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Paclitaxel-Loaded Microparticles for Intratumoral Administration via the TMT Technique: Preparation, Characterization, and Preliminary Antitumoral Evaluation

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In our pursuit to develop suitable therapeutic particulate systems for intratumoral delivery by the targeted multi-therapy (TMT) technique, we describe the preparation of paclitaxel-loaded poly(D,L-lactic-co-glycolic) acid (PLGA) microparticles (MPs) (drug loading 35–38%, wt/wt; size 0.7–5 μm). Magnetite (15%, wt/wt) was also incorporated in some preparations for a future magnetic resonance imaging (MRI)-guided delivery. X-ray diffraction (XRD) and differential scanning calorimetry (DSC) experiments showed that paclitaxel was not encapsulated in its initial crystalline form. The paclitaxel in vitro release pattern showed a biphasic tendency with a burst effect followed by a sustained release (28% released amount after 1 month), which was accompanied with MP erosion and degradation signs as confirmed by scanning electronic microscopy (SEM) micrographs. The paclitaxel-loaded MPs demonstrated a dose-dependent antitumor effect on human uterine cancer cells, with an IC_{50} value relatively close to that of commercial Taxol®. This paclitaxel delivery system represents a potent antiproliferative and radiosensitizer agent for intratumoral administration via the TMT technique.

Keywords paclitaxel; PLGA; solid tumor; TMT; microparticle; release

INTRODUCTION

Paclitaxel is a potent anticancer agent with well-established antiproliferative, antimetastatic, and antiangiogenic properties (Huang et al., 2002; Sindermann et al., 2002; Stearns & Wang, 1992). It acts by a microtubule stabilizing mechanism and selectively blocks cells in the two most radiosensitive cell cycle phases: G_2 and M (Dhanikula & Panchagnula, 1999). Therefore, several investigations concluded that paclitaxel has a radiosensitizing effect (Steren et al., 1993; Tishler, Schiff, Geard, & Hall, 1992). This useful effect has been proposed to surmount the failure of standard radiotherapy regimens and their inability to control the tumor evolution which is partially attributed to radioresistant hypoxic cells within solid tumors (Milas et al., 1999).

Clinical studies investigating the potential of paclitaxel have mostly focused on systemic administration of the drug formulated in a vehicle of Cremophor® EL, a mixture of 50% alcohol and 50% polyethoxylated castor oil. These studies reported complications due to paclitaxel side effects on normal rapidly dividing cells (bone marrow and oral mucosa) and hypersensitivity reactions at the injection site induced by the vehicle (He, Wang, & Zhang, 2003; Rowinsky et al., 1989). In this context, localized chemotherapy via intratumoral drug administration becomes a method of first choice to improve paclitaxel delivery to solid tumors. A delivery system loaded with paclitaxel at tumor resection site can provide high and sufficient local drug levels in a defined release manner to eradicate malignant cells that may have survived surgery and conventional chemotherapy and furthermore, more particularly, to radiosensitize radioresistant cells withstanding conventional radiotherapy.

Recently, we have described the elaboration of radioactive Rhenium-186-, Rhenium-188-, and Holmium-166-loaded nanoparticles (Hamoudeh, Salim, Barbos, Paunoiu, & Fessi, 2007c; Hamoudeh, Fessi, Salim, & Barbos, 2007a) for intratumoral radiotherapy via a novel multimodal administration technique, invented by the group of Dr. Henri Mehier (Cerma, France), called targeted multi-therapy (TMT) (Hiltbrand et al., 2003;

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Roux et al., 2006). The first purpose of this technique is to treat solid tumors via thermoablation by locally applying hot vaporized water at 400°C under high pressure, attainable via a hydropneumatic pump. The pulses of hot water vapor are injected through a microtube perforated with narrow halls of around 50 μm diameter at its distal end which is administered in the tumor (Hiltbrand et al., 2003). The efficacy of TMT technique in inducing thermonecrosis has already been proven for treatment of cancers in animal models (Hiltbrand et al., 2003, 2004).

Our overall planned objective is to use this TMT technique as an administration method for the local treatment of solid tumors, with a cocktail of therapies comprising, in a first step, the tumor thermoablation by water vapor; in the second step, beneficiation of paclitaxel-loaded microparticles (MPs) due to paclitaxel anticancer properties; and in the third step, radiosensitization of radio resistant tumoral cells by using paclitaxel-loaded MPs before the administration of radioactive rhenium- or holmium-loaded nanoparticles. Furthermore, paclitaxel-loaded MP delivery can be guided by magnetic resonance imaging (MRI) if a suitable contrast agent as magnetite is simultaneously incorporated into the particles (Hamoudeh & Fessi, 2006; Hamoudeh & Fessi et al., 2007). We hypothesize that the combination of these three successive treatments by the same TMT administration technique would be more efficacious to achieve a total eradication of solid tumor.

