Journal of
Nanoscience and Nanotechnology
Vol. 6, 1–8, 2006

Lyoprotected Nanosphere Formulations for Paclitaxel Controlled Delivery

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The preparation and technological characterization of nanosphere formulations (NS) containing the anticancer drug paclitaxel (PTX) are reported. Poly(lactide) (PLA) and poly(lactide-*co*-glycolide) (PLGA) nanospheres (NS) were prepared by a solvent displacement method. They showed a mean particle size in the range 150–300 nm, with a high homogeneity (polydispersity index <0.3). For long term stability, NS require additional procedures, such as freeze-drying. In this study, the effect on NS particle size and surface charge of different lyoprotectants (mono- and disaccharides, polyalcohols, and hydroxypropyl-ß-cyclodextrin) at various concentrations was tested by means of light scattering size analysis. The formulations freeze-dried with the addition of 10% glucose (w/v) showed interesting characteristics after freeze-drying. They were chosen for specific studies on drug encapsulation efficiency, *in vitro* drug release and biological activity on the human anaplastic thyroid carcinoma cell line 8305C. The PLGA NS, in particular, showed a cell growth inhibitory activity comparable to the free drug.

Keywords: Paclitaxel, Nanoparticles, PLA, PLGA, Lyoprotectants, Freeze-Drying, Stability, 8305C Cell Line.

RESEARCH ARTICLE

1. INTRODUCTION

Nanoparticles or nanospheres (NS) offer many therapeutic advantages in the pharmaceutical field.[1,2] Their clinical application is often limited by problems related to the thermodynamic instability of the colloidal suspension for prolonged times, that induces particle aggregation and precipitation.[3,4]

Polyester polymers, such as poly(D,L-lactide-*co*-glycolide) (PLGA) and poly(D,L-lactide) (PLA), have raised great interest as materials to form pharmaceutical NS, due to their physicochemical and biological properties (e.g., biocompatibility and biodegradability).[5]

To improve the stability of these colloidal delivery systems upon storage, removal of moisture is essential. Freeze-drying is one of the most efficient techniques in the pharmaceutical industry to generate anhydrous materials. However, several physico-chemical phenomena, such as air adsorption and modification of nanoparticles surface during the various steps of the process[6] may lead to difficulty in redispersion of NS in aqueous media for the subsequent administration. Protection of NS formulations against stress induced by freeze-drying can be achieved by the addition of inert additives such as sugars. Monosaccharides have been used in the lyoprotection of colloidal dispersions, including SLN,[7] PLA-based nanoparticles,[8] and even highly fragile unilamellar liposomes.[9]

The natural anti-tumour drug paclitaxel (PTX, Fig. 1) was chosen as a model drug in this study. PTX is used for the treatment of solid cancers such as primary epithelial ovarian carcinoma, breast, colon, and head cancer, non-small cell lung cancer, and AIDS-related Kaposi's sarcoma.[10–14] PTX and taxanes in general possess a unique mechanism of action, promoting the polymerization of tubulin.[15]

Due to poor aqueous solubility (less than 1 μg/ml),[16,17] PTX requires the use of solvents, such as a blend of ethanol and polyethoxylated castor oil for i.v. administration. To the latter additive adverse events like neurotoxicity, nephrotoxicity, anaphylactic cryses, hyperlipidemia, and cytotoxicity have been ascribed.[17–19] As a consequence, many non-conventional formulations have been

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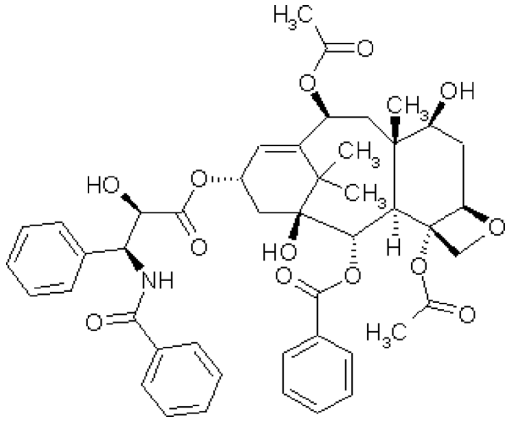

Fig. 1. Chemical structure of PTX.

investigated including liposomes,[20–23] polymeric prodrugs,[24] and micro- and nanoparticles.[25–32]

The encapsulation of PTX in colloidal systems would access selective targeting to cancer cells, resulting in a safer and more efficacious dosage form, and a modified release of the drug, with the final outcome of reducing the drug dose and frequency of administration.

In this paper we report the effects of different carbohydrate lyoprotectants in freeze-drying/reconstitution cycles on NS made of PLA and PLGA and loaded with PTX. Lyoprotectants were chosen from among monosaccharides (glucose, fructose), disaccharides (lactose, trehalose), oligosaccharides such as hydroxypropyl-β-cyclodextrin (HP-Cyd), and polyalcohols (mannitol and sorbitol). Photon correlation spectroscopy (PCS) was used to measure the particle size of reconstituted NS suspensions. The effectiveness of the lyoprotectant additives against the adverse effects of freeze-drying on NS redispersibility was also evaluated based on particle size measurements.

