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Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity

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Abstract

The main objective of this study was to develop a polymeric drug delivery system for paclitaxel, intended to be intravenously administered, capable of improving the therapeutic index of the drug and devoid of the adverse effects of Cremophor® EL. To achieve this goal paclitaxel (Ptx)-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (Ptx-PLGA-Nps) were prepared by the interfacial deposition method. The influence of different experimental parameters on the incorporation efficiency of paclitaxel in the nanoparticles was evaluated. Our results demonstrate that the incorporation efficiency of paclitaxel in nanoparticles was mostly affected by the method of preparation of the organic phase and also by the organic phase/aqueous phase ratio. Our data indicate that the methodology of preparation allowed the formation of spherical nanometric (<200 nm), homogeneous and negatively charged particles which are suitable for intravenous administration. The release behaviour of paclitaxel from the developed Nps exhibited a biphasic pattern characterised by an initial fast release during the first 24 h, followed by a slower and continuous release. The in vitro anti-tumoral activity of Ptx-PLGA-Nps developed in this work was assessed using a human small cell lung cancer cell line (NCI-H69 SCLC) and compared to the in vitro anti-tumoral activity of the commercial formulation Taxol®. The influence of Cremophor® EL on cell viability was also investigated. Exposure of NCI-H69 cells to 25 μg/ml Taxol® resulted in a steep decrease in cell viability. Our results demonstrate that incorporation of Ptx in nanoparticles strongly enhances the cytotoxic effect of the drug as compared to Taxol®, this effect being more relevant for prolonged incubation times.
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1. Introduction

Paclitaxel has been shown to exhibit a significant activity against a variety of solid tumors, including breast cancer, advanced ovarian carcinoma, lung cancer, head and neck carcinomas, and acute leukemias [1–4]. However, the success of its clinical application is mainly limited by its low therapeutic index and low solubility in water as well as in many other pharmaceutical solvents acceptable for in-

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travascular (i.v) administration [5]. Presently, the only available formulation for clinical use (Taxol®) consists of a solution of paclitaxel (6 mg/ml) in a vehicle composed of Cremophor® EL and dehydrated alcohol at a 50:50 (v/v) ratio. This vehicle has been associated with severe hypersensitivity reactions and has shown incompatibility with common PVC intravenous administration sets [6–10]. In order to eliminate the Cremophor® based vehicle and in an attempt to increase the therapeutic efficacy of the drug, alternative dosage forms have been suggested, including parenteral emulsions [11,12], liposomes [13–15], nanoparticles [16–20] and microspheres [21–24].

Among the new drug delivery systems, polymeric nanoparticles have been considered as promising carriers for anticancer agents. In fact, it has been demonstrated that a significant improvement in drug specificity of action can be reached upon its incorporation into nanoparticles, this effect being mainly attributed to changes in tissue distribution and pharmacokinetics [25,26]. These modifications may consequently result in a reduction in the side-effects and toxicity of the drug and in an increase in its therapeutic efficacy. Furthermore, it has been demonstrated that nanoparticles can escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and thus accumulate in certain solid tumors [27,28]. More recently, it was also reported that nanoparticles can overcome the multidrug resistance phenotype mediated by glycoprotein-P leading to an increase in drug content inside the neoplastic cells [29]. This finding is of great importance for the particular case of paclitaxel since acquired resistance to the drug has already been reported [30]. Other important advantages associated with the use of nanoparticles include the ease of their preparation with well-defined biodegradable polymers (ex: PLGA) and their high stability in biological fluids and during storage [31].

Hence, the main goal of this work was to develop a polymeric drug delivery system for paclitaxel aiming at avoiding the use of Cremophor® EL and improving the anti-tumoral efficacy of the drug.

For this purpose nanoparticles of PLGA containing paclitaxel were prepared by the interfacial deposition (nanoprecipitation) method [32]. PLGA was used in this study since the demonstration of biocompatibility and biodegradation of the polyesters makes them suitable candidates for pharmaceutical purposes [33]. Recently, some work on the use of PLGA nanoparticles as carriers for paclitaxel has been published [19,20,23]. Nevertheless, to our knowledge there are no reports on the literature regarding the incorporation of paclitaxel into PLGA nanoparticles using the nanoprecipitation method. Besides being the simplest method for Nps preparation, involving only one step for dispersion of the non-toxic organic phase in the aqueous phase, thus avoiding any purification procedure, it provides high yields of encapsulation of hydrophobic compounds, and the formation of particles exhibiting adequate features for i.v. administration. The resulting formulations were extensively characterized regarding their size, morphology and charge. In vitro drug release studies were also performed using the most promising formulation developed in this work. Finally, in vitro anti-tumoral activity of paclitaxel incorporated in the nanoparticles was evaluated using a human small cell lung cancer cell line (NCI-H69).

2. Materials and methods

2.1. Materials

Paclitaxel was a gift from Bristol Myers Squibb (Portugal). PLGA copolymers (50/50; Resomer RG 502H, MW 6000 and RG 502, MW 14 500) and the 75/25 PLGA copolymer (Resomer RG 755, MW 63 600) were purchased from Boheringer Ingelheim. Acetone pro analisi® was supplied by Merck. Poloxamer 188 (Symperonic® F68) was obtained from ICI (ICH, France). Dichloromethane (DCM), acetonitrile and phosphoric acid (HPLC grade) were purchased from Merck. The tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma. Cremophor® EL was kindly provided by BASF (BASF, Portugal). Water was purified by reverse osmosis (Milli-Q, Milipore®).

