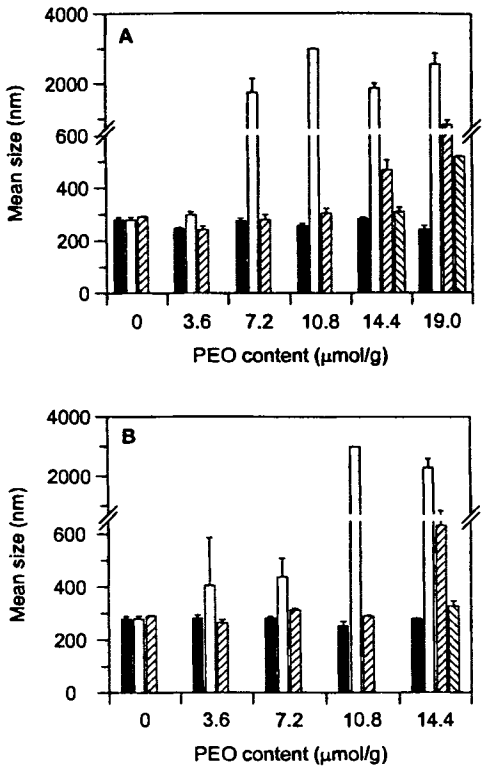

Fig. 1. Mean size of PLA nanoparticles (control), PLA-PEO2000 nanoparticles (A) and PLA-PEO5000 nanoparticles (B) before freeze-drying (closed bar), after freeze-drying without trehalose (open bar), after freeze-drying with 1/1 (left dashed bar) or 2.5/1 (right dashed bar) trehalose/nanoparticle ratio (w/w). (Mean ± SD, n = 3).

PEO polymers exhibit particular and unique solution properties in aqueous media (3). Inherent to these properties, a commonly exploited effect is the steric stabilization of aqueous colloidal dispersions, through which colloid aggregation is prevented (18). PEO coatings also stabilize colloids in blood or plasma by reducing the interactions with biological macromolecules (e.g. opsonins) and cells (1,2). Another illustration of the protective effect of PEO polymers is their extensive use as co-solute in protein solutions, to prevent protein denaturation during freeze-thawing processes (19,20). In particular, these studies have highlighted a fundamental difference in the PEO protective efficiency, depending on whether the protein/PEO solution is undergoing a freeze-thawing or a freeze-drying process. Whereas PEO polymers are very efficient in protecting proteins during freeze-thawing, they fail to protect proteins

during the solvent removal step of freeze-drying (19,20). In other words, PEO polymers can be termed as potent cryoprotectants but not as lyoprotectants. The reason for this differential behaviour has been attributed to the tendency of PEO to crystallize upon freezing (19,21). Whereas PEO protect proteins during freezing by the so-called thermodynamic preferential exclusion mechanism (20,22), they become unable, after crystallization, to serve as a protective "water substitute" when the hydration shell of the protein is removed during the drying step of freeze-drying (19,20). These basic principles established in the field of protein cryoprotection and lyoprotection could provide a possible explanation for the problems encountered with PLA-PEO nanoparticles after freeze-drying. It can be assumed that, as a result of covalent attachment of PEO to the surface and close proximity of the particles, intra- and interparticular bridges of crystallized PEO might have formed during freezing, resulting in aggregated particles after water removal. This effect appeared to be PEO-concentration dependent, the rationale for this being that crystallization is more likely to occur as the number of chains in contact between the particles is increased (Fig. 1). The length of the PEO chains, however, did not appear to have any influence on the aggregation effect, since no real difference could be observed between the aggregation pattern of PLA-PEO2000 and PLA-PEO5000 nanoparticles (Fig. 1).

To our knowledge, there is no publication thoroughly considering the redispersibility of PEO surface-modified nanoparticles after freeze-drying. In most studies, the nanoparticles were tested directly after simple purification of the raw suspensions, eluding the implications of a freeze-drying step (6,10,23). Some publications (8,9,12) have reported freeze-drying of PEO surface-modified nanoparticles without any mention of aggregation problems or lyoprotection necessity. It is possible that, in these studies, the equivalent amount of PEO in the tested formulations was below 3.6 μmol/g, although this could not be verified from the data available (8,9,12). Because the methods reported in the literature for the preparation and freeze-drying of PEO surface-modified nanoparticles are scarcely comparable with each other, it is difficult to draw common conclusions for all systems. However, as it was shown with other nanoparticulate systems made of different materials (24), the use of a lyoprotectant should be systematically considered, especially in the presence of PEO, because of its particular thermal behaviour during freeze-drying.