Therefore, in thisarticle, we describe the preparation of paclitaxel-loaded MPs with small sizes suitable for the TMT technique. Paclitaxel was encapsulated in poly(D,L-lactic-co-glycolic) acid (PLGA) polymer by an emulsion-solvent evaporation method. These MPs were characterized by different methods including Fourier transformed infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), and X-ray diffraction (XRD). The paclitaxel release and MP degradation in vitro were investigated. Finally, the antitumoral effect of these MPs was evaluated in vitro on a human uterine sarcoma cell model.

MATERIALS AND METHODS

Materials

The PLGA polymer (Resomer RG 502H) was purchased from Boehringer-Ingelheim, Ingelheim Germany. Paclitaxel was supplied by Chengdu Kejie Hi-tech Development Co, Chengdu China. Polyvinyl alcohol (PVA, MW = 31 KDa, hydrolyzation degree = 88%), deuterated chloroform ($CDCl_3$), red nile, and potassium bromide were from Sigma Aldrich, Lyon France. Dichloromethane (DCM) and acetonitrile were from Laurylab, Lyon France. Nitric acid (65%) and sulfuric acid (95%) were purchased from Carlo-erba, Val de Reuil, France.

Paclitaxel-Loaded Microparticle Preparation

Paclitaxel-loaded MPs were prepared by an oil-in-water (O/W) simple emulsion-solvent evaporation method as described by Gupte and Ciftci (2004) with modification. Briefly, the O/W emulsion consisted of the following:

- Organic phase: Paclitaxel was mixed with the polymer in DCM.
- Aqueous phase: PVA was used as a stabilizer in the external aqueous phase at a concentration ranging from 1 to 3% (wt/vol). The organic phase was then added to the aqueous phase under mechanical stirring between 1,000 and 6,500 rpm for 5 min.

After the emulsion formation, DCM was evaporated by a rotative evaporator (R-144, Buchi, Essen Switzerland) at 100 rpm under vacuum. The prepared MPs were separated by ultracentrifugation (Beckman, Fullerton, CA, USA) and then washed with water for several times to eliminate the PVA excess. Finally, MPs were freeze-dried.

Magnetic Microparticle Preparation

Magnetic MPs were prepared using the same mentioned protocol except that oleic-acid-coated magnetite (magnetite = 65%, wt/wt), being synthesized as described in our previous work (Hamoudeh & Fessi, 2006), was added to the organic phase before emulsification.

Drug Encapsulation Efficiency

Paclitaxel loading in the polymeric MPs was assessed by high-performance liquid chromatography (HPLC). Briefly, 5 mg of freeze-dried loaded MPs was dissolved in 1 mL DCM and vortexed for 1 min. Then, 5 mL of acetonitrile:water (50:50 vol/vol) mixture was added and the all was vortexed for 2 min. DCM evaporation was accelerated by a short sonication (around 3 min) in an ultrasonic bath at 35°C. After a clear solution has been obtained, the sample volume was completed to 30 mL by the acetonitrile:water (50:50 vol/vol) mixture. The mixture was analyzed using HPLC system. The drug encapsulation efficiency (EE%) was expressed as the percentage of the drug amount found in MPs to that initially used in formulation. The drug extraction from different MP batches, of the same preparation formula, was performed in triplicate.

In Vitro Release

Freeze-dried paclitaxel-loaded MPs (3 mg) were put in screw-capped tubes and dispersed in 50 mL phosphate buffer solution (PBS, pH 7.4). The tubes were put in an orbital shaker water bath and vibrated at 100 rpm at 37°C. At designated time intervals, the tubes were taken out and centrifuged at 20,000 rpm for 10 min. The supernatant containing the released drug was then vortexed with 10 mL of DCM for 5 min and left thereafter under magnetic stirring overnight. DCM inferior layer was then separated and vortexed with 10 mL of acetonitrile:water (50:50 vol/vol). DCM was evaporated as explained in the Section “Drug Encapsulation Efficiency” and the

volume was completed up to 25 mL. The released drug amount was determined by HPLC in triplicate.