2. MATERIALS AND METHODS

2.1. Materials

Resomer® polylactic acid R 203 (MW 16,000), R 207 (MW 209,000), and 50:50 polylactide-co-glycolide acid (RG 502H) were purchased from Boehringer Ingelheim GmbH (Germany). Polyoxyethylene sorbitan monoleate (Tween 80®), PTX and all the lyoprotectant agents were purchased from Sigma-Aldrich Chimica Srl (Milan, Italy). HP-Cyd was obtained from Cyclolab (Budapest, Hungary). All other chemicals and solvents were of analytical reagent grade. De-ionized double-distilled water was used throughout the study.

2.2. Preparation of PTX-Loaded Nanospheres

PLA and PLGA NS were prepared by solvent displacement followed by polymer deposition.[33,34] PTX (0.5%, 1%, or 3%, w/v) and the chosen polymer (75 mg) were dissolved in acetone (20 ml). The organic phase was poured into 40 ml of a water/ethanol solution (1:1, v/v) containing 0.5% (w/v) Tween 80, under magnetic stirring, obtaining a milky colloidal suspension. The organic solvent was then evaporated off under high vacuum at 40 °C. The different formulations were purified from untrapped PTX and unadsorbed surfactant by means of centrifugation ($15000 \times g$) for 1 h at 5 °C, using a Beckman (Fullerton, CA) J2-21 model centrifuge equipped with a Beckman JA-20.01 fixed-angle rotor. The obtained surfactants were collected and the pellets resuspended in water (50 ml), then centrifuged using the same conditions described above. This operation was repeated 3 times. After washing, the NS were resuspended in 5 ml of filtered water (0.22-μm Sartorius membrane filters) and characterized for size distribution and surface chemistry.

2.3. Size and Zeta Potential Evaluation

PLA and PLGA NS mean size was determined by photon correlation spectroscopy (PCS) (Zetamaster, Malvern Instruments Ltd., Worcs, England). The experiments were carried out using a 4.5-mW laser diode operating at 670 nm as light source. Size measurements were carried out at a scattering angle of 90°. To obtain the mean diameter and polydispersity index of colloidal suspensions, a third-order cumulant fitting correlation function was performed by a Malvern PCS submicron particle analyzer. The real and imaginary refractive indexes were set at 1.59 and 0.0, respectively. The following parameters were used for experiments: Medium refractive index 1.330, medium viscosity 1.0 mPa·s and a dielectric constant of 80.4. Each sample (100 μl) was suitably diluted with filtered water (2 ml) to avoid multi-scattering phenomena and placed in a quartz cuvette. The size analysis of a sample consisted of 30 measurements, and the results are expressed as mean size $\pm$ SD.

Zeta potential distribution was measured with a Zetamaster particle electrophoresis analyzer setup equipped with a 5-mW HeNe laser (633 nm). Each NS sample (100 μl) was suitably diluted with 20 ml of filtered water. Zeta limits ranged from -120 to 120 V. Strobing parameters were set as follows: Strobe delay -1.00, on time 200.00 ms, off time 1.00 ms. A Smoluchowsky constant F (Ka) of 1.5 was used to achieve zeta potential values from electrophoretic mobility.

2.4. Preparation of Lyoprotected NS

Unloaded PLGA RG502H and PLA R 203 NS were freeze-dried in the presence of different amounts of glucose (5, 10, 15, or 25%, w/v), HP-Cyd (0.5, 5, 10, or 15%, w/v) or 10% (w/v) fructose, trehalose, lactose, mannitol, or sorbitol. NS pellets coming from the washing steps (see above) were resuspended in 5 ml water containing the required amount of lyoprotectant. The resulting

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suspensions were frozen by immersion in liquid nitrogen for 45 min and then immediately freeze-dried using an Edward Modulyo apparatus, at a pressure of 1.3×10^{-6} atm and -50 °C for 24 h. Control samples were obtained without using any lyoprotectant.

The freeze-dried cakes were reconstituted with 5 ml of MilliQ water with manual shaking; the resulting colloidal suspensions were then submitted to particle size and zeta potential analysis, as described above. The ratios between the final and initial particle size (S_f/S_i) was calculated.

To assess the drug encapsulation efficiency, *in vitro* drug release and biological activity, PTX-loaded NS formulations were prepared as previously described and freeze-dried in the presence of 10% (w/v) glucose.

2.5. Surface Morphology

Scanning electron microscopy (SEM) was performed to evaluate the surface morphology of NS using a SEM XL-30 (Philips, Eindhoven, the Netherlands). NS samples were fixed by means of bi-adhesive tape on a glass disk applied to an aluminum stub (TAAB, Laboratories Equipment, Berks, UK) and evaporated under vacuum overnight. Before the SEM analysis the samples were metallized under argon atmosphere to 10 nm gold palladium thickness (EMITECH-K550 Sputter Coater, Houston, Texas, USA).