Freeze-Drying with a Lyoprotectant

Trehalose, a non-reducing disaccharide of glucose, was chosen as lyoprotectant in this study because it is commonly and successfully used to protect colloids and biomolecules (19,25,26). It can be seen from Fig. 1 and Table II that the redispersibility of PLA-PEO particles was markedly improved when trehalose was added to the nanoparticulate suspensions prior to freeze-drying. Complete redispersion (RI ~ 1) could be obtained when trehalose was used at a weight ratio of 1/1 (1 g trehalose/1 g nanoparticles), except for batches 5, 6 and 10 for which trehalose had to be used at a ratio of 2.5/1. Only batch 6 could not be fully redispersed (RI ≥ 2), even at a 3.5/1 ratio (data not shown). All the rehydrated samples exhibited particle sizes acceptable for intravenous administration.

A similar mechanism to that described for the lyoprotection of proteins and liposomes (19,25,26) may be proposed to explain the protective effect of trehalose on PLA-PEO nanoparticles during freeze-drying. By forming an amorphous matrix with water around the particles during freezing, trehalose may maintain the PEO chains in a pseudo-hydrated state through intramolecular hydrogen-bonding. It can be assumed that when sufficient hydrogen-bonding is formed (i.e., when the sugar/PEO ratio is optimal), crystallization of the PEO chains may be prevented in the frozen sample. Supporting this hypothesis, a recent study has demonstrated that crystallization of PEO could be partially or totally prevented in different frozen PEO/sugar/water systems (21). Selection of trehalose as a lyoprotectant in the present study appeared to be appropriate given the almost perfect recovery of the PLA-PEO particle sizes after rehydration. Complete redispersion of batch 6 could not be achieved with a trehalose/nanoparticle weight ratio of 2.5/1. Using a 3.5/1 ratio did not lead to any improvement, indicating that the lyoprotective activity of trehalose might be saturated

Table II. Redispersibility Index (RI) of the Freeze-Dried PLA-PEO Nanoparticles, Expressed as the Ratio of the Mean Size of the Rehydrated Particles to that of the Original Particles Before Freeze-Drying

			RI after freeze-drying (PI[a])		
Batch #	Composition	PEO content (μmol/g)	Without trehalose	With trehalose 1/1[b]	With trehalose 2.5/1[b]
1	PLA	0	1.0 (3)	1.0 (1)[c]	—
2	PLA-PEO2000	3.6	1.2 (3)	1.0 (2)[c]	—
3		7.2	6.3 (7)	1.0 (3)[c]	—
4		10.8	>11	1.2 (5)[c]	—
5		14.4	6.6 (5)	1.7 (5)	1.1 (4)[c]
6		19.0	10.6 (6)	3.4 (6)	2.3 (5)[c]
7	PLA-PEO5000	3.6	1.4 (6)	0.9 (2)[c]	—
8		7.2	1.5 (6)	1.1 (3)[c]	—
9		10.8	>11	1.1 (3)[c]	—
10		14.4	8.2 (8)	2.3 (6)	1.2 (4)[c]

[a] PI: Polydispersity index of the size distribution after rehydration (expressed using a 0–9 scale).
[b] Trehalose/nanoparticle ratio (w/w).
[c] Aliquots selected for *in vitro* testing.

at a ratio of 2.5/1. However, the residual particle aggregation was slight relative to that without trehalose, with the final particle size remaining in the nanometer range (Fig. 1 and Table II).