High-Performance Liquid Chromatography Analysis

HPLC determination was carried out at room temperature in an HPLC unit (Thermosystems, inc., Lombard, IL, USA) consisting of a set of a Spectra System P1000XR pump, a Spectra System AS 300 autosampler, and a Spectra System UV 6000LP diode array detector. Twenty microliters of samples or calibration standards was injected directly into a C18 guard column, Kromasil (5 μm, 250 mm × 4.6 mm). The mobile phase was an acetonitrile:water (50:50 vol/vol) mixture and degassed with helium flow before use. The total run time was 25 min at a flow rate of 1 mL/min. The detection wavelength (λ_{max}) was 227 nm and the retention time of paclitaxel was 17 min. The assay was linear between 0 and 100 μg/mL with a correlation coefficient of .999. Each determination was carried out in triplicate.

Particle Size and Zeta Potential Determination

The size of paclitaxel-loaded particles was determined by dynamic light scattering using Zetasizer 3,000 HSa (Malvern, Worcestershire England) at 25°C. The zeta potential was determined in diluted particles suspensions. Each measurement (size or zeta potential) was performed in triplicate.

Scanning Electronic Microscopy

MPs were deposited on a metallic probe and then metallized with gold/palladium with a cathodic pulverizer (technics Hummer II, 6 V, 10 mA). Imaging was realized on a FEG Hitachi S800 SEM at an accelerating voltage of 15 kV.

X-Ray Diffraction

X-ray powder diffractometry analysis was carried out using Siemens D500 apparatus operating with Cu Kα X radiation, a voltage of 40 kV, and a current of 30 mA. The scans were conducted at a scanning rate of 1°/min in the 2θ range from 5 to 65°.

Fourier Transformed Infrared Spectroscopy

The MPs, the polymer, paclitaxel, and their physical mixture were characterized by infrared spectroscopy using a Unicam Mattson 5000 FTIR spectrometer at room temperature. The spectra were taken in KBr discs in the range of 4,500–400 cm^{-1}.

Nuclear Magnetic Resonance Characterization

^{1}H and ^{13}C NMR spectra were acquired on a Bruker DMX-300 SB spectrometer in $CDCl_3$ for the polymer, paclitaxel, and loaded MPs. In each analysis, about 25 mg of samples was placed in 5 mm NMR tubes and then dissolved in 2 mL of $CDCl_3$.

Differential Scanning Calorimetry

Thermal analysis was performed using a differential scanning calorimeter DSC TA 125 (TA instruments, Newcastle, DE, USA). About 10 mg of samples was introduced into aluminum pans and hermetically sealed. All samples were heated at a 10°C/min scanning rate between 25 and 250°C after a 5 min stabilization plate under nitrogen atmosphere. The instrument was calibrated with indium for melting point and enthalpy heat of melting heat.

Gel Permeation Chromatography

Polymer molecular weights were determined on a Waters GPC system equipped with an isocratic pump (Waters 515) operated at a flow rate of 1 mL/min with tetrahydrofuran (THF), an autosampler (Waters 717 plus), a column oven, and a refractive-index (RI) detector Model (Waters 410) with integrated temperature controller maintained at 35°C. Calibration was carried out using narrow distributed (ND)-polystyrene standards. The mobile phase was THF (HPLC grade). MPs samples were dissolved in THF. Chromatography was carried out after sample filtration through a 0.45-μm filter. Toluene (internal standard) was added to standards and samples as a flow rate corrector.

Thermogravimetric Analysis

The analysis was carried out on a TA 2950 (TA instruments). Samples were analyzed in closed platinum cups at a temperature range of 30–700°C (heating rate 10°C/min) in a nitrogen atmosphere (flux of 5 mL/min).

Cellular Uptake of Microparticles

The cellular uptake of PLGA-based particles was further studied using fluorescence microscopy. HeLa cells (cervical cancer) were grown on coverslips at a density of 1×10^5 for 24 h in a culture plate at 37°C. Cells were propagated in Dulbecco's minimum essential medium (DMEM, Cambrex, East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum (FBS, Cambrex, Cergy Pontoise, France), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen). The cells were then incubated for 24 h with the fluorescent particles (size between 0.7 and 1.5 μm) labeled with red nile, a hydrophobic fluorescent agent added in DCM during their preparation. The selected MPs concentration was relatively small (10 μg/mL) to enable better fluorescence visualization and to prevent medium saturation with MPs. Furthermore, a control that consisted of Hela cells without a treatment with MPs was used. After rinsing with PBS (pH 7.4) twice, the cells were fixed with formaldehyde (5% [vol/vol] in PBS) containing 2% sucrose, washed again with PBS

twice, and then permeabilized for 10 min in a PBS solution containing 1% Triton (Sigma Lyon). Coverslips were then washed at least three times with PBS and mounted using Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA). Cells were observed using fluorescence microscopy and photographed using a digital camera equipped with Zeiss fluorescence microscope.