2.6. Encapsulation Efficiency and *In Vitro* Release of PTX-Loaded NS

The pellet obtained from the washing steps (see above) was resuspended in water containing 10% (w/v) glucose and freeze-dried. Fifty milligrams of the resulting solid were dissolved in 1 ml of acetonitrile, filtered through 0.45-μm nylon membrane filters (Whatman) and subjected to HPLC analysis to assess the drug concentration. The encapsulation efficiency was calculated as the mass ratio of the entrapped drug in NS to the amount used in their preparation.

The *in vitro* release studies were carried out according to a published method.[35] Aliquots of 50 mg of PTX-loaded NS freeze-dried with 10% glucose were placed in a screw-capped tubes and suspended in 5 ml of isotonic pH 7.4 phosphate buffer solution (PBS, Sigma). The tubes were placed in a water bath at 37 ± 0.5 °C under magnetic stirring. At fixed time intervals, three tubes for each batch were removed and centrifuged at 14,000 rpm for 1 h. The pellets were resuspended in 5 ml of fresh PBS and replaced in the water bath to continue the drug release test. The supernatants were extracted with 3×5 ml-aliquots of dichloromethane. The solvent aliquots were pooled, evaporated under nitrogen, and the residue was dissolved in 500 μl acetonitrile. The resulting solutions were analyzed by HPLC to determine the drug concentration.

The efficiency of the extraction process was evaluated in triplicate using PBS solutions (5 ml) containing different known concentrations of pure PTX and submitted to the same extraction procedure described above, and was found to be $93.9 \pm 0.4\%$.

2.7. HPLC Analysis

HPLC analysis was performed at room temperature using a 1050 Hewlett Packard apparatus on a 5 μm HP Hypersil ODS cartridge (125×4 mm i.d.) equipped with a 5 μm HP Hypersil 100 RP-18 guard cartridge (4×4 mm i.d.) and eluted isocratically with acetonitrile/water (60/40, v/v). Flow rate was set at 1 ml/min and UV detection was made at 230 nm. The linear regression coefficient, determined in the range 0.33–33 μg/ml, was 0.9992 ($n = 6$). The method sensitivity was 3 ng/ml (signal to noise ratio, 3:1).

2.8. Cell Lines and Culture Conditions

The human anaplastic thyroid carcinoma cell line 8305C was kindly provided by Dr. P. Vigneri, Department of Biomedical Sciences, University of Catania, Italy. Cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100U/ml penicillin-streptomycin.

2.9. MTS Assay and Cell Viability

Cell viability was determined using a colorimetric method, the CellTiter 96® AQ_{ueous} One solution Cell Proliferation Assay (TB245, Promega Corporation). The assay uses a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent, phenazine ethosulfate (PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium.[35] This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Since the quantity of formazan produced, as measured by the absorbance at 490 nm, is proportional to the number of living cells in culture, the intensity of the produced colour is a good indication of the viability of the cells.

Cells were seeded into 96-well plates at 5×10^3 cells/200 μl/well and allowed to adhere to the plate overnight. The next day the cells were treated with 2.5 or 5 μM of PTX or equivalent amount NS formulations. Dilutions were made using the culture medium from a 5-mM stock solution of the drug in ethanol or NS suspensions, respectively. The above concentrations were chosen based on a cell growth curve made using a 0.62–10 μM range of drug concentrations for different incubation times (24–72 h) (data not shown). However, in our own experiments (unpublished data) as well in the literature,[36] PTX showed

a good growth inhibitory activity against 8305C cell only beyond 24 h of incubation.

After 48 or 72 h of incubation the culture medium was aspirated and cells were washed with PBS (pH 7.4); 100 μl of fresh culture medium without drugs and 20 μl of MTS were added to each well and the cells were incubated for 3 h. The plates were read on a Microplate Reader (Synergy HT, BIO-TEK).

Survival was expressed as the percentage of viable cells in the treated sample compared to untreated control cells. Each experiment was repeated three times in triplicate.

3. RESULTS AND DISCUSSION

3.1. Characterization of PTX-Loaded NS

Drug-loaded NS were prepared by a solvent deposition method to evaluate the effect of different polymers on mean particle diameter, size distribution, and drug loading capacity. The nanosuspensions were stabilized using a non-ionic surfactant to reduce the dynamic interfacial tension. Tween 80® was used at 0.5% (w/v), a concentration reported to be sufficient to obtain small PLA and PLGA nanoparticles and to allow a complete removal of the excess surfactant by centrifugation and washing.[34] The microscopic evaluation showed spherically shaped NS with a smooth surface and no signs of aggregation. In Figure 2 the SEM picture of PLGA NS is shown, but similar results were obtained for the other systems (data not shown).