2.2. Preparation of paclitaxel-loaded nanoparticles

Nanoparticles were prepared by the interfacial deposition method as previously described by Fessi

et al. [32]. Briefly, an organic solution of PLGA (100 mg) and paclitaxel (0.4 or 1 mg) in acetone (10 ml) was added to an aqueous poloxamer 188 solution (10 or 20 ml, 0.25%, w/v) under magnetic stirring at room temperature. Following 15 min of magnetic stirring the acetone was removed under reduced pressure. To remove the non-incorporated drug, the obtained nanosuspension was filtered (S&S 'Filter paper circles', pore size 1 μm) and ultracentrifuged twice at 61 700×g for 1 h at 4 °C (Beckman L-80 ultracentrifuge equipped with a Ti-70 rotor). The supernatant containing the free drug was discarded and the pellet was freeze-dried for 24 h (Labconco Freeze Dry System—Freezone 6 Liter, Kansas City, MO, USA).

Drug-free nanoparticles were prepared according to the same procedure.

In an attempt to investigate the influence of various formulation parameters on drug incorporation efficiency, different experimental conditions were evaluated (Fig. 1). Two distinct methods for the preparation of the organic phase were tested: (i) a specific amount of paclitaxel powder was added directly to the polymer (also added as a powder) and this mixture was then solubilized in 10 ml of acetone (method A); (ii) a solution of paclitaxel in acetone was prepared (final concentration of 0.4 or 1 mg/ml) and 1 ml of this solution added to the polymer, being the resulting mixture vortexed vigorously until complete dissolution of the polymer. The volume was completed to 10 ml with acetone immediately before the addition of the organic phase to the aqueous phase (method B). In addition, the effect of using copolymers of PLGA with different molecular weights and different organic/aqueous phase ratios were also investigated.

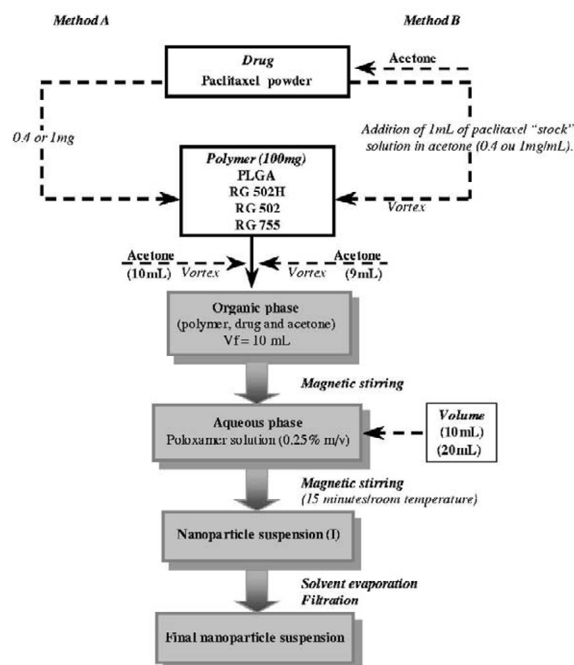

Fig. 1. Diagram of the methodology and experimental conditions used to prepare the different formulations.

2.3. Determination of paclitaxel content in the nanoparticles

The paclitaxel content in the PLGA nanoparticles was assayed by HPLC. For this purpose, optimization and validation of a method proposed by Wang et al. [21] were performed (data not shown). Briefly, a specific amount of lyophilized nanoparticles was dissolved in 1 ml DCM and mixed with 5 ml of an acetonitrile:2 mM phosphoric acid solution (50:50, v/v). The mixture was then vortexed vigorously for 5 min and DCM was evaporated under a nitrogen stream until a clear solution was obtained. The final solution was diluted with acetonitrile:2 mM phosphoric acid (50:50, v/v) and used for paclitaxel analysis.

For HPLC analysis, a reverse-phase Lichrocart® RP 18 column (125×3 mm i.d., pore size 5 μm Merck, KGaA, Purospher®) was used. The column was protected with a Lichrocart® RP 18 pre-column (4×4 mm i.d., pore size 5 μm Merck, KGaA, Purospher®). The column temperature was maintained at 40 °C with a column oven. The mobile phase, a mixture of acetonitrile:2 mM phosphoric acid (50:50, v/v), was delivered at a flow rate of 1.2 ml/min (Hewlett-Packard 1050 pump series). Samples were filtered by Acrodisc LC PVDF (13 mm diameter and 0.45 pore size) and sonicated before injection. Paclitaxel was quantified by UV detection (λ=227 nm, Hewlett-Packard 1050 series). The area of each eluted peak was integrated (Integrator Hewlett-Packard 1050 series) and used for paclitaxel quantification. Drug incorporation efficiency (I.E.) (%) was expressed as the percentage of the drug in

the produced nanoparticles with respect to the initial amount (mg) used for the preparation of nanoparticles (Eq. (1)).

$$\text{I.E.}(\%) = (\text{amount of drug in Nps}_{(\text{mg})} / \text{initial amount of drug}_{(\text{mg})}) \times 100 \quad (1)$$

2.4. Physicochemical characterization

2.4.1. Particle size distribution and morphology

Particle size distribution (mean diameter and polydispersity index) was determined by photon correlation spectroscopy (PCS) using a Malvern Autosizer 2c (Malvern Instruments, UK). The analysis was performed at a scattering angle of 90° at a temperature of 25 °C using samples appropriately diluted with ultra-purified water. For each sample, the mean diameter of three determinations was calculated. Values reported are the mean ± standard deviation of at least three different batches of nanoparticles.

The morphology of the nanospheres was ascertained by Transmission Electronic Microscopy (TEM) (CM-12, Philips, The Netherlands). A drop of the nanoparticles suspension (10 μl) was placed on copper electron microscopy grids (Formvar filmed) and stained with a 2% (w/v) phosphotungstic acid solution (Sigma). After 30 s the sample was washed with ultra-purified water and the excess fluid removed with a piece of filter paper. The dried sample was then examined.

