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New Biocompatible Aliphatic Polyesters as Thermosensitive Drug Nanocarriers. Application in Targeting Release Pharmaceutical Systems for Local Cancer Treatment

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Abstract

In the present study a new drug delivery system for the treatment of local cancer was developed. Two aliphatic polyesters namely poly(propylene adipate) (PPAd) and poly(propylene pimelate) (PPPim), were used as carriers in order to prepare nanoparticles loaded with paclitaxel. The starting materials as well as the nanoparticles were characterized with DSC, SEM and WAXD techniques. The nanoparticles had a mean particle size of 160-190nm and characterized for drug loading content, efficiency and in vitro dissolution at 37°C and 42°C in two different pH buffer solutions (pH 7.4 and pH 6.0). Results showed enhanced release rate of paclitaxel at 42°C compared to 37°C in both pH conditions. The degree of crystallinity plays also an important role to paclitaxel release. The cytotoxicity of the prepared paclitaxel/polyester nanoparticles was studied in comparison with control samples using two cancer cell lines like Human hepatoma (HepG2) cells and Human Cervical Adenocarcinoma Cells (HeLa). In both cases it was found that cells are in the phase of necrosis or apoptosis after 20h of incubation. Finally, the temperature is also an important factor since this behaviour is faster in 42°C than in 37°C, indicating that the studied polyesters could act as thermosensitive carriers.

Introduction

Until now, much research has been done on pharmaceutical drug delivery systems able to deliver the API directly to the local environment of the pathology concerning cancer therapy [1,2]. Today's research in cancer therapy focuses mainly on pharmaceutical systems which are able to reduce the side effects of anti-cancer APIs (high cytotoxicity) and target tumour tissues by taking advantage of their physiology under specific conditions. Such systems include nanoparticles prepared by thermosensitive polymers.

It is well documented that in a temperature range of 39°C to 43°C the blood flow increases in the tumour tissues leading to increased vascular permeability compared to normal tissues which remain unaffected [3]. Several attempts have been made in order to develop site-specific anticancer drug delivery systems where folic acid, transferrin, heparin and albumin were chemically conjugated on nanoparticles [4-13]. Other studies reported magnetic nanoparticles for targeting tumour tissues by applying a topical magnetic field [14-17], while few attempts focused on pH-sensitive drug carriers [18,19]. Kong et al. [20] studied the effect of particle size in combination with mild hyperthermia showing that nanoparticles with mean diameter from 100-400nm are more effective for cancer treatment.

Aliphatic polyesters are biodegradable and biocompatible polymers which are nowadays commercially available in a variety of types [21,22]. From previous studies it was found that the physical properties of such polyesters, (e.g, melting point or degree of crystallinity), are directly affecting drug release behaviour [23,24]. Hence, it is suggested that these polyesters could be used as thermosensitive drug carriers since the drug dissolution rate was found to be higher when aliphatic polyesters with melting points in the range 40-44°C, are prepared [23]. Furthermore, it was found that aliphatic polyesters with lower degree of crystallinity show enhanced drug dissolution rate due to higher mobility of the macromolecular chains [24].

The purpose of the present study is to prepare a thermosensitive nanoparticulate system which in combination with mild hyperthermia can be used for the treatment of cancer tumour tissues which are topically heated in a temperature range of 39°C to 43°C. Aliphatic polyesters were synthesized and used for the nanoencapsulation of paclitaxel. Two different molecular weights of PPAd and PPPim were tested (Figure 1). paclitaxel, a cytotoxic anticancer mitotic inhibitor, was used as API.

Experimental Section

Materials

Adipic and Pimelic acids were purchased from Aldrich Chemical Co. 1,3-Propanediol (1,3-PD) (CAS Number: 504-63-2, Purity: > 99.7 %) was kindly supplied by Du Pont de Nemours Co. Tetrabutyl Titanate (TBT) of analytical grade, used as catalyst, was purchased from Aldrich Chemical Co. Polyphosphoric acid (PPA), used as heat stabilizer, was supplied from Fluka. Paclitaxel, a white, odourless, crystalline powder with melting point of 210-220°C and 853.92 Da molecular weight, was purchased from Indena SPA, Italy. All the other materials and solvents used were of analytical grade.

Synthesis of polyesters

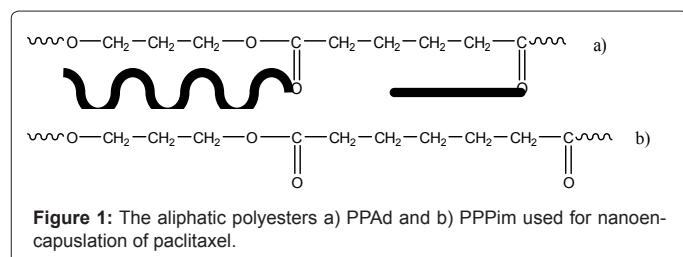Synthesis of aliphatic polyesters was performed according to

Figure 1: The aliphatic polyesters a) PPAd and b) PPPim used for nanoencapsulation of paclitaxel.

a two-stage melt polycondensation method (esterification and polycondensation) in a glass batch reactor [25]. In brief, during esterification the proper amount of adipic acid (ADA) or pimelic acid (PA) and 1,3-PD in a molar ratio 1/1.1 along with TBT (3×10^{-4} mol TBT/ mol ADA) were charged into the reaction tube of a polycondensation apparatus. The apparatus was evacuated several times and filled with argon. The reaction mixture was heated to 190°C under argon atmosphere and stirred at 500 rpm. Almost all theoretical amount of H_2O was removed from the reaction mixture by distillation and collected in a graduate cylinder. In the second stage of synthesis (polycondensation), PPA was added (5×10^{-4} mol PPA/mol ADA). Vacuum (5.0 Pa) was applied slowly over a time period of about 30 min, to avoid excessive foaming and to minimise oligomer sublimation. The temperature was slowly increased to 230°C, while stirring speed was also increased to 720 rpm. The polycondensation continued for about 30 or 60 min in order to attain polyesters with low and high molecular weights respectively. After the end of polycondensation, the polyesters were easily removed, milled and washed with methanol.