In Table I the properties of PTX-loaded NS are summarized. All the three polymer matrices produced small nanoparticles, with a mean size below 200 nm. No significant difference among the various polymers was observed, neither in terms of size homogeneity nor surface charge. PTX entrapment appeared to be proportional to the initial drug loading; the last, in fact, ranged from 20% to about 35–40% when the NS were obtained in the presence of 0.5 or 3% of drug, respectively. Only limited differences were measured in terms of drug encapsulation efficiency among the various polymer matrixes; these results indicated that the variables involved (e.g., molecular weight and/or the chemical composition of the polymer networks) did not affect significantly the solubility and hence dispersion of PTX in the polymer matrix.

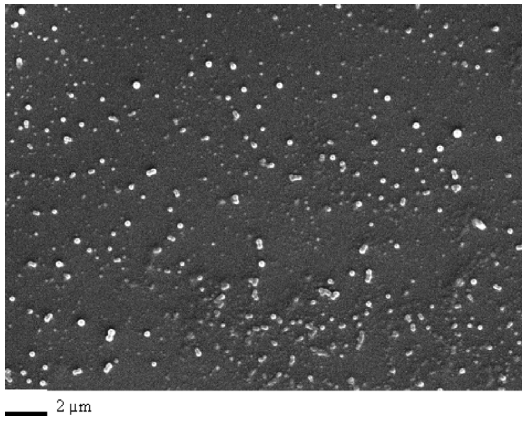

Fig. 2. Scanning electron microscopy of PLGA nanoparticles loaded with 3% (w/v) PTX.

The *in vitro* release of PTX from NS is shown in Figure 3. All the three polymers slowly released the entrapped drug, but some differences were observed among them, also as a function of initial drug loading. In particular, at 3% drug loading (corresponding to about 1% actual drug content, cf. Table I) the two PLA polymers R 203 and R 207 behaved in a different way, with the latter being able to retard the diffusion of the drug in the external medium. Conversely, drug release from PLA R 207 NS had an almost linear profile, whereas the other two systems showed a more rapid leakage during the first two days of testing, followed by a more constant release of PTX. Both PLA R 203 and PLGA RG 502H NS released all the entrapped drug within 15 days, while the PLA R 207 matrix released less than 60% of the encapsulated drug during the same time. At the lower 1% drug loading, which, according to Table I, corresponds to about 0.2% drug content, PTX release profiles were similar: PLA R 203 and PLGA NS gave a higher drug leakage, whereas PLA R 207 nanoparticles released the entrapped drug very slowly during all the experiment.

The different behaviour shown in Figure 3 was interesting, although not novel for these systems; in fact, it allows the choice of the right polymer or polymer blend to obtain the desired sustained release of encapsulated active compounds.

Based on the above release tests, the NS formed by PLA R 203 and PLGA RG 502H were selected for the following biological studies.

3.2. Freeze-Drying of NS and Effects of Lyoprotectants

Freeze-drying of NS represents a possible, valid means of preserving their physical stability and incorporation of drugs for long periods of time, compatible with commercial purposes. The possibility of freeze-drying already filter-sterilized solutions increases the validity of this technique, also including parenteral formulations.

Since colloidal systems can be negatively affected by the freezing step of the aqueous external environment, adjuvants commonly called lyoprotectants are usually added to the solution with the aim of preserving the structure and morphology of colloidal particles and their following reconstitution in water or physiologically compatible aqueous media.

The feasibility of different lyoprotectants on PLA and PLGA NS loaded with 3% PTX to maintain their properties after freeze-drying was then tested. Their effects on

Table I. Properties of PTX-loaded PLA and PLGA NS before freeze-drying.

NS batch	PTX-polymer ratio (%, w/w)	Size (nm)	PI[a]	Encapsulation efficiency, %
PLA R 203	0.5	153.8 ± 6.8	0.204 ± 0.030	20.54
PLA R 203	1	195.2 ± 7.1	0.239 ± 0.020	20.97
PLA R 203	3	188.0 ± 12.6	0.138 ± 0.161	39.30
PLA R 207	0.5	122.5 ± 1.9	0.261 ± 0.040	19.45
PLA R 207	1	128.4 ± 3.4	0.403 ± 0.030	22.36
PLA R 207	3	157.2 ± 2.9	0.118 ± 0.023	33.78
PLGA RG 502H	0.5	141.7 ± 7.5	0.223 ± 0.501	20.97
PLGA RG 502H	1	172.8 ± 8.6	0.239 ± 0.613	28.23
PLGA RG 502H	3	192.5 ± 18.4	0.296 ± 0.071	35.59

[a]Polydispersity index.