2.4.2. Surface charge

Nanoparticles were also characterized with respect to electrophoretic mobility and zeta (ζ) potential using a Coulter DELSA 440 (Coulter Corporation, Miami, FL). Samples from the prepared suspensions were diluted in ultra-purified water and placed in the measurement cell, with its position adjusted to cover the previously determined stationary layer. The electric field applied was 10 V.

2.5. In vitro release studies

The in vitro release profile of paclitaxel from PLGA-Nps prepared with the Resomer RG 502 copolymer and containing 1% (w/w) of the drug was assessed by determining the residual amount of paclitaxel present in the nanospheres. For that purpose, several aliquots (1 ml) of the same nanoparticle suspension were diluted with phosphate-buffered saline solution (PBS), pH 7.4 (final volume of 20 ml) in a capped conical flask. The flasks were incubated at 37 °C and shaken horizontally at 160 strokes/min. At preselected time intervals, two flasks were withdrawn and nanospheres were separated by ultracentrifugation. After removing the supernatant, the pellet was washed twice with distilled water and lyophilized. The amount of residual paclitaxel in the nanoparticles was determined by HPLC using the same procedure as described above (Section 2.3).

2.6. Cells

NCI-H69 cells, a human small cell lung cancer (SCLC) cell line, were obtained from America Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom, Berlin) and antibiotics (100 μg/ml of streptomycin and 100 unit/ml of penicillin, Sigma) at 37 °C in a balanced air humidified incubator with an atmosphere of 5% CO_2. Cells were maintained in an exponential growth phase by periodic dilutions with fresh medium.

2.7. In vitro anti-tumoral activity

Briefly, 8×10^4 viable cells/well were seeded in 100 μl of growth medium in 96-well microtitre plates (Costar, Cambridge, MA) [34]. Cells were then incubated with different concentrations of Taxol® or paclitaxel-loaded nanoparticles, after appropriate dilution of these formulations in 100 μl of culture medium, for 24, 72, 120 or 168 h. In order to determine the cytotoxic effect of the vehicle Cremophor® EL and of the polymer used to prepare the nanoparticles, cells were also incubated with different dilutions of these reagents for the same periods of time.

The effect of the different treatments on cell viability was assessed by the tetrazolium dye assay of Mosmann [35]. This assay depends on the cellular reductive capacity to metabolise the yellow tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide dye (MTT), to a highly

coloured formazan product. Briefly, at the end of the incubation period with the different formulations, cells were washed twice with phosphate-buffered saline solution (PBS, pH adjusted to 7.4) by centrifugation (10 min, 1200 × *g*). Cells were then incubated with 100 μl of a MTT solution (0.5 mg/ml) in RPMI culture medium without FCS and phenol red for 4 h at 37 °C. One hundred microlitres of an isopropanol acidic solution (isopropanol–HCl 0.04 N) were then added in order to dissolve the formazan crystals formed. The UV absorbance of the solubilized formazan crystals was measured spectrophotometrically (ELISA—Mediators phL, Mediators Diagnostika, Vienna, Austria) at 570 nm. Cell viability was determined by the following equation, in which $Abs_{test\ cells}$ and $Abs_{control\ cells}$ represent the amount of formazan determined for cells treated with the different formulations and for control cells (non-treated), respectively (Eq. (2)).

$$\text{Cell viability (\%)} = (Abs_{test\ cells}/Abs_{control\ cells}) \times 100 \quad (2)$$

3. Results

3.1. Incorporation efficiency of paclitaxel in PLGA-Nps: effect of different experimental parameters

In an attempt to optimise the amount of paclitaxel incorporated into PLGA nanoparticles, different experimental conditions for the preparation of these particles were evaluated. The influence of the preparation of the organic phase on the incorporation efficiency (I.E.) of paclitaxel in PLGA-Nps is illustrated in Fig. 2A. As can be observed, using the method A only about 15% of the initial drug loading is incorporated in the nanoparticles, in contrast to the 100% of incorporation obtained when the organic phase was prepared by the method B. Therefore, this latter method was selected to prepare all the other formulations.

Fig. 2B illustrates the influence of the copolymer molecular weight and composition on the I.E. of paclitaxel. Nps were prepared from PLGA with different molecular weight (MW) and with different lactic acid/glycolic acid (LA/GA) molar ratio. As can be observed, for the two initial drug loadings used (0.4 and 1%) the incorporation efficiency of Ptx was very high (≥90%) and independent of the molecular weight and composition of the copolymers tested.

Finally, the dependence of the I.E. of the drug on the organic to aqueous phase ratio was also studied. Data presented in Fig. 2C clearly show that doubling