Polymer Characterization

Intrinsic viscosity measurement

Intrinsic viscosity measurements on the isolated polymers were performed using an Ubbelohde capillary viscometer at 25°C in chloroform at a solution concentration of 1 wt%.

Gel Permeation Chromatography (GPC)

GPC analysis was performed using a Waters 150C GPC equipped with differential refractometer as detector and three ultrastyragel (103, 104, 105 A) columns in series. CHCl3 was used as the eluent (1 ml/ min) and the measurements were performed at 35°C. Calibration was performed using polystyrene standards with a narrow molecular weight distribution.

Differential Scanning Calorimetry (DSC)

DSC study of polyesters was performed on a Perkin–Elmer, Pyris Diamond DSC differential scanning calorimeter, calibrated with high purity standards. A Perkin Elmer Intracooler 2P cooling accessory was used. Samples of 5.0 ± 0.1 mg were sealed in aluminium pans and scanned under nitrogen atmosphere. A cyclic scanning procedure was followed according to the following steps: (a) heat from 0 to 40°C above the melting point of each sample at a heating rate 20°C/min, (b) hold at this temperature for 2 min in order to erase any thermal history of the sample, (c) rapid cooling to -65°C and equilibration, (d) reheat at a heating rate of 2.5°C/min from -65°C to 40°C (e) hold for 2 min (f) final cooling at a cooling rate 10oC/min down to -50°C.

Wide Angle X-Ray Diffraction (WAXD)

X-ray diffraction measurements of the samples were performed by an automated powder diffractometer Rigaku Mini Flex II with Bragg-Brentano geometry (θ-2θ), using CuK$_\alpha$ radiation (λ=0.154 nm) in the angle 2θ range from 5 to 50 degrees.

Cytotoxicity Study of the Prepared Polyesters

Cell culture

The human umbilical vein endothelial cells (HUVEC) was grown routinely in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS), 15 mg ECGS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 2.5 μg/ml amphotericin B. Cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

In vitro cytotoxicity study

The cytotoxicity of aliphatic polyesters, in comparison to biocompatible poly(lactic acid) (PLA), was evaluated by measuring the viability of HUVE cells in the presence of different concentrations of the polymers. Cell viability was determined by the ''3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay. HUVEC were seeded in 24-well plates at a density of 30.000 cells per well in 500 μl cell culture medium. Twenty-four hours after plating, different amounts of aliphatic polyesters in the form of nanoparticles (suspended in culture medium) were added in the wells. After 24 hours of incubation at 37°C, 50 μl of MTT solution (5 mg/ml in PBS pH 7.4) were added into each well and plates were incubated at 37°C for 2 hours. The medium was withdrawn and 200 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were added in each well and agitated thoroughly to dissolve the formed crystals. The solution was transferred to 96-well plates and immediately read on a microplate reader (Biorad, Hercules, CA, USA), at a wavelength of 490 nm. The experiments were performed in triplicate. Biocompatibility of polymers was expressed as % cell viability, which was calculated from the ratio between the number of cells treated with the nanoparticles and that of non-treated cells (control).

Preparation of paclitaxel loaded polyester nanoparticles

Water-oil (w/o) emulsification and solvent evaporation technique was used for paclitaxel nanoencapsulation in polyester matrices. In brief, 50 mg of polyester and 5mg of paclitaxel were dissolved in 2 ml of dichloromethane. The polymer-drug solution was added in 6 ml of 12 mM sodium cholate aqueous solution and the mixture was sonicated for 1 min. Sodium cholate was added to prevent drug particle aggregation during solvent evaporation. As a result drug-loaded polymer is dispersed in the form of nanoparticles. The emulsion formed was gently stirred until the evaporation of the organic solvent was completed. Nanoparticles were purified by centrifugation (9000 rpm for 15min). The samples were reconstituted with deionized water. Polymer aggregates were removed by filtering the suspension through a 1.2 μm pore size microfilter. Nanoparticles were maintained at room temperature under vacuum to remove the remaining water traces.

Characterization of Drug-Loaded Nanoparticles

Nanoparticle yield, drug loading and entrapment efficiency

Drug loading content was determined with HPLC analysis using a Shimadzu HPLC (model LC-20AD). 3mg of nanoparticles were added in 50ml of water/ACN 50/50 v/v and stirred with a magnetic stirrer till complete dissolution. A clear solution was obtained which was filtered through 45μm ready for HPLC analysis. The column used was a Eclipse XDB-C18, 5μm, 250 x 4.6 mm. The flow rate was 1 ml/min and the column temperaure was 25°C. A diode array detector was used at 227 nm, and quantification of the API was based on a calibration curve created by diluting with mobile phase a stock solution of 20 μg/

ml paclitaxel in water/ACN 50/50 v/v to concentrations 20, 10, 5, 2.5, 1 and 0.5 µg/ml. Nanoparticle yield, drug loading and drug entrapment efficiency were calculated from equations (1)-(3), respectively:

$$\text{Nanoparticles Yield(\%)} = \frac{\text{weight of nanoparticles}}{\text{weight of polymer and drug fed initially}} \times 100 \qquad (1)$$

$$\text{Drug Loading(\%)} = \frac{\text{weight of nanoparticles}}{\text{weight of nanoparticels}} \times 100 \qquad (2)$$

$$\text{Entrapment Efficiency (\%)} = \frac{\text{weight of drug in nanoparticles}}{\text{weight of drug fed initially}} \times 100 \qquad (3)$$

Scanning Electron Microscopy (SEM) measurements

The morphology of the prepared nanoparticles was examined with a Scanning Electron Microscope (JEOL, JMS–840). The samples were coated with carbon black to avoid charging under the electron beam. Operating conditions were: accelerating voltage 20 KV, probe current 45 nA, and counting time 60 s.