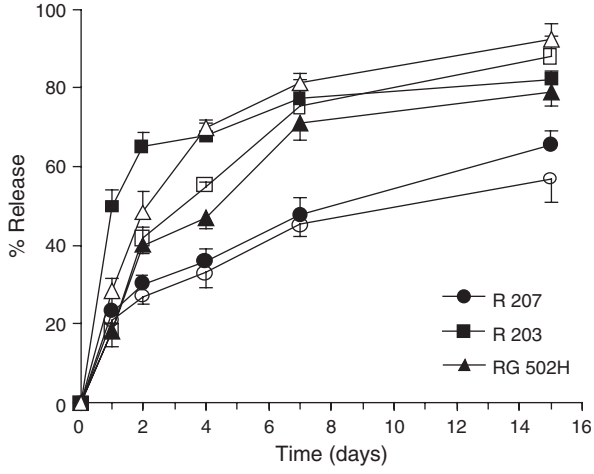

Fig. 3. *In vitro* release profiles in PBS (pH 7.4) at 37.0 of PTX from PLA R 203, PLA R 207, and PLGA RG 502H NS. The initial drug loading was 1% (empty symbols) or 3% (w/v) (filled symbols), the actual drug content is reported in Table I.

the particle size and zeta potential of NS are reported in Table II.

The presence of any of the tested lyoprotectants reduced the particle aggregation during freeze-drying, as the S_f/S_i ratio values reported in Table II show. Trehalose seemed to have the best lyoprotective effect among simple sugars in terms of S_f/S_i ratio; however, re-dispersion of NS pellet in the presence of this sugar did not give small nanoparticles, along with a high PI value and a small zeta potential, therefore it was not examined further. The redispersion of pellet in the presence of glucose gave NS with a relatively higher mean size (around 400 nm), but with a high homogeneity and the more negative zeta potential value (Table III and Fig. 4). Therefore, we chose this lyoprotectant agent for the further *in vitro* and biological tests, as a good compromise between the final size and the other technological properties of freeze-dried nanoparticles.

The adsorption of polyalcohol-type lyoprotectants onto the NS surface can be hindered by the number of hydroxyl groups; as a consequence, the initial particle size measured using 10% mannitol, lactose or sorbitol is comparable with that obtained when the pellet was resuspended in water. However, after freeze-drying the nanoparticles appeared to be aggregated, with an almost doubled PI value.

HP-Cyd and also dimethyl-ß-cyclodextrin (data not shown) were the most efficacious lyoprotectants, even at lower concentrations than the other agents (Table II and Fig. 5); the cyclic structure of these oligoglucoside compounds ensured a better adsorption onto the NS surface during the sublimation of the aqueous phase, with a following easy reconstitution of the dried material.

As Figure 4 shows, when the NS pellet obtained from the centrifugation step (see par. 2.2.) was resuspended in the presence of glucose a reduction of nanoparticles size was observed, in particular from and above a 10% (w/v) concentration of sugar. After freeze-drying, NS displayed a slight

Table II. Mean particle size, size distribution (polydispersity index, PI), and zeta-potential of PLA R 203 NS loaded with 3% PTX before and after freeze-drying in the absence or presence of different lyoprotectants.

	Before freeze-drying			After freeze-drying			
Lyoprotectant (10%, w/v)	Size (nm)	PI	Zeta (mV)	Size (nm)	PI	Zeta (mV)	S_f/S_i
None	188.7 ± 12.6	0.138 ± 0.161	−9.6 ± 2.0	1054.8 ± 186.8	0.932 ± 0.118	−3.6 ± 1.3	3.55
Glucose	140.1 ± 20.0	0.126 ± 0.029	−5.6 ± 3.6	408.4 ± 23.1	0.383 ± 0.093	−16.9 ± 4.2	2.03
Fructose	248.1 ± 22.9	0.131 ± 0.011	−5.0 ± 1.8	435.3 ± 127.1	0.585 ± 0.365	−2.7 ± 0.1	1.75
Trehalose	376.0 ± 23.1	0.569 ± 0.209	−9.6 ± 9.4	529.8 ± 98.6	0.569 ± 0.209	−3.8 ± 0.6	1.41
Lactose	141.2 ± 2.6	0.170 ± 0.011	−5.8 ± 2.8	379.1 ± 12.2	0.370 ± 0.011	−5.8 ± 2.8	2.68
Mannitol	147.0 ± 1.1	0.183 ± 0.013	−8.2 ± 5.7	367.0 ± 6.3	0.337 ± 0.022	−12.2 ± 5.5	2.50
Sorbitol	153.8 ± 6.8	0.204 ± 0.030	−3.7 ± 1.0	342.0 ± 15.2	0.441 ± 0.018	−6.0 ± 2.4	2.22
5% HP-Cyd	194.6 ± 22.0	0.354 ± 0.014	−30.9 ± 4.0	165.2 ± 3.2	0.344 ± 0.004	−18.1 ± 5.1	0.85
10% HP-Cyd	197.1 ± 7.2	0.229 ± 0.021	−32.8 ± 2.6	235.8 ± 55.2	0.287 ± 0.07	−30.5 ± 2.7	1.19

Table III. Mean particle size, size distribution (polydispersity index, PI), and zeta-potential of PLA and PLGA NS loaded with 3% PTX, before and after freeze-drying without or in the presence of 10 or 25% (w/v) glucose.