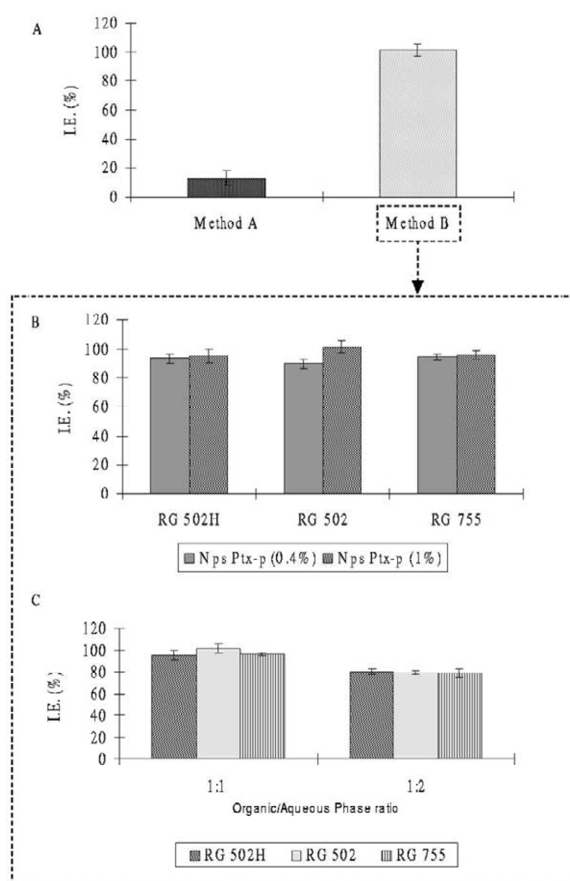

Fig. 2. Incorporation efficiency (%) of paclitaxel in PLGA nanoparticles. (A) Influence of the method of preparation of organic phase; (B) influence of copolymer MW and composition (RG 502H: MW 6000; LA/GA 50:50; RG 502: MW 14 500; LA/GA 50:50; RG 755 MW 63 600; LA/GA 75:25); (C) Influence of organic/aqueous phase ratio. Ptx-PLGA-Nps were prepared by the nanoprecipitation method. The free drug was separated by ultracentrifugation and the amount of paclitaxel incorporated in nanoparticles determined by HPLC as described in Section 2. Values reported are the mean ± standard deviation of at least three different batches of nanoparticles.

the volume of the external aqueous phase, while maintaining constant the volume for the organic phase, resulted in a reduction of the incorporation efficiency of the drug for all the copolymers tested. This reduction was approximately 15% for Resomer RG 502H and approximately 20% for Resomer RG 502 and Resomer RG 755 as compared to the formulation obtained with 1/1 organic/aqueous phase ratio.

3.2. Physicochemical characterization

Aiming at predicting the in vivo behaviour of the developed Nps, the physicochemical properties of Ptx-Nps, in terms of their mean diameter, polydispersity index, morphology, electrophoretic mobility and ζ potential, were evaluated. The effect drug incorporation on such properties was also studied by comparing the results obtained with those for drug-free PLGA-Nps (Table1). The MW and the composition of the copolymers used to prepare nanoparticles are also presented in order to facilitate the interpretation of the obtained results. With respect to particle size analysis and as illustrated in Table 1, all systems prepared were nanometric (mean diameter <200 nm) and exhibited a narrow size distribution (polydispersity index <0.1). The same conclusion can be withdrawn from the TEM analysis. Representative TEM microphotographs obtained with the PLGA-Nps (Resomer RG 502) are presented in Fig. 3A,B showing the spherical shape of the nanoparticles as well as their homogeneous particle size distribution.

The presence of paclitaxel did not affect the size of nanoparticles when considering the same copolymer. However, the characteristics of the copolymer used were found to slightly affect the size of both drug-free PLGA-Nps and Ptx-PLGA-Nps, this effect being dependent on the copolymer MW.

All formulations exhibited a net negative charge with ζ potential values ranging from −33.4 to −23.1 mV. Drug-free Nps prepared with the copolymers RG 502H and RG 502 showed similar values for the ζ potential, while a significant reduction in this parameter was observed when the copolymer RG 755 was used. Regarding the formulations prepared in the presence of paclitaxel, drug incorporation did not affect the ζ potential of the nanoparticles prepared with the copolymers RG 502H and RG 755. However, a slight increase was observed for nanoparticles prepared with the polymer RG 502.

3.3. In vitro release studies

The in vitro release behaviour of paclitaxel from PLGA-Nps prepared with the polymer RG 502 with an initial drug loading of 1% (w/w) is shown in Fig. 4. As can be observed, the release behaviour of paclitaxel from the polymer matrix exhibited a biphasic pattern characterised by a fast initial release during the first 24 h, followed by a slower and continuous release.

Table 1
Physicochemical characteristics of various nanoparticles formulations prepared without (drug-free PLGA-Nps) or with paclitaxel (Ptx-PLGA-Nps). The Ptx-PLGA-Nps were prepared by the methodology B and the initial drug loading used was 1% (w/w)

Polymer	RG 502H		RG 502		RG 755	
MW:	6000		14 500		63 600	
Ratio LA/GA:	50/50		50/50		75/25	
Parameters	Drug-free PLGA-Nps	Ptx-PLGA Nps	Drug-free PLGA-Nps	Ptx-PLGA Nps	Drug-free PLGA-Nps	Ptx-PLGA Nps
MD (nm)	117±2	122±3	132±2	133±2	159±3	160±2
PI	0.06±0.02	0.05±0.01	0.1±0.03	0.09±0.01	0.03±0.02	0.08±0.01
EM (μm·cm/V·S)	−2.35±0.5	−2.48±0.5	−2.6±0.1	−2.0±0.3	−1.83±0.3	−1.79±0.3
ζ (mV)	−30.2±6.5	−31.9±6.2	−33.4±1.8	−25.6±3.5	−23.4±3.1	−23.1±3.7

After appropriate dilution of the different formulations obtained, mean diameter (MD) and polydispersity index (PI) were measured by PCS and electrophoretic mobility (EM) and ζ potential were determined by DELSA as described in Section 2.