Particle size distribution

Particle size distribution of the paclitaxel/polyester nanoparticles was determined by dynamic light scattering (DLS) using a Zetasizer Nano instrument (Malvern Instruments, Nano ZS, ZEN3600, UK) operating with a 532 nm laser. A suitable amount of nanoparticles was dispersed in distilled water creating a total concentration 1‰ and was kept at 37ºC under agitation at 100 rpm. Particle size was measured at different time intervals after sample introduction into the disperse medium. All measurements were performed in triplicates and the results were reported in terms of mean diameter ±SD.

In-vitro drug release studies

The release rate of paclitaxel from nanoparticles was measured in a DISTEK 2100B apparatus, equipped with an autosampler using the basket method (USP I method). Nanoparticles corresponding to 1.5 mg of paclitaxel were dispensed and placed in a dialysis tubing cellulose membrane. The test was performed at 37±1ºC and 42±1ºC for two phosphate buffers (pH 7.4 and pH 6.0, 500 ml dissolution medium) with a rotation speed of 100 rpm. At predetermined time intervals, samples of 3 ml were withdrawn from the dissolution medium, filtered through 45µm UHMW Polyethylene filters and analyzed using an HPLC the HPLC method described above. An equal volume of fresh dissolution medium was transferred to the vessel after sample withdrawal. The phosphate buffers contained 0.1% v/v Tween 80 in order to ensure sink conditions. All measurements were performed in triplicate.

As reported previously by various scientists, Paclitaxel shows high tendency in degradation in phosphate buffers and the highest stability is observed in a pH 3.0-5.0 region [26-28]. According to Liggins et. al., [29] Paclitaxel which has a solubility of 3.5µg/ml in water is possible to recrystallize in aqueous solutions as a stable dihydrate with lower solubility. Other scientists reported some ways to increase Paclitaxel stability and solubility in aqueous solutions using either cyclodextrins or Tween 80 [30,26]. In this study the phosphate buffers of the dissolution medium contained 0.1% v/v Tween 80 in order to ensure that the solubility of Paclitaxel will be higher than its maximum concentration in dissolution medium.

Cytotoxicity of the Prepared Nanoparticles in Cancer Cells

Cell culture

Two human cancer cell lines were studied, the Human hepatoma HepG2 cells and Human Cervical Adenocarcinoma Cells (HeLa Line). The cells were cultured in GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin, at 37ºC and 5%-CO_2. For the study at 42ºC the incubation temperature of the cell cultures was increased 30 minutes before the sample addition.

In vitro cytotoxicity study in cancer cells

The cytotoxicity of the prepared nanoparticles, was studied in comparison with control and placebo samples. For the placebo nanoparticles, a sample of 50mg was reconstituted in 1ml of phosphate buffered saline pH 7.4 while for the paclitaxel loaded nanoparticles samples corresponding to 500µg of paclitaxel was reconstituted in 1 ml medium. The cells were seeded in 6-well plates at a density of 300.000 cells per well in 2 ml cell culture medium and the appropriate amount of nanoparticle suspension was added on the cells in order to achieve the desired paclitaxel maximum concentrations of 500nM in the culture medium. The control, placebo and paclitaxel loaded samples were observed by reverse phase microscopy and photos were taken at the time point that the effect on the cancel cells was clear. In the case of HepG2 cell cultures Trypan Blue was added after the incubation in order to obtain the cells that are in the necrosis or apoptosis stage. Cytotoxicity of the nanoparticles was expressed as % cell viability, which was calculated from the ratio between the number of cells treated with the nanoparticles and that of non-treated cells (control).
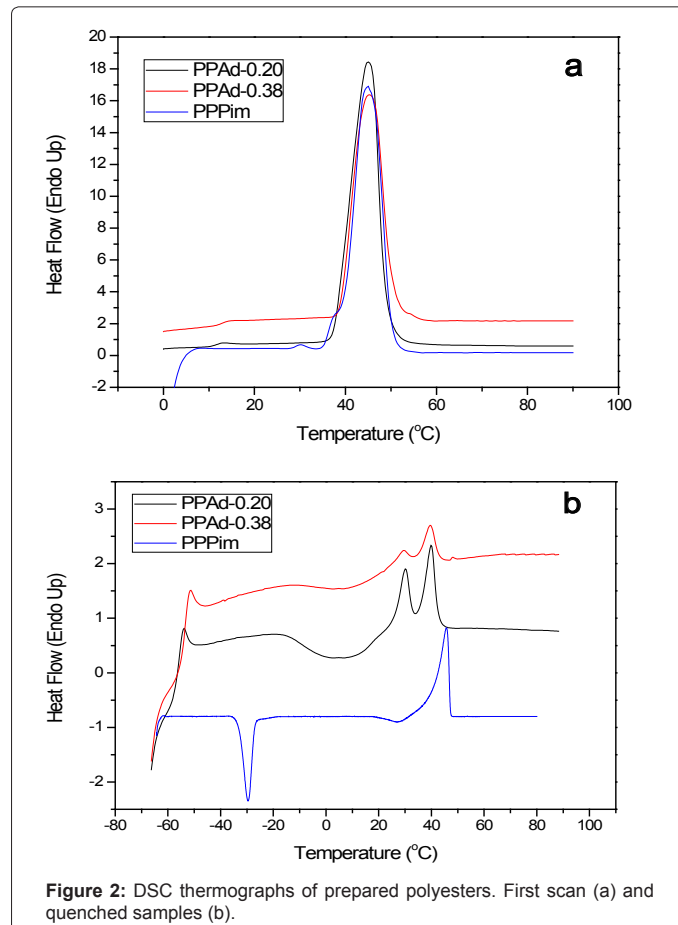

Figure 2: DSC thermographs of prepared polyesters. First scan (a) and quenched samples (b).