Effect of Storage on the Redispersion of Freeze-Dried Nanoparticles

Because the different batches could not be produced simultaneously, they had to be stored until the *in vitro* experiments could start. To prevent PLA degradation (27), the dried aliquots were immediately stored at −25°C. For each *in vitro* experiment, the samples were thawed at room temperature and frozen again at −25°C. Figure 2 presents the mean particle sizes of the dried samples after 12 months of storage and several encountered freeze-thawing stresses, compared to the initial mean sizes after freeze-drying. It seemed that, under these storage conditions, slight recrystallization of the PEO chains had occurred, with an almost linear relationship between particle aggregation and the amount of PEO in the formulations (Fig. 2, Table III). A possible explanation may lie in the presence of residual water in the dried samples, originating from the entrapment of a certain amount of "unfrozen water" in the sugar matrix during the freezing step of freeze-drying (21,28,29). It can be suggested that, upon storage at −25°C, water molecules might have progressively diffused and recrystallized out of the trehalose matrix in the dried frozen samples. After a few months, such a phenomenon might have altered the hydrogen-bonding network between the PEO chains and trehalose. The cryoprotective effect of trehalose appeared to be partially but not completely lost: the particle sizes still remained in the nanometer range after rehydration and the highest RI value did not exceed 3.5 (Table III). Admittedly, the freeze-thawing stresses inherent to the repeated nanoparticle withdrawals from the containers might have also contributed to the destabilization of the PEO chains, i) by thermodynamically promoting the diffusion of the water molecules through the trehalose matrix, ii) by promoting moisture contamination of the sample. In this study, it appeared that in addition to the lyoprotection feature, storage is another critical feature which should be carefully considered when long-term stability of freeze-dried PLA-PEO nanoparticles is desired. First, to avoid the presence of residual water in the dried samples, a secondary drying step might be recommended during freeze-drying (30). In addition, to avoid any freeze-thawing stress and moisture contamination, the dried samples should be aliquoted. Finally, the containers should be vacuum sealed or filled with argon and opened only after reaching perfect temperature equilibrium.

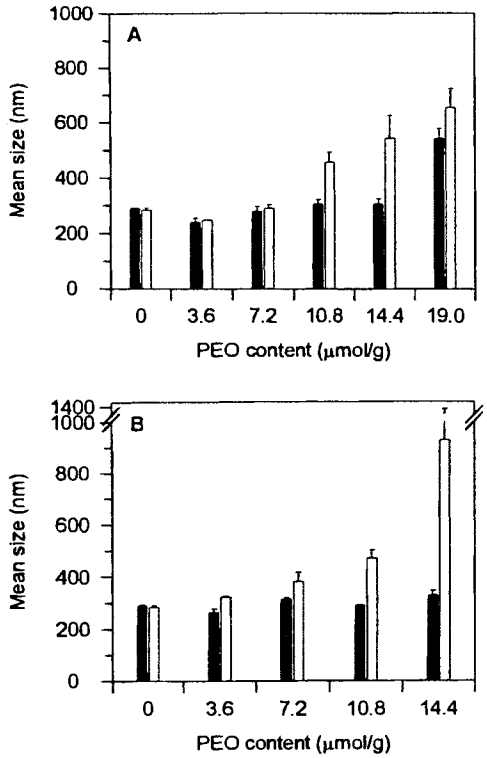

Fig. 2. Mean size of freeze-dried PLA nanoparticles (control), PLA-PEO2000 nanoparticles (A) and PLA-PEO5000 nanoparticles (B) before storage (closed bar) and after storage at −25°C with repeated freeze-thawing stresses over a 12 months period (open bar). (Mean ± SD, n = 3).

In Vitro Cell Uptake Experiments

As indicated in Table II, the aliquots which were shown to exhibit the best redispersibility (i.e., the lowest RI) after freeze-drying were selected for the *in vitro* cell uptake study. Since the aliquots were tested early within the 12 months period of storage, the RI were not significantly different between the consecutive experiments. In a first instance, the optimal experimental conditions were determined. For this purpose, the saturability of the system was studied by incubating a number of cells in plasma with variable concentrations of PLA (Batch 1) and PLA-PEO5000 nanoparticles (Batch 10) at 37°C during 30 min (Fig. 3). With PLA nanoparticles, saturation of monocyte uptake occurred at a concentration of 2000 nanoparticles/cell as indicated by the flattening of the curve at this concentration. For subsequent experiments, incubation conditions were set at

Table III. Influence of Storage on the Redispersibility of the Aliquots Selected for *In Vitro* Testing

Batch #	Composition	PEO content (μmol/g)	RI[a] (PI[b]) Before storage	RI[a] (PI[b]) After storage[c]
1	PLA	0	1.0 (1)	1.0 (1)
2	PLA-PEO2000	3.6	1.0 (2)	1.0 (1)
3		7.2	1.0 (3)	1.1 (3)
4		10.8	1.2 (5)	1.8 (6)
5		14.4	1.1 (4)	1.9 (7)
6		19.0	2.3 (5)	2.7 (5)
7	PLA-PEO5000	3.6	0.9 (2)	1.1 (3)
8		7.2	1.1 (3)	1.4 (5)
9		10.8	1.1 (3)	1.8 (6)
10		14.4	1.2 (4)	3.3 (8)