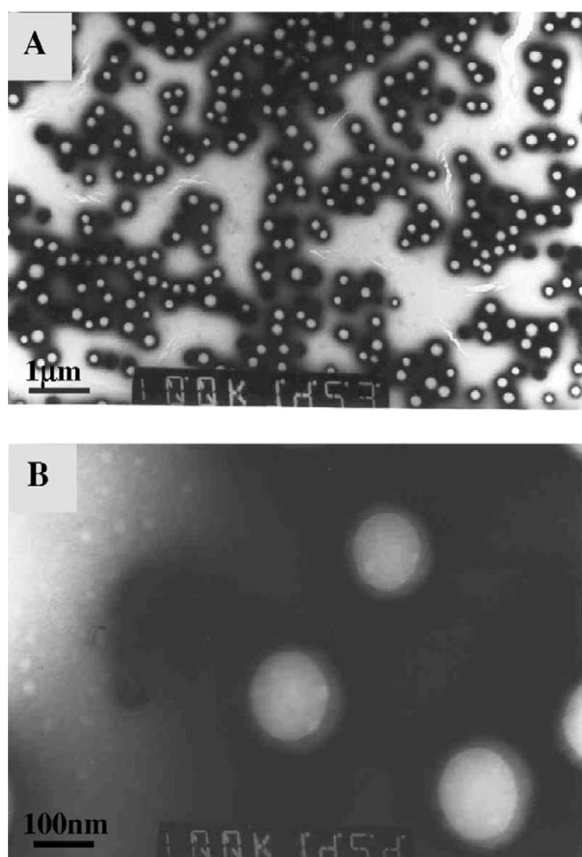

Fig. 3. Transmission electronic microphotographs of PLGA (Resomer RG 502) nanoparticles. (A) ×8800; (B) ×88 000.

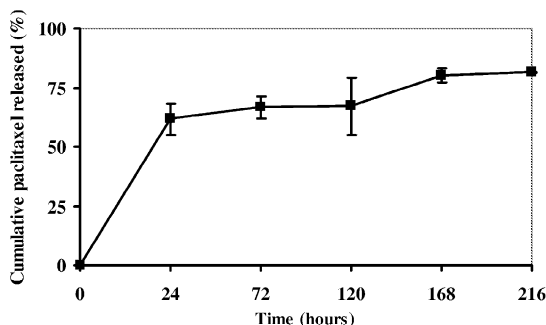

Fig. 4. Cumulative in vitro release of paclitaxel from PLGA-Nps (Resomer RG 502). PLGA-Nps containing paclitaxel 1% (w/w) were diluted in PBS, incubated at 37 °C and shaken horizontally. At preselected time intervals, the released drug was separated by ultracentrifugation and the residual amount of paclitaxel present in the nanospheres determined by HPLC as described in Section 2. Each point represents the mean±standard deviation obtained from triplicates of two samples.

3.4. In vitro anti-tumoral activity

The cytotoxic activity of paclitaxel, both formulated in Cremophor® EL (Taxol®) (Fig. 5A) and loaded in PLGA nanoparticles (Fig. 6), was evaluated by assessing cell viability by the MTT assay using the NCI-H69 cell line. Cells were incubated with concentrations of paclitaxel ranging from 0.025 to 25 µg/ml. This range of concentrations was selected because it corresponds to plasma levels of the drug achievable in humans [36].

As can be seen in Fig. 5A, a marked reduction in cell viability was observed when NCI-H69 cells were incubated with 25 µg/ml Taxol® for 24 h at 37 °C. At this concentration, the cell growth was almost totally inhibited after 72 h of incubation, this effect

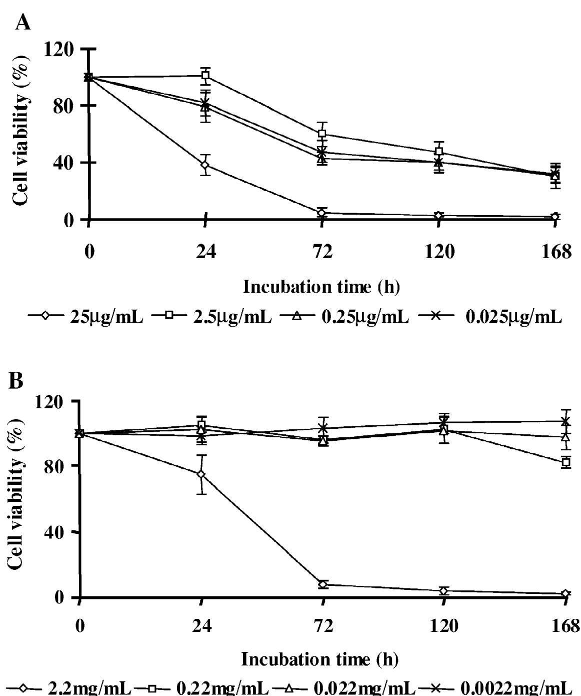

Fig. 5. Viability of NCI-H69 cells incubated with Taxol® (A) or with the excipient Cremophor®EL (B). Cells were seeded into 96-well plates and incubated with different concentrations of both formulations for 24, 72, 120 or 168 h at 37 °C as described in Section 2. Following the incubation, cell viability was determined by the MTT assay and expressed as a percentage of control wells (cells without treatment). Results shown in this figure represent the mean±standard deviation obtained for at least three independent experiments performed in triplicate.