In Vitro Antitumor Activity

Here, the MESSA human uterine sarcoma cells were chosen to evaluate the antitumor activity of paclitaxel-loaded MPs in comparison with commercial Taxol® (6 mg/mL). Cells were grown for 24 h at 37°C in 5% CO_2 in a culture medium of RPMI medium (RPMI, Gibco, Invitrogen) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen). Thereafter, 100 μL of cells (cell suspension at a density of 1×10^4) was added to wells in a 96-well plate and incubated 24 h to allow cell attachment. The cells were incubated with the paclitaxel-loaded MP suspension or Taxol® at paclitaxel concentrations ranging from 0 to 500 nM for 72 h at 37°C in 5% CO_2. Then, the medium was removed and the wells were washed with PBS for three times. Culture medium (90 μL) and 20 μL of MTT reagent (5 mg/mL in PBS) were added to the wells. After incubation for 3 h, the culture solution was removed, leaving the precipitate. About 100 μL of isopropanol/HCl (90/10) mixture was then added to the wells and the optical density (OD) of obtained solutions was measured by using a microplate reader at 492 nm. Cell viability was calculated using the following equation:

$$\text{Cell viability}\,(\%) = \left(\frac{Int_s}{Int_{\text{control}}}\right) 100$$

where Int_s is the OD of the cells incubated with the MPs suspension or Taxol® and Int_{control} is the OD of the cells incubated with the culture medium only. IC_{50}, the drug concentration at which inhibition of 50% cell growth is observed in comparison with that of the control, was calculated by the curve fitting of the cell viability data.

RESULTS AND DISCUSSION

Size, Zeta Potential, and Paclitaxel Encapsulation Efficiency Percentage

Figure 1 shows an SEM micrograph of obtained MPs at a stirring speed of 2,000 rpm and a PVA concentration of 2% (wt/vol). As it can be noticed from this figure, the MPs size was relatively polydisperse but ranged between 0.7 and 3 μm, a size range being adequate for the TMT technique administration with a microtube perforated with narrow halls of only 50 μm, as mentioned above. Hence, we have chosen to encapsulate paclitaxel in relatively small MPs ~1–5 μm) rather than larger MPs described elsewhere (Elkharraz et al., 2006; Jackson, Hung, Letchford, & Burt, 2007; Liggins & Burt, 2004) and obtained by emulsification-solvent evaporation similar method. Different TMT injection tests of particles were carried out in our laboratory and showed to efficaciously inject a particle suspension at a 25% (wt/vol) concentration in water, in agarose gel, or in vivo intramuscularly in a mice model (Hamoudeh et al., 2007 Hamoudeh et al., 2007a; Hamoudeh et al., 2007; Hamoudeh, Fessi, Mehier, AlFaraj, & Canet-Soulas, 2007b).

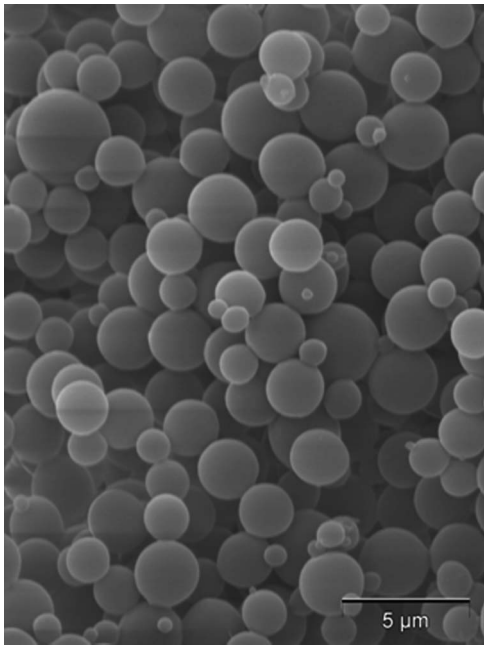

FIGURE 1. Scanning electronic microscopy (SEM) micrograph of paclitaxel-loaded microparticles (MPs) prepared at stirring speed of 2,000 rpm and 2% (wt/vol) of polyvinyl alcohol (PVA) (bar = 5 μm).