[a] RI: redispersibility index (ratio of the mean size of the rehydrated nanoparticles to that of the original nanoparticles before freeze-drying).
[b] PI: polydispersity index of the size distribution after rehydration (expressed using a 0–9 scale).
[c] Storage at −25°C during 12 months with repeated freeze-thawing stresses.

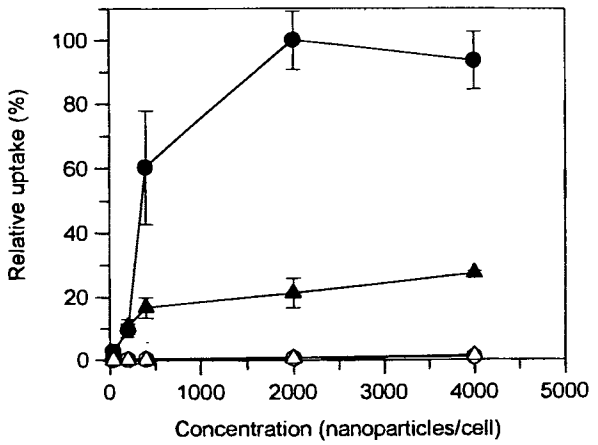

Fig. 3. Relative uptake of PLA nanoparticles (circle) and PLA-PEO5000 nanoparticles (Batch 10) (triangle) by human monocytes (closed symbol) and lymphocytes (open symbol) as a function of nanoparticle concentration, after 30 min incubation at 37°C in plasma. (Mean ± SD, n = 3).

400 nanoparticles/cell, because this concentration was in the non-saturated portion of the curve for both PLA and PLA-PEO5000 nanoparticles. From these preliminary results, it was also shown that PLA-PEO5000 nanoparticles containing 14.4 μmol/g PEO were taken up by the monocytes to a much lesser extent than the PLA nanoparticles, approximately three time less at the concentration of interest. As expected, the lymphocyte-associated signals were very weak, these cells being known as "non-professional phagocytes" (14,16,17).

In the subsequent experiments involving the different batches of nanoparticles in parallel, only signals associated to the monocytes were considered for discussion. Two incubation media were used, namely PBS and plasma, for rational understanding of the mechanisms regulating the uptake of the particles. As shown in Fig. 4, in PBS (where no opsonins are present) the particle uptake was always very low compared to particle uptake in plasma. Moreover, no difference between the various formulations could be demonstrated in this medium, suggesting that the PEO layers may principally act by interfering with the opsonization process (14,16). As a matter of fact, in plasma, a clear relationship between the PEO content and the decrease of uptake was demonstrated with PLA-PEO2000 nanoparticles (Fig. 4A). Compared to the plain PLA nanoparticles, up to 80% uptake decrease was observed with batch 6. The protective effect of the PEO chains in plasma appeared to be generally less pronounced with the PLA-PEO5000 nanoparticles (Fig. 4B). When PLA-PEO2000 nanoparticles (Batch 5) and PLA-PEO5000 nanoparticles (Batch 10) were tested simultaneously, PLA-PEO2000 nanoparticles again showed their superiority in avoiding monocyte uptake (Fig. 5).

Whatever the mode of designing PEO surface-modified nanoparticles, efficiency against MPS recognition has been shown to be dependent on the PEO chain length (i.e., PEO molecular weight) as well as on the density of the chains at the particle surface. It is still not clear, however, which of these two parameters is the most dominant (1). It has been suggested that the higher the PEO surface density, the greater the repulsive effect for proteins (opsonins) (31). The results obtained in the