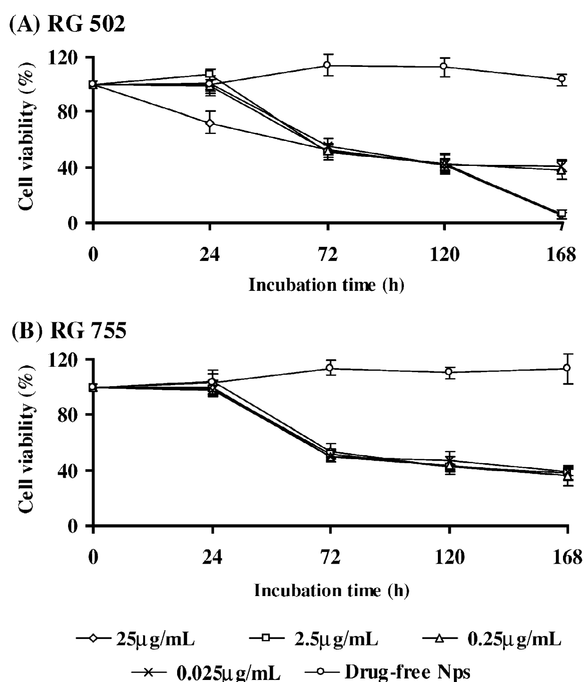

Fig. 6. Viability of NCI-H69 cells incubated with drug-free Nps or Ptx-PLGA-Nps prepared with the RG 502 copolymer (A) or with RG 755 (B). Cells were seeded into 96-well plates and incubated with different concentrations of both formulations for 24, 72, 120 or 168 h as described in Section 2. Cell viability was determined by the MTT assay and expressed as a percentage of control wells (cells without treatment). Results shown in this figure represent the mean ± standard deviation obtained for at least three independent experiments performed in triplicate.

being maintained for the other incubation times tested. It is interesting to note that no toxicity was observed when cells were incubated with 2.5 μg/ml Taxol® for 24 h. However, for lower concentrations (0.025 and 0.25 μg/ml Taxol®) a 20% reduction in cell viability could be seen. For longer incubation times no significant differences in cytotoxicity were observed among the different concentrations tested (0.025, 0.25 and 2.5 μg/ml Taxol®). However, as expected, for each of these concentrations an enhancement in cytotoxicity with increasing time of incubation was observed. Nevertheless, it should be mentioned that cell growth was never completely inhibited (approximately 30% of viable cells were determined even after 168 h of exposure).

Since it is well recognized that Cremophor® EL (the solvent employed in the Taxol®) has some biological activity [37–39], the possible contribution of this excipient to the in vitro cytotoxicity observed when NCI-H69 cells were exposed to Taxol® was also investigated. As can be shown in Fig. 5B, a significant reduction in cell viability was also observed when cells were exposed to 2.2 mg/ml of this excipient (added at the same concentration of Cremophor® EL present in a solution of 25 μg/ml Taxol®). Similar to what was observed with Taxol® (Fig. 5A), this reduction was more pronounced following 72 h of incubation, cell growth being almost totally inhibited following 120 h of incubation. Still regarding Cremophor® EL, it should be noted that at the lowest concentrations (0.022 and 0.0022 mg/ml) no cytotoxic effect was observed for the longest incubation times.

Fig. 6A,B illustrates the results obtained when paclitaxel-loaded nanoparticles were incubated with the NCI-H69 cells for different times at 37 °C. With respect to the dependence of cytotoxicity on the type of the copolymer used, it should be mentioned that for the Ptx-PLGA-Nps prepared with the copolymer RG 502 (Fig. 6A), a reduction of about 30% in cell viability was observed when NCI-H69 cells were incubated for 24 h with 25 μg/ml of incorporated drug. For the same incubation time, no significant effect was observed for the other concentrations tested. Although for higher incubation times (72 and 120 h), cell viability decreased to 55 and 43%, respectively, this effect was shown to be independent of the concentration used. Nevertheless, for the longest incubation time (168 h) a marked cytotoxic effect could be observed for both 2.5 and 25 μg/ml. Under these conditions, Ptx-PLGA-Nps were able to mediate similar levels of toxicity to those observed for 25 μg/ml of the commercially available formulation of paclitaxel (Taxol®).

Paclitaxel loaded-nanoparticles obtained with the RG 755 copolymer, showed a different behaviour (Fig. 6B). In this case, the incubation time seems to be the most critical parameter for paclitaxel activity. After 24 h of incubation, no cytotoxic effect could be observed for any of the concentrations tested. Increasing times of incubation resulted in an enhancement of Ptx-PLGA-Nps cytotoxicity. After 168 h of incubation with this formulation, a reduction of approximately 63% in cell viability was detected for all concentrations tested.

In order to determine any possible cytotoxic effect attributed to the copolymers used to prepare the nanoparticles, cell viability was also evaluated upon incubation of the cells with different concentrations of drug-free nanoparticles. According to our results no cytotoxic effect was observed upon incubation of these formulations with cells. The results presented reflect only the effect of the highest copolymer concentration used (2.5 mg/ml), since similar results were obtained for the other concentrations tested (data not shown).

A comparison of the results obtained for both formulations (Fig. 6A,B), shows that after 168 h of incubation with either 2.5 or 25 μg/ml of incorporated drug, Ptx-PLGA-Nps prepared with the RG 502 copolymer were able to mediate higher levels of toxicity than Ptx-PLGA-Nps prepared with the RG 755.

4. Discussion

Paclitaxel is one of the most promising drugs currently available for cancer therapy. Nevertheless, taking into account the problems associated with the clinical use of the only available paclitaxel formulation (Taxol®), it can be deduced that the development of a suitable carrier system for this drug is considered to be of prime importance. The main goal of this work was to develop a polymeric delivery system for paclitaxel intended to be intravenously administered, capable of increasing the therapeutic index of the drug and devoid of adverse effects.