Results and Discussion

Polymer characterization

Aliphatic polyesters derived from the reaction of 1,3-propanediol and different dicarboxylic acids were synthesized and fully characterized in a previous study [31]. Similar polyesters, like poly(propylene dicarboxylates) have been used earlier in order to prepare nanoparticles loaded with a water soluble drugs [23]. These studies showed that the melting point of the used polyesters was a critical parameter concerning drug dissolution behavior. In the present study similar polyesters like were chosen for nanoencapsulation of paclitaxel and their effectiveness as thermosensitive carriers was evaluated. Results concerning polyester intrinsic viscosities, molecular weights, thermal properties and degree of crystallinity are presented in Table 1. The prepared PPAd polyesters had different intrinsic viscosities 0.22 and 0.38dL/g, and thus different molecular weights. PPPim has higher molecular weight than PPAd but much lower degree of crystallinity.

As it can be seen from Table 1 the synthesized polyesters have similar melting points, which range from 44.8 to 45.2°C. Furthermore, DSC thermographs in Figure 2 showed that the examined polyesters started to melt at around 37°C After melting in DSC, the samples were rapidly cooled down to -65°C and a second scan was performed in the quenched samples in order to record the glass transition and cold-crystallization (Tcc) of the amorphous polyesters. PPPim showed a lower glass transition temperature (Tg) (-64.3°C), while PPAd-0.38 showed the highest (-51.4°C). However, these small differences are not expected to affect the paclitaxel release behaviour. In order to record the cold-crystallization of the quenched polyesters slow heating rate was used in the second scan (2.5°C/min). Thermographs revealed that

Sample	[η] (dL/g)	M_n (Da)	M_w/M_n	T_m (°C)	T_g (°C)	T_{cc} (°C)	ΔH_m (J/g)	X_c* (%)
PPAd-0.20	0.22	5000	2.06	45.0	-53.9	3.0	48.1	52.3
PPAd-0.38	0.38	9000	2.25	45.2	-51.4	2.8	47.3	48,7
PPPim	0.71	19 000	2.22	44.8	-64.3	-29.8	53.7	29.1

Xc* WAXD measurement

Table 1: Intrinsic viscosity [η], average molecular weight (M_n), melting point (T_m), glass transition temperature (T_g), cold-crystallization temperature (T_{cc}), heat of fusion (ΔH_m) and degree of crystallinty (X_c) for the aliphatic polyesters used in this study
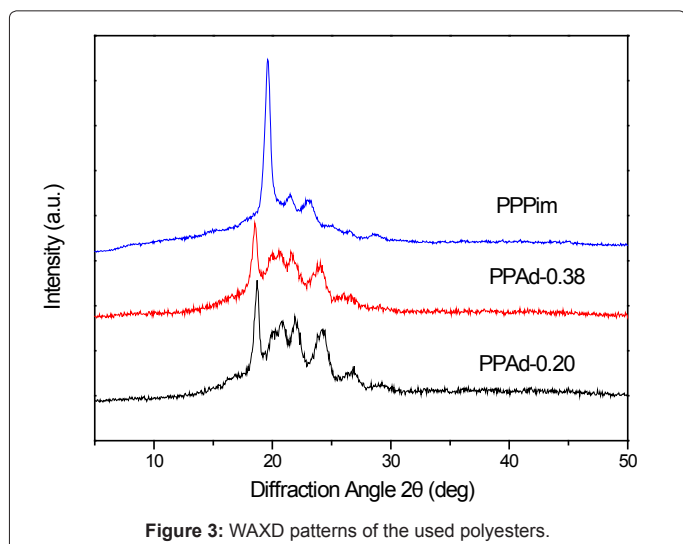

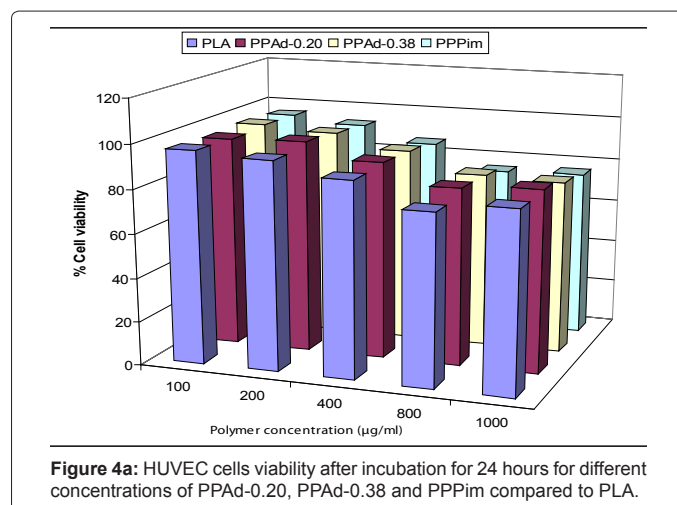**Figure 3:** WAXD patterns of the used polyesters.

Figure 4a: HUVEC cells viability after incubation for 24 hours for different concentrations of PPAd-0.20, PPAd-0.38 and PPPim compared to PLA.

PPAd polymers crystallize at about 3.0°C while PPPim, which had higher chain flexibility, showed a cold crystallization temperature at -29.8°C.