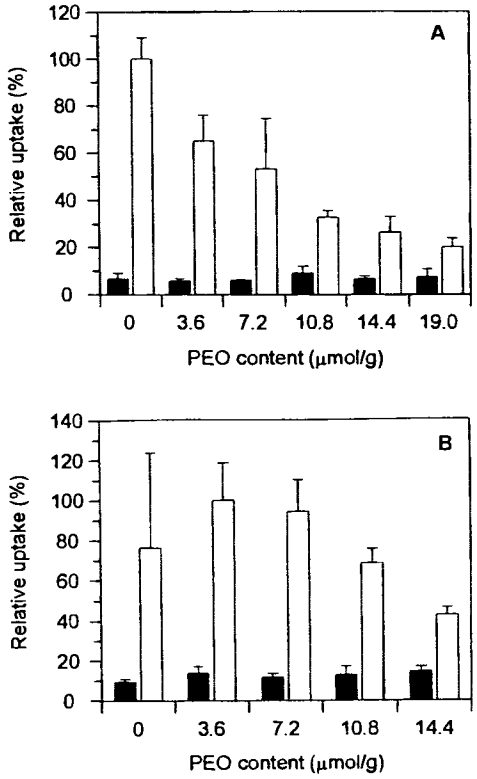

Fig. 4. Relative uptake of PLA nanoparticles (control), PLA-PEO2000 nanoparticles (A) and PLA-PEO5000 nanoparticles (B) by human monocytes as a function of PEO content after 30 min incubation at 37°C in PBS (closed bar) or plasma (open bar). (Mean ± SD, n = 3).

present study tend to support this hypothesis (Fig. 4). However, for equivalent PEO chain densities, the lower protective effect generally observed for PLA-PEO5000 nanoparticles relative to PLA-PEO2000 nanoparticles (Figs. 4 and 5) was somewhat unexpected, considering the commonly reported superiority of high molecular weight PEO over lower molecular weight PEO in providing protection against MPS uptake (32). Several hypothesis can be put forward, including a higher conformational change of the PEO5000 chains upon freeze-drying and/

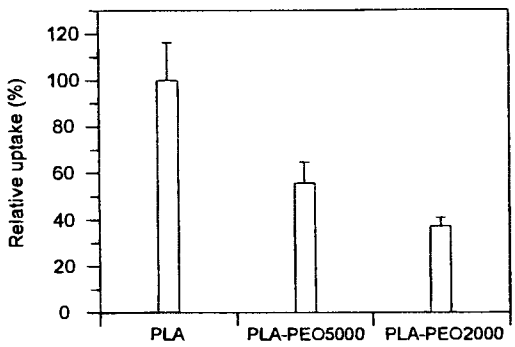

Fig. 5. Relative uptake of PLA-PEO5000 and PLA-PEO2000 nanoparticles containing an equivalent amount of PEO (14.4 μmol/g) compared to PLA nanoparticles (control) after 30 min incubation at 37°C in plasma. (Mean ± SD, n = 3).

or storage conditions. Indeed, if freeze-drying and storage influence was shown to be similar for PLA-PEO2000 and PLA-PEO5000 particles when assessed on the basis of particle size analysis (Figs. 4 and 5), distinct conformational changes might have eventually occurred in the two types of systems.

CONCLUSIONS

PEO surface-modified systems have received increased attention over the last few years due to their increased plasmatic half-life *in vivo*. It was shown in this study that PEO surface-modified nanoparticles could be produced in a tailored way by the salting-out procedure using different blends of PLA and PLA-PEO copolymers. Freeze-drying is probably one of the most useful methods to obtain dry products with long-term stability and practical pharmaceutical handling. Because particle aggregation upon rehydration would severely limit clinical use and safety of freeze-dried systems, the redispersibility of each PLA-PEO nanoparticulate formulation was evaluated after freeze-drying. It appeared that the presence of PEO could severely impair redispersion of the nanoparticles. This effect was attributed to the well known tendency of PEO to crystallize upon freeze-drying and was circumvented using an appropriate lyoprotectant additive. The influence of storage conditions on the particle size integrity was also related to the specific thermal behaviour of the lyoprotective additive. This study showed that, although rarely addressed, these pharmaceutical features should be systematically considered at an early stage before any further development of PEO surface-modified nanoparticles. Further work is ongoing in our laboratory in order to i) define optimal handling conditions of these systems, ii) clarify, through precise physico-chemical characterization, the relative importance of PEO molecular weight and surface density with regard to cell uptake *in vitro*. Obviously, the successful design of PEO surface-modified nanoparticles should result from a rational compromise between conditions maintaining their physical stability (first during freeze-drying, then during storage) and their ability to escape cell uptake in biological environments.

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