		Before freeze-drying			After freeze-drying		
Batch	Glucose (% w/v)	Size (nm)	PI	Zeta (mV)	Size (nm)	PI	Zeta (mV)
PLA R 203	—	188.0 ± 12.6	0.138 ± 0.161	−9.6 ± 2.0	1054.8 ± 186.8	0.932 ± 0.120	−3.6 ± 1.3
	10	140.1 ± 20.0	0.126 ± 0.029	−5.6 ± 3.6	408.4 ± 23.1	0.383 ± 0.090	−16.9 ± 4.2
	25	273.8 ± 5.8	0.09 ± 0.008	−8.5 ± 3.4	319.9 ± 6.3	0.094 ± 0.007	−5.9 ± 0.4
PLA R 207	—	157.2 ± 2.9	0.118 ± 0.023	−11.8 ± 12.2	763.2 ± 210.4	0.913 ± 0.150	−2.9 ± 1.4
	10	298.1 ± 65.9	0.680 ± 0.509	−4.8 ± 0.4	614.3 ± 71.9	0.998 ± 0.003	−11.8 ± 6.4
	25	420.3 ± 84.8	0.533 ± 0.310	−5.2 ± 1.8	853.6 ± 67.5	0.873 ± 0.232	−2.4 ± 1.9
PLGA RG 502H	—	192.5 ± 18.4	0.296 ± 0.071	−2.2 ± 0.1	786.4 ± 295.0	0.685 ± 0.550	−2.6 ± 0.2
	10	361.1 ± 24.2	0.202 ± 0.191	−2.0 ± 0.1	422.7 ± 93.8	1.000	−5.3 ± 1.0
	25	353.2 ± 38.3	0.208 ± 0.085	−2.3 ± 0.1	565.6 ± 99.3	0.943 ± 0.099	−2.7 ± 0.1

increase of the mean size, up to 350–400 nm (Fig. 4A) in the presence of 10% or more of such a lyoprotectant. The formulation containing 10% glucose was chosen for further experiments, including the biological assays, because it also showed the maximum increase of zeta potential after freeze-drying and reconstitution of NS (Fig. 4B). The negative value of zeta potential (around −17 mV) can be in fact useful to stabilize the suspension and avoid or retard particle aggregation phenomena.

Figure 5 shows the effect of increasing concentrations of HP-Cyd in the aqueous phase used to resuspend the NS pellets and the following freeze-drying of the suspensions. Such a lyoprotectant was effective at lower concentrations with respect to glucose and other sugars and showed a better ability to maintain the size of NS at around 200 nm after the exsiccation process, in particular at 5 and 10% (w/v) concentration (Fig. 5A). Similarly, starting from 1% HP-Cyd the reconstituted NS showed a more negative surface charge (around −35 mV) (Fig. 5B) and hence could represent an even more valid lyoprotectant means for these systems. It should be noted that HP-Cyd is one of the few cyclodextrins approved for parenteral uses. Although it could cause an increase of costs for large-scale

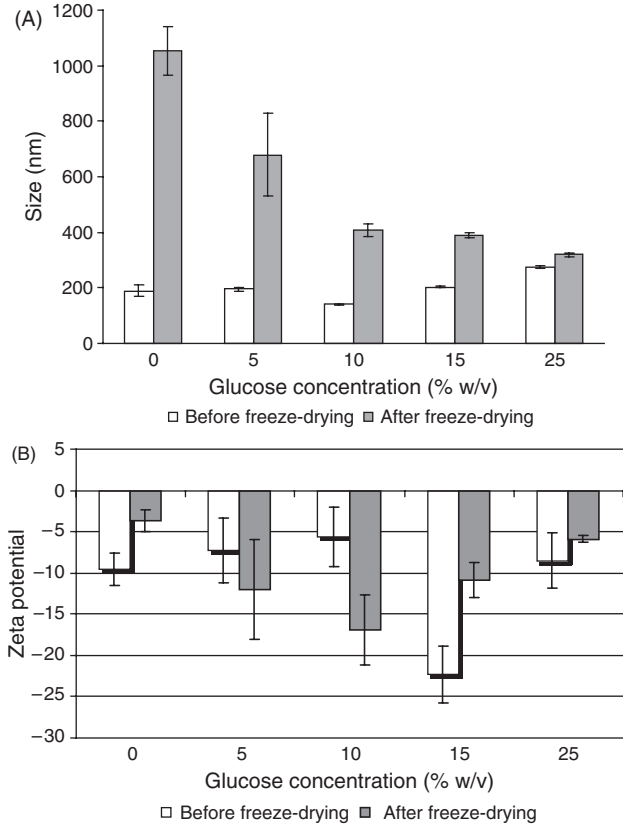

Fig. 4. Changes of the mean size (A) and zeta potential (B) of PLA R 203 NS loaded with 3% PTX before and after freeze-drying in the presence of different concentrations of glucose as lyoprotectant. Values are the mean of five experiments ± S.D.