DSC analysis revealed only small variations in melting points and glass transition temperatures for the selected aliphatic polyesters. However, important differences were observed in the degree of crystallinity. PPPim had the lowest degree of crystallinity (29.1%) while PPAd-0.20 had the highest (52.3%). PPAd-0.38 which had higher molecular weight than PPAd-0.20 showed slightly lower degree of crystallinity, which is in accordance with literature [32-34]. Differences in the degree of crystallinity, also affect drug release behavior [24]. Degree of crystallinity was calculated from WAXD patterns shown in Figure 3 using the relative areas under the crystalline peaks, Ac, and the amorphous background, Aam, using the equation (4) according to Lu and Hay [35]. Results are summarized in Table 1.

$$X_c = \left(1 + \frac{A_{am}}{A_c}\right)^{-1} \tag{4}$$

In vitro cytotoxicity of aliphatic polyesters

Although the prepared polyesters exhibit good thermosensitive characteristics in order to be able to be used as anticancer drug delivery systems, they should exhibit also low cytotoxicity. Figure 4a demonstrates the HUVEC cells viability after incubation for 24 hours for both PPAd and PPPim polyesters. Results showed that both polyesters exhibited low toxicity against HUVEC cells, with appreciable cytotoxicity (higher than 20% reduction of cell viability) being observed only after exposing the cells at high nanoparticle concentrations, i.e. higher than 800 μg/ml. Based on polymer toxicity using HUVEC cells, the biocompatibility of polyesters was comparable to that of PLA, which is a polymer of high biocompatibility and is widely used in biomedical applications [26]

Nanoparticle characterization

The paclitaxel loaded nanoparticles where characterized with multiple methods in order to determine the physical parameters which can affect the performance of these systems during the in vitro dissolution studies. Nanoparticle yield, drug loading content, entrapment efficiency, as well as nanoparticle size are characteristics which are related with the preparation method and the physical and chemical properties of the used materials. Several factors may affect
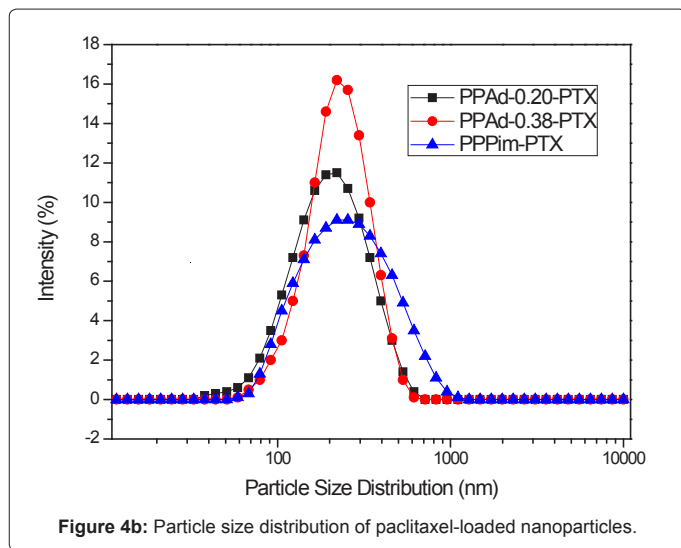
Figure 4b: Particle size distribution of paclitaxel-loaded nanoparticles.

Polyester	Nanoparticle yield (%)	Drug loading content (%)	Entrapment efficiency (%)	Mean Particle size (nm)	Polydispersity index (pdi)
PPAd-0.20	62±3	6.7±0.3	46±3	162±2	0.209
PPAd-0.38	76±2	8.1±0.4	65±2	168±2	0.126
PPPim	69±3	8.5±0.3	66±3	189±5	0.236

Table 2: Yield, drug loading content and entrapment efficiency of the nanoparticles prepared with the used polyesters (all measurements were performed in triplicate).

these parameters like the hydrophobicity of the polymer matrix, drug solubility in water, drug-drug interaction etc. [36,37]. As can be seen from Table 2 nanoparticle yield is very high, ranging from 62 to 76%, depending mainly on the used method of preparation and not on polymer characteristics. Furthermore, paclitaxel which is a lypophilic API is expected to be entrapped easily within the nanoparticles. Generally, estimated drug loading values are satisfactory in all polyesters, and close to the theoretical drug loading content (9.1%). Results also showed that drug loading content and entrapment efficiency increased by increasing the molecular weight of the used polyester. PPPim which, had the highest molecular weight, showed the highest drug loading and entrapment efficiency.

The mean particle size of nanoparticles, as well as their distribution, was measured by light scattering (Figure 4b). The particle size of the prepared nanoparticles is one of the most important parameters since it can affect the drug release, the physical stability and the cellular uptake [29]. Especially for the purpose of this study it was desired to develop nanoparticulate systems with a mean particle size above 100nm, in order to take advantage of the physical changes which occur in the tumor tissue when they are topically heated. According to reported research, the vascular permeability of the tumor is enhanced during the hyperthermia while normal tissues remain unaffected [3,20]. Therefore for nanoparticles with particle size above 100 nm the entrance in normal cells should be limited compared to cancer cells, reducing the side effects and increasing the effectiveness of the chemotherapy. As can be seen in Figure 4 the prepared nanoparticles show a unimodal size distribution for all polyesters. The mean nanoparticle diameter varied from 160 to 190 nm, which is desired for the application in targeted delivery of paclitaxel. Nanoparticles with the same particle sizes were also observed in a previous study using similar aliphatic polyesters for the encapsulation of Ropinirole HCl [24]. In the present

study it was found that increasing molecular weight of polyesters led to increasing mean particle size of nanoparticles, which was in accordance with another study previously reported using PLGA [38]. PPPim which had the highest molecular weight showed the highest mean particle size (189 nm), while PPAd-0.20, (with the lowest molecular weight), showed also the lowest particle size. However, it is expected that the
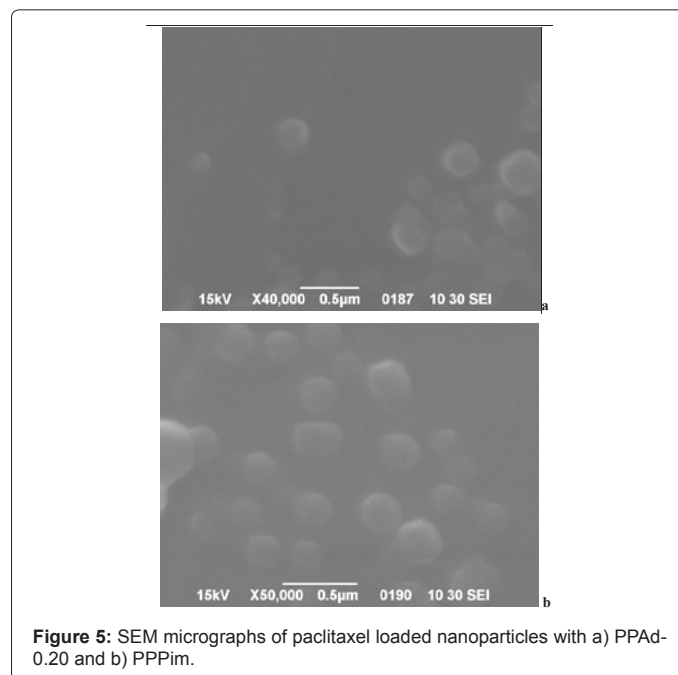