The interfacial deposition method (nanoprecipitation) is recommended for the incorporation of hydrophobic drugs into polymeric nanoparticles [32,40]. Nevertheless, as described by several authors and also as demonstrated in this work, the establishment of a protocol that allows Nps precipitation, while avoiding extensive diffusion of the drug along with the solvent aiming at obtaining high values of drug encapsulation is a challenging issue [41]. Taking into account that both of the organic solutions tested have the same composition and the same concentration of paclitaxel at the moment of their addition into the aqueous phase, one possible explanation for the discrepancy of the encapsulation efficiency results obtained from the two methods explored in this work may be that a different interaction between paclitaxel and the polymer occurs when they are both solubilized, thus leading to a different incorporation of the drug in the nanoparticles. In fact, paclitaxel has a complex structure and previous studies have provided evidence that its conformation in solution, as well as its molecular interactions, may be dependent on the type of solvent and on its concentration in the organic phase [42,43]. Balasubramanian et al. [43], reported differences in paclitaxel conformation and molecular interactions when its concentration varied from 10^{-4} to 10^{-3} M, values that are in the same range as those employing methods A and B, respectively. Besides, the polymer concentration is also 10 times higher using method B which may also favours its interaction with the drug.

The organic/aqueous phase ratio is among the most critical parameters for the spontaneous formation of colloidal particles by the nanoprecipitation method [44]. Although the effect of this parameter on the particle size distribution has been previously investigated, there is still a lack of fundamental information on its influence on the incorporation efficiency of drugs in nanoparticles prepared by this methodology. In this work, an increase in the volume of the external aqueous phase (1/2 ratio) caused a decrease in the incorporation efficiency of paclitaxel in PLGA nanoparticles. Preliminary studies performed in our laboratory also revealed that this ratio led to a significant decrease in the nanoparticles size as compared to that obtained when the 1/1 ratio was used (data not shown). Based on our findings and on the results reported in the literature, it is reasonable to consider that the increase in the specific surface area caused by the formation of smaller nanodroplets during the emulsification stage of the process, may facilitate the diffusion of the drug to the external phase along with the solvent, leading to lower incorporation efficiencies [45,46]. It is also possible that the smaller the size of the Nps, the lower the capacity of the polymer matrix to incorporate the drug.

The physicochemical characteristics of colloidal systems, namely size and surface charge, are recognized to affect their physical stability and to significantly influence their interaction with the biological milieu after in vivo administration, as well as the release rate of an incorporated substance and

their interaction with cells [47,48]. The method used in this work allowed the instantaneous and reproducible formation of Nps exhibiting diameters below 200 nm and low polydispersity indexes, indicating an homogeneous size distribution. As mentioned above, these findings were confirmed by the TEM data, which also showed that nanospheres were spherical in shape. Nps were shown to exhibit a negative surface charge, which can be attributed to the type of polymer used and more specifically to the presence of polymeric carboxylic groups on the nanoparticle surface [49,50]. Nevertheless, the results presented slightly differ from those reported by other authors [51–53], which can be attributed to different conditions of Nps preparation (concentration of the polymer, concentration and type of drug and concentration and type of surfactant) and to the composition of the analysis medium used.

With respect to drug-free nanoparticles, a slight effect of the copolymer composition on the surface charge was observed. Nanoparticles prepared with RG 502H and RG 502 copolymers (at a 50/50 LA/GA ratio) showed similar values of ζ potential, while nanoparticles obtained with the RG 755 copolymer exhibited a lower ζ potential. It has been described that the surface charge of nanoparticles prepared with copolymers may depend on the proportion of the different monomers [54]. It is also accepted that the presence of poloxamer 188, the emulsifier used during the preparation of nanoparticles, can contribute to a reduction in their surface charge [54,55]. In fact, it is known that this emulsifier tends to bind to the nanoparticles surface through hydrophobic interactions involving its polyoxypropylene chains, while the hydrophilic polyoxyethylene chains protrude into the surrounding medium, thus masking the negative surface charges present in the nanoparticles. In the light of these considerations, it is possible that a stronger interaction is established between poloxamer 188 and the RG 755 copolymer-based Nps (which has a more hydrophobic surface due to the higher proportion of LA/GA), as compared to the other copolymer used, thus justifying the lower values of ζ potential observed.

The release behaviour of paclitaxel from the polymer matrix exhibited a biphasic pattern characterised by a fast initial release during the first 24 h, followed by a slower and continuous release of the drug. Similar patterns have been previously observed for the release of paclitaxel from other PLGA polymeric systems [20,56]. The burst release of paclitaxel may be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix, while the slower and continuous release may be attributed to the diffusion of the drug localised in the PLGA core of the nanoparticles. It should be noted that the amount of drug released from the Nps developed in this work is within the range of the values reported for other PLGA systems containing paclitaxel [20,56].

In this study a small cell lung cancer cell line was used to determine the anti-tumoral activity of paclitaxel, either incorporated in PLGA-nanoparticles or in the commercial formulation Taxol®. The results obtained clearly demonstrated that both the incubation time and concentration play a major role in the in vitro cytotoxicity of paclitaxel. Cell toxicity was higher for longer periods of incubation with the drug. This finding is consistent with previous reports on the in vitro cytotoxicity of paclitaxel against other tumour cell lines and is in agreement with the mechanism of action of paclitaxel [36,37]. In fact, for longer incubation periods a larger number of cells enter the G2 and M cell cycle phases during which paclitaxel is more active [57]. This result suggests that a drug delivery protocol that could maintain a therapeutic concentration of paclitaxel for an extended period would be desirable in order to maximize its clinical efficacy.