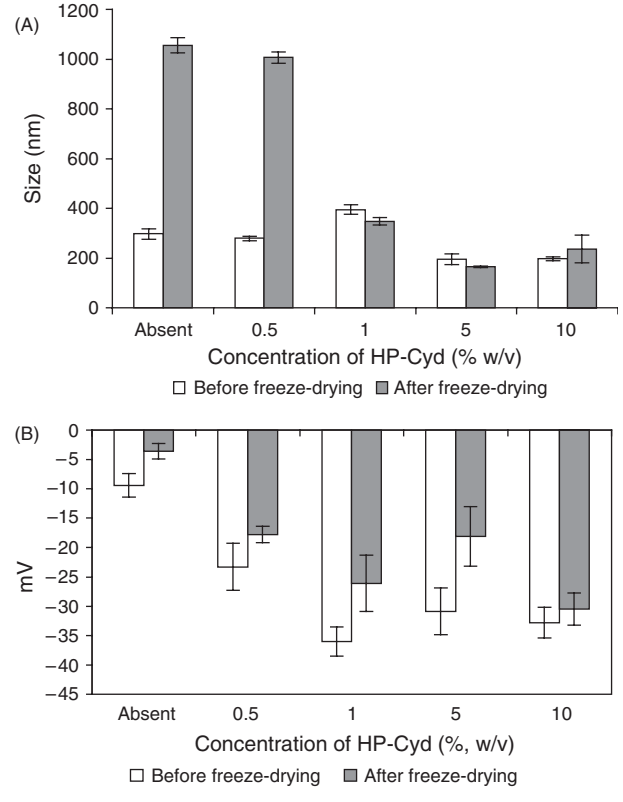

Fig. 5. Changes of the mean size (A) and zeta potential (B) of PLA NS loaded with 3% PTX after freeze-drying in the presence of different concentrations of HP-Cyd as lyoprotectant. Values are the mean of five experiments ± S.D.

production, HP-Cyd can be considered for stabilization by freeze-drying of PLA and PLGA NS.

3.3. Biological Assay

To evaluate the therapeutic potential of PLA and PLGA NS, some of them were submitted to an *in vitro* cytotoxicity study, using the MTS test. A human anaplastic thyroid carcinoma cell line (8305C) was used as a biological substrate; it represents the more resistant thyroid tumour form, for which simple surgical ablation is not usually sufficient. Very often, pharmacological or radiological treatment is required to improve the general conditions of the patient.

Plain (unloaded) NS were initially tested on 8305C cells at a polymer concentration range (4.68 to 0.117 mg/ml) resembling that needed for drug-loaded nanoparticles to achieve the desired PTX concentrations in the cell cultures. Figure 6 shows a concentration-dependent specific cell toxicity by PLA R 203 NS, whereas the NS made from the PLGA copolymer were much less toxic to these cells. The different cytotoxicity of PLGA and PLA R 203 was probably due to the amount of lactic acid monomer that they contain (50% versus 100%, respectively). Compared to the copolymer, in fact, PLA could saturate the Krebs cycle of tumour cells leading to their death. Due to the above behaviour, the PTX-loaded PLGA NS were submitted to the cell growth inhibition assays.

Cell cultures were then exposed to PTX-loaded NS using two different concentrations of drug, i.e., 2.5 and 5 μM. The higher concentration represents the PTX dosage equivalent to those reached during cancer treatment; the lower was included to verify the efficacy of the carrier system for PTX against the tested tumour cell line, with respect to the free drug.

As Figure 7 shows, the encapsulation of PTX in NS preserved the cell growth inhibitory activity of the free drug. Cells exposed to free PTX at both the tested concentrations

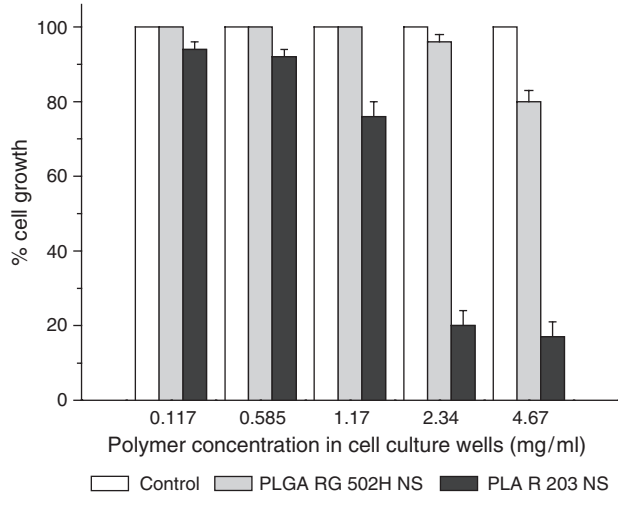

Fig. 6. Cytotoxicity curves of unloaded PLA and PLGA NS against the 8305C cell line.