Figure 5: SEM micrographs of paclitaxel loaded nanoparticles with a) PPAd-0.20 and b) PPPim.
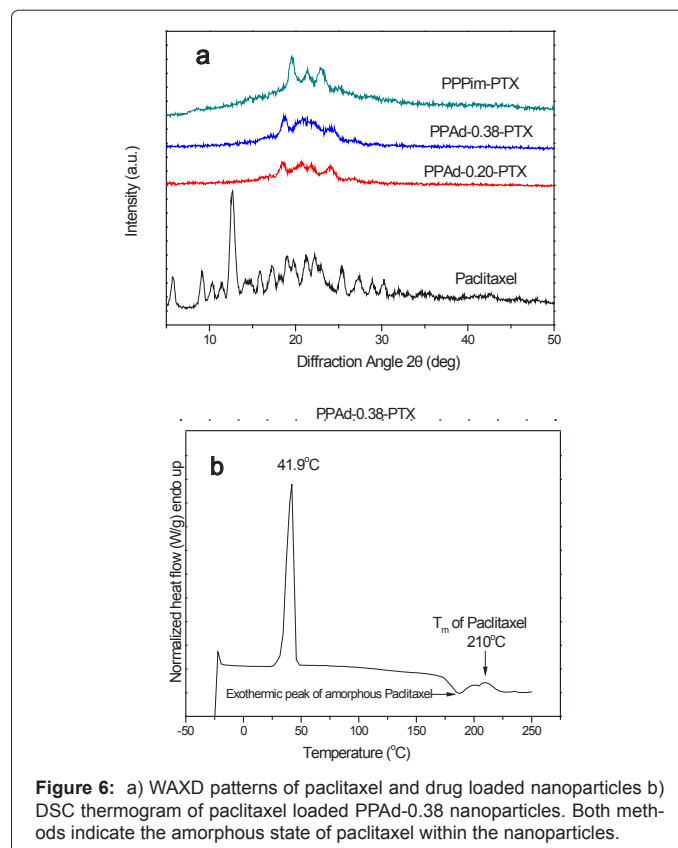

Figure 6: a) WAXD patterns of paclitaxel and drug loaded nanoparticles b) DSC thermogram of paclitaxel loaded PPAd-0.38 nanoparticles. Both methods indicate the amorphous state of paclitaxel within the nanoparticles.

above small differences in the mean particle size (20-30 nm) would not have a serious effect on paclitaxel release.

The shape and particle size of the prepared aliphatic polyester nanoparticles loaded with paclitaxel are also studied with SEM. The collected micrographs are shown in Figure 5. As can be seen the drug-loaded nanoparticles in both polyesters have a discrete spherical shape with sizes ranging from 70-80 up to 300-350 nm. These results are in accordance with the results obtained from DLS measurements. Some small differences are due to the different procedure used in both techniques. SEM analysis was used in samples after solvent evaporation and thus nanoparticles with smaller sizes could be detected. Similar results have been also reported previously [24,39,40].

In order to identify the physical state of the drug incorporated in the polymeric nanoparticles, WAXD and DSC measurements were performed. The WAXD patterns of pure paclitaxel and drug-loaded nanoparticles are presented in Figure 6a. PPAd showed several characteristic peaks at 2θ 18.65, 20.81, 21.89, 24.15 and 26.75º while PPPim at 19.61, 21.41, 23.01 and 25.30º (Figure 3). WAXD pattern of pure paclitaxel showed a large number of sharp diffraction peaks and according to the literature its crystalline form is the hydrated one which can be formed at ambient temperature and relative humidity above 43% [29]. Hence, WAXD analysis in all prepared nanoparticles showed that the drug was in amorphous state. Also the patterns of nanoparticles were identical to that of the pure polyesters, indicating that the nanoencapsulation of paclitaxel into the polyester nanoparticles does not change their crystal form. Furthermore, DSC thermograph of the prepared nanoparticles showed an exothermic peak at 180ºC (Figure 6b), which, according to Liggins et al. [29], is attributed to the

crystallization of the amorphous paclitaxel. At a higher temperature a small endothermic peak recorded at 210ºC is atributed to the melting point of the formed drug crystals.

In-vitro drug release

As showed from the previous analysis, paclitaxel was successfully entrapped in the prepared biocompatible aliphatic polyesters, which had similar melting points (near to human body) but different degrees of crystallinity and molecular weights. The resulted paclitaxel loaded nanoparticles are designed for intravenous administration, therefore their dissolution behavior was studied in pH 7.4. The measured particle size of prepared nanoparticles was found appropriate for cellular uptake, especially in the case of tumour cells due to the enhanced vascular permeability during mild hyperthermia. Therefore, the dissolution behavior of the prepared nanoparticles was studied in pH 6.0 as well, in order to simulate the second stage of the nanoparticles route through the endosomes. The results of the dissolution studies are presented in Figure 7. Dissolution profile analysis showed that in all used polyesters and at different studied conditions (temperature and pH) the release behaviour of paclitaxel was similar. Initially, in all samples, there is a burst effect since high amounts of drug are released within the first hour. This behaviour was observed in almost all nanoparticles and was attributed to the API located in the surface and the outer area of nanoparticles (initial diffusion step). After that time sustained release profiles were observed , especially in pH 7.4.