Although a marked reduction in cell viability was observed when NCI-H69 cells were incubated with 25 μg/ml Taxol®, cell growth being almost totally inhibited for longer periods of incubation (72 h), these results should be analysed together with those observed for the Cremophor® EL. In fact, an intriguing similarity in the extent of cell toxicity was observed upon incubation of the cells with either Taxol® (25 μg/ml) or the excipient Cremophor® EL (used at a concentration equivalent to that existing in a Taxol® dose of 25 μg/ml). This result suggests that the reduction in cell viability obtained with this concentration is not only due to paclitaxel activity but also to the presence of the Cremophor® EL. Curiously, when cells were incubated with 2.5 μg/ml Taxol® for 24 h no cytotoxic effect was observed,

while for the lowest concentrations (0.25 and 0.025 μg/ml Taxol®), a decrease in cell viability was observed, a fact that can also be related to the presence of Cremophor® EL in the medium. In fact, it has been reported that, above certain concentrations (but below toxic concentrations), Cremophor® EL is able to block cells at the G1 phase of the cell cycle preventing them to enter G2/M phases and thus hampering the cytotoxic effect of paclitaxel [37–39].

For the higher concentrations tested, significant differences in the cytotoxic effect of paclitaxel-loaded nanoparticles were observed depending on the copolymer used, the RG 502 causing a larger extent of cell death than RG 755. This effect was particularly pronounced for the 24- and 168-h incubation times, which may be attributed to the different characteristics of the copolymers used to prepare the Nps (i.e., hydrophobic/hydrophilic balance and MW) that are known to influence the release rate of a drug from a polymeric system [58,59].

In this work, a correlation can be established between the cytotoxicity results and the in vitro release kinetics of the drug from the PLGA-Nps. In fact, following 24 h of incubation a significant amount of paclitaxel was released and thus available to mediate some cytotoxicity. This effect increases with incubation time most likely due to the drug mechanism of action which requires cell division to occur.

The advantages of incorporating paclitaxel in the developed PLGA nanoparticles are illustrated in Fig. 7. Incubation of the cells with 2.5 μg/ml of free paclitaxel (Taxol® formulation) contributed to only 70% reduction in cell viability, while the same concentration of the drug provided as Ptx-PLGA-Nps allowed a cytotoxic effect of almost 100%. It is important to mention that for this latter condition the cytotoxic effect was similar to that observed when cells were incubated with 25 μg/ml Taxol®, meaning that the same cytotoxic effect can be observed with a 10-fold reduction in paclitaxel concentration.

For the higher concentrations (25 μg/ml), similar levels of toxicity were observed for Taxol® and Ptx-PLGA-Nps. Nevertheless, it should be emphasized that in the case of Taxol® a significant effect is attributed to the diluent Cremophor® EL (absent in the Nps), whereas in the case of Ptx-PLGA-Nps the

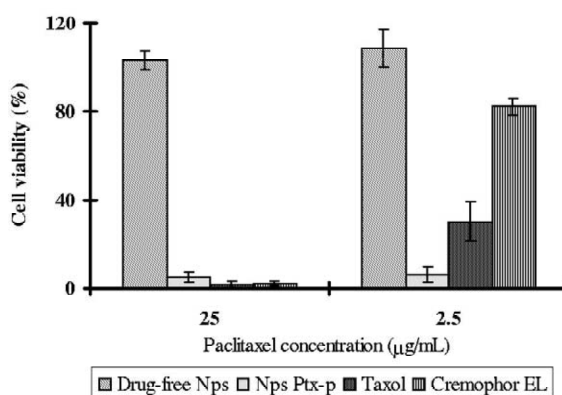

Fig. 7. Viability of NCI-H69 cells incubated with Taxol®, Cremophor® EL, drug-free Nps or Ptx-PLGA-Nps. Cells were seeded into 96-well plates and incubated with the different formulations for 168 h as described in Section 2. Cell viability was determined by the MTT assay and expressed as a percentage of control wells (cells without treatment). Results shown in this figure represent the mean ± standard deviation obtained for at least three independent experiments performed in triplicate.

cytotoxicity observed is only attributed to the amount of paclitaxel released (since empty Nps are non-toxic). The fact that total inhibition of cell growth was only observed for the highest concentration tested suggests that a threshold concentration needs to be reached in order that paclitaxel can develop 100% of cell death.

The enhancement of paclitaxel activity mediated by its incorporation into Nps can be explained by the fact that these systems can act as a reservoir for paclitaxel, protecting the drug from epimerization and hydrolysis [60,61] and providing not only a sustained release of paclitaxel but also contributing to the maintenance of its activity. In addition, it is reasonable to consider that cells become more prone to paclitaxel activity when the drug is delivered in the absence of Cremophor® EL (which is the case for Nps) since as reported above, this compound can antagonize paclitaxel activity [37–39]. The mechanism of drug release from the nanoparticles is also of crucial importance for the extent of paclitaxel activity. In this regard, two distinct but not exclusive pathways can justify the enhancement of cytotoxic activity of anti-tumoral drugs incorporated into Nps: (i) Nps can adsorb onto the cell membrane, leading to an increase in drug concentration near the cell surface, thus generating a concentration gradient that

would favour a drug influx into the cell [62]; (ii) tumoral cells (which in many situations exhibited enhanced endocytotic activity) can internalise polymeric nanoparticles allowing the drug to be released into the interior of the cells, thus contributing to an increase of the drug concentration near its site of action [27].

5. Conclusions

The results obtained showed that the methodology selected in this work allowed the instantaneous and reproducible formation of nanometric (<200 nm), spherical and homogeneous PLGA nanoparticles that exhibit high incorporation efficiencies of paclitaxel. Moreover, it was shown that incorporation of paclitaxel in the PLGA nanoparticles strongly enhances its anti-tumoral efficacy as compared to the free drug (Taxol®), this effect being more relevant for prolonged incubation times with cells. Based on these results, it can be concluded that the formulations developed in this work may be considered promising systems for in vivo paclitaxel delivery.

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