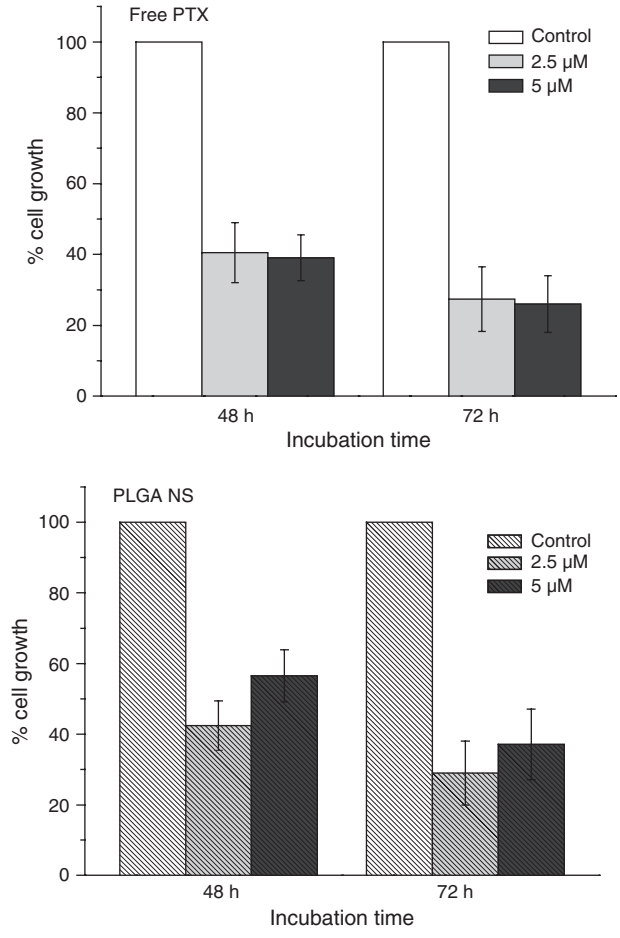

Fig. 7. Growth inhibition of 8305C cell line by different concentration of free PTX or drug-loaded PLGA NS after 48 or 72 h of incubation. The indicated concentrations refer to the actual drug concentration added to the culture wells. The significance (Student's *t*-test) was set at $p < 0.05$.

showed the same vitality after 48 h (40%) or 72 of incubation (26–27%), indicating that the inhibitory effect of the drug was independent of its concentration within the tested range, while it was affected by the incubation time.

At 48 h, the PTX-loaded PLGA NS formulation containing 5 μM drug showed a little lower cell growth inhibition (about 44%) than that one measured with the equivalent dose of the free drug (61%). After 72 h of incubation, however, the inhibitory activity raised to 63%, approaching the effect shown by the free PTX (74% cell growth inhibition).

Using a 2.5 μM PTX concentration, the growth inhibition exerted by the NS formulation reached 58% and 71% after 48 and 72 h of incubation, respectively, values very close to those displayed by the free drug.

The slight difference observed in the inhibitory growth activity shown by PTX-loaded PLGA NS at the two drug concentrations suggested that the drug exerted different biochemical properties in the cellular system used in our experiments (anaplastic thyroid tumour), as already described in the literature on different cell lines and primary thymocytes.[37] Such an interpretation would suggest

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that the release pattern of the drug from the NS inside the tumour cells is different and, in absolute terms, slower than the release profile registered in the *in vitro* dissolution tests (Fig. 3). Pushkarev et al.[38] showed some differences in the biological activity of free PTX: It in fact induced classical apoptosis in thyroid cancer cells only when used at relatively low concentration (6–50 nM), whereas in a drug excess, other forms of cell death may take place, such as necrosis, mitotic catastrophe, and others.

In conclusion, we have described the preparation of PTX-loaded PLA and PLGA nanospheres, using an alternative preparation method compared to those described in the literature for similar systems. Using a suitable amount of a lyoprotectants agent, in particular 10% glucose or 5 or 10% HP-ß-cyclodextrin, the colloidal carrier systems can be freeze-dried and stored for longer periods of time, ensuring good physico-chemical properties upon reconstitution. In preliminary *in vitro* biological assays, the PTX-loaded PLGA NS showed to keep the drug growth inhibitory activity against a thyroid tumour 8305C cell line.

Since nanoparticle systems are known to distribute to tumour sites to a greater extent, due to a passive targeting phenomenon, their use as carriers of PTX could be advantageous, since a better selectivity of action, together with a reduction of side effects could be achieved. Moreover, the dispersion of the drug within a polymer network avoids using ethanol and Cremophor EL® in the dosage forms.

Based on the results of the present study, further work is in progress on PLGA NS, also in pegylated formulations, to complete the study of their *in vitro*/*in vivo* anticancer activity on thyroid tumour cell lines.

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Received: 14 December 2005. Revised/Accepted: 28 February 2006.