Comparative analysis of the dissolution behaviour in all samples showed that the paclitaxel released was higher at 42ºC than 37ºC. This was due to the fact that as the dissolution temperature increases (42ºC) the carrier becomes softer since the most of the crystalline structure is destroyed and larger amounts of macromolecules are in the melt state. Thus, the drug is released easier [41]. The temperature effect is more detectable in PPAd samples. Furthermore, comparing the dissolution results at 37ºC and 42ºC it was found that PPPim showed the highest dissolution rates in both pHs, while the two PPAd polymers showed the lowest rates. This was attributed to PPPim's lower degree of crystallinity, compared with PPAd. Hence, PPPim (with low degree of crystallinity) shows the highest % dissolved API in 72h at 37ºC (88%), while PPAd-0.20 and PPAd-0.38 released the 46.8% and 45% of the API after 72h at 37ºC, respectively. Thus, the degree of crystallinity plays also an important role in paclitaxel release behaviour from the prepared nanoparticles. This is in accordance with our previous findings where the dissolution was increased by decreasing the degree of crystallinity [24].

Furthermore, molecular weight variations and variations in particle size of prepared polyesters may also affect the release rates of paclitaxel. According to a previously published study, it was found that drug release decreased by increasing particle size of the nanoparticles [42]. However, PPPim which has the highest molecular weight and thus nanoparticles with high particle sizes were prepared compared with PPAd samples, showed the highest release rates in both pHs. It is believed that this was due to the greater impact of different degrees of crystallinity compared to the mean particle size. The high crystallinity of the PPAd matrix leads to reduced dissolution rates, as the lamellae acts as a barrier during drug diffusion. Bigger and more perfectly shaped crystalline lamellae should reduce the overall release. Hence, the diffusion of an active substance through the amorphous matrix is easier due to the higher mobility of polyesters macromolecular chains in the amorphous state, and thus easier penetration of the water through them and, consequently, a faster drug release [43-45]. However, this high release in anticancer drugs is not always the desired
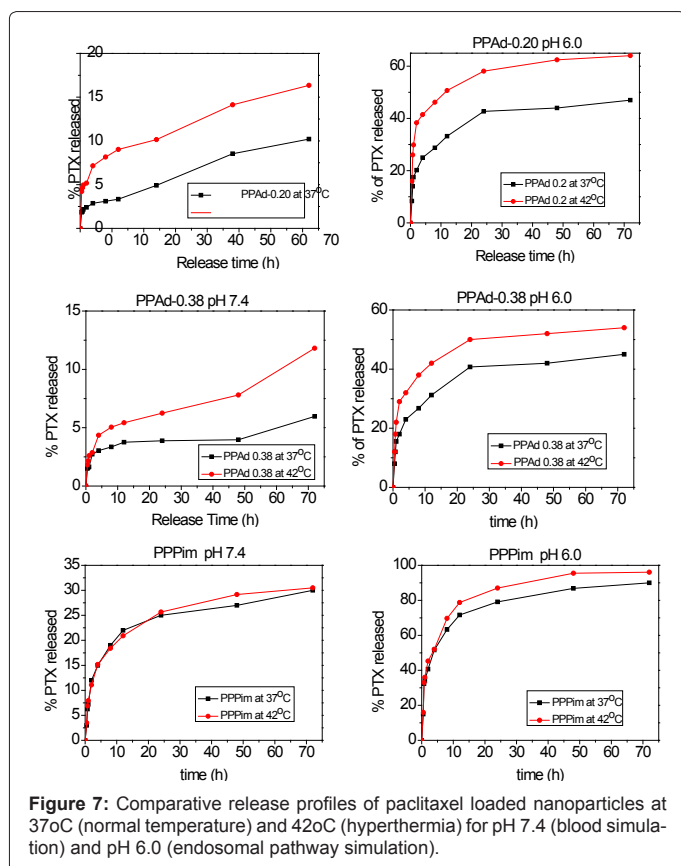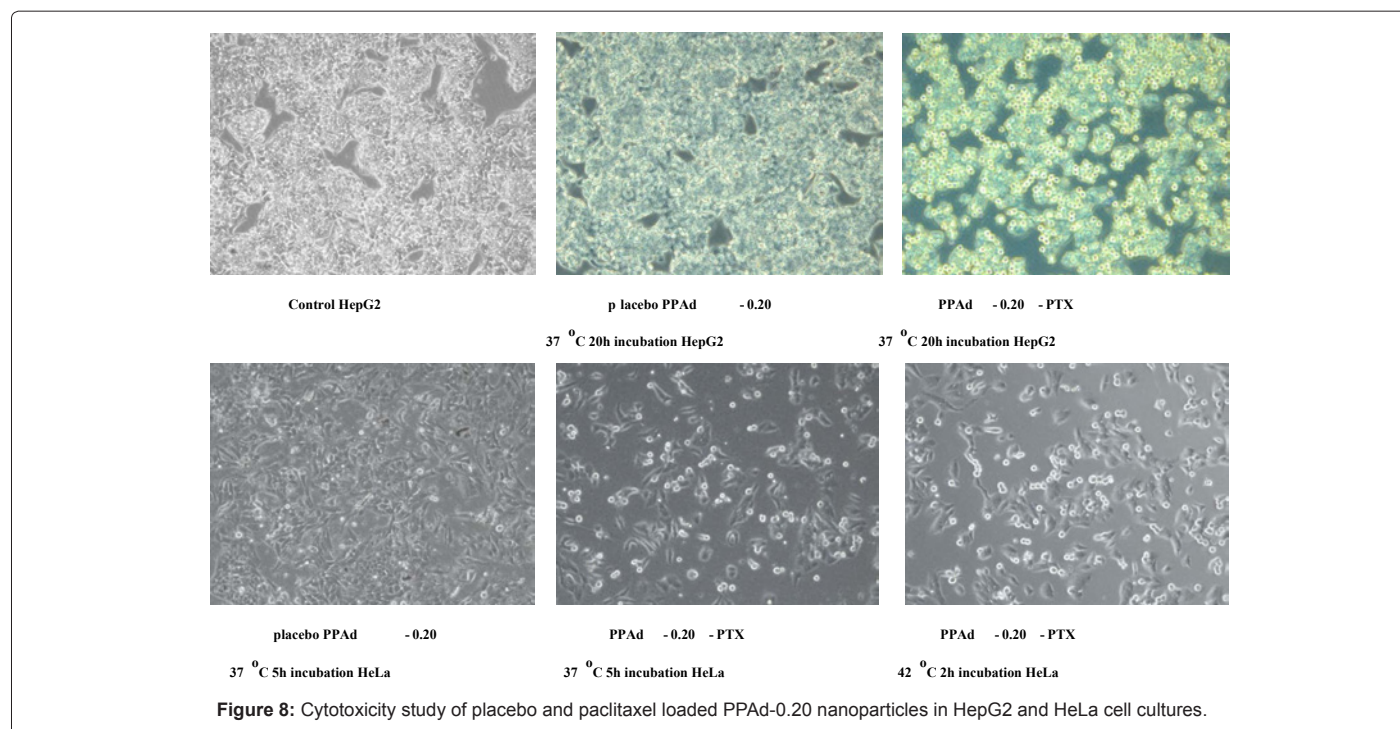

Figure 7: Comparative release profiles of paclitaxel loaded nanoparticles at 37ºC (normal temperature) and 42ºC (hyperthermia) for pH 7.4 (blood simulation) and pH 6.0 (endosomal pathway simulation).

Figure 8: Cytotoxicity study of placebo and paclitaxel loaded PPAd-0.20 nanoparticles in HepG2 and HeLa cell cultures.

behaviour and slower rates are appropriate [46]. Hence, it is realized that in order to achieve a desirable release profile by thermosensitivity using aliphatic polyesters, polymers with high degree of crystallinity like PPAd polyesters should be used. These samples exhibit lower release rates at normal body temperature conditions but show higher response and increased API release at higher temperatures which are locally applicated during chemotherapy in combination with mild hyperthermia.

Figure 7, also showed that paclitaxel exhibits higher release rates at pH 6.0 than pH 7.4. Analytically, the maximum release of paclitaxel from PPAd-0.20 nanoparticles at 42°C and pH 7.4 was almost 16% (after 72h) while at the same conditions for pH 6 was over 60%. The same behaviour was observed for both PPAd-0.38 and PPPim in both temperatures.

The pH dependant dissolution behavior of the paclitaxel loaded nanoparticles could not be attributed to the used API, as it is widely reported that pH variations affect only the stability of the API and not it's solubility [26-29]. This is the first time that a pH dependant variation in dissolution rates is been documented for aliphatic polyesters. A possible explanation is that in slightly acidic conditions the hydrolysis of the aliphatic polyesters is accelerated [47]. Furthermore, pH responsive nanoparticles based on PLA were recently reported [18]. Finally, it is important to note that the dissolution release mechanism form the prepared nanoparticles was a combination of diffusion and slight erosion of the polymer matrix.

In vitro cytotoxicity study in cancer cells

The cytotoxicity of PPAd-0.20 paclitaxel loaded nanoparticles were studied in comparison with control and the relevant placebo sample at cell culture medium incubated at 37 and 42°C. As shown in Figure 8 the effect of the nanoparticles is higher on the HeLa cells compared to HepG2 cells indicating that paclitaxel is maybe more effective in HeLa cells. In both cases the treated cells obtain a spherical shape and detach

from the well plates. About 50 % of the HepG2 cells were found to be in the phase of necrosis or apoptosis after 20h of incubation in both temperatures 37 and 42°C. Thermosenisivity was shown for PPAd-0.20 paclitaxel loaded nanoparticles since over 80% of the HeLa cells were found to be in the phase of necrosis after only 2h for the case of 42°C and after 5h for the case of 37°C. Furthermore taking into account that the release of paclitaxel from PPAd-0.20 nanoparticles is around 7% in 42°C after 2 hours in the dissolution medium (Figure 7) probably this effect shown in Figure 8 is caused by very low concentration of paclitaxel (below 50nM) in the culture medium, indicating that these nanoparticulate systems could be applicable in lower concentrations.

Conclusions

PPAd and PPPim aliphatic polyesters showed low cell toxicity and thus they could be used as drug delivery systems. Water-oil (w/o) emulsification and solvent evaporation techniques were appropriate for the preparation of paclitaxel/polyester nanoparticles with spherical sizes ranging from 160 to 190nm, satisfactory drug loading content and high entrapment efficiency. WAXD analysis showed that paclitaxel was in amorphous form within the nanoparticles.

In vitro dissolution studies showed that paclitaxel release is mainly depended on the experimental temperature, the degree of crystallinity for the used polyesters and the pH of dissolution medium. Drug release was higher at 42°C compared to 37°C since in the first case the temperature was closer to the melting point of the tested polyesters and thus the macromolecular chains are more flexible. Furthermore, as the degree of crystallinity increases the drug release decreases. It was also shown that the used polyesters present higher drug dissolution rate in pH 6.0 compared to pH 7.4. This was attributed to the increased hydrolysis of the polymer's ester bond in slightly acidic conditions. The release mechanism of paclitaxel from the prepared nanoparticles in pH 6.0 was a combination of diffusion and erosion, while in pH 7.4 the release was mainly controlled through diffusion.

The in vitro cytotoxicity studies for the prepared nanoparticles in HepG2 and HeLa cancer cell lines proved that they are effective in low drug concentrations and that effect can be accelerated in higher temperatures. Furthermore, it was found that the effect of paclitaxel/polyester nanoparticles is higher on the HeLa cells compared to HepG2 cells.

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Disclosure

The authors have no conflicts of interest to report in this work.

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