To prepare MPs with the most appropriate size range for the TMT administration technique, we investigated the influence of stirring speed and PVA percentage in the aqueous phase on the particle size. The prepared MP size showed to decrease in correlation with both the applied stirring speed (Figure 2) and PVA percentage in the aqueous phase (data not shown) in accordance with other studies (Dong & Feng, 2005; Elkharraz et al., 2006; Hamoudeh & Fessi, 2006; Seung-Jun et al., 2005).This can be explained by the fact that the higher the stirring speed, the smaller the dispersed organic droplets (Seung-Jun et al., 2005). In addition, the increase in PVA concentration renders the external aqueous phase more viscous, which magnifies the shear forces' influence on the organic phase and allows obtaining smaller droplets (Sahoo, Panyam, Prabha, & Labhasetwar, 2002).

The MPs zeta potential was found around –23 mV regardless of paclitaxel incorporation, suggesting that paclitaxel did not significantly contribute to the particle surface charge (Lee, Lim, & Kim, 2007).

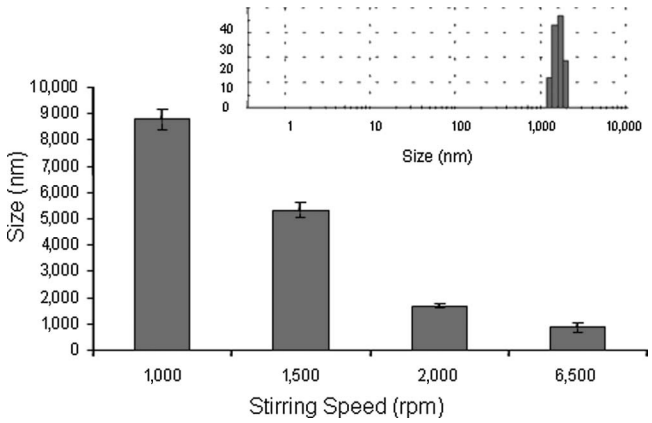

FIGURE 2. Microparticles (MPs) size distribution and the stirring speed effect on MPs size.

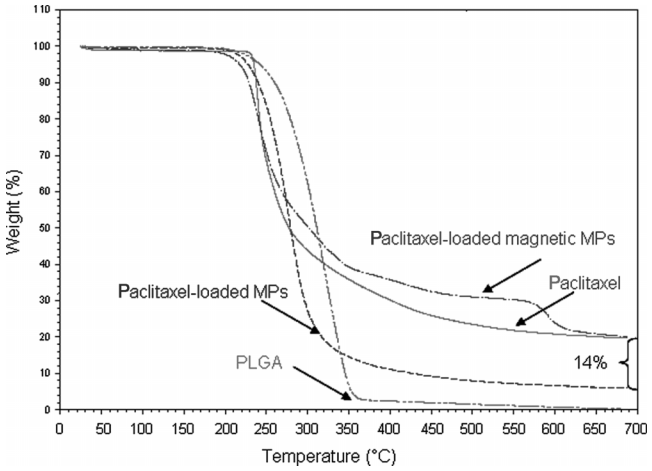

FIGURE 3. Thermogravimetric analysis (TGA) results of paclitaxel, poly(D,L-lactic-co-glycolic) acid (PLGA), paclitaxel-loaded microparticles (MPs), and paclitaxel-loaded magnetic MPs.

Thereafter, the paclitaxel encapsulation efficacy was found to be very high ranging between 88 and 95%, which yielded MPs loaded with around 35–38% (wt/wt) of paclitaxel. However, there was no clear effect of stirring speed and PVA percentage on the encapsulation efficacy despite insignificant small decrease of paclitaxel loadings in smaller MPs (data not shown). Elkharraz et al. (2006) attributed this paclitaxel loading decrease in smaller MPs to a decrease in the length of the diffusion pathways and, consequently, to an increase in drug loss in the external aqueous phase during MPs preparation. Furthermore, the decrease in particle size is accompanied by an increase in the MP surface area per unit of volume and by consequence a probable enhanced drug molecules loss by diffusion toward the external aqueous phase (Hamoudeh et al., 2007c).

Magnetite Loading

As it has been indicated in the Introduction, the incorporation of oleic-acid-coated magnetite was performed to render paclitaxel-loaded particles visualized by MRI to enable an imaging-guided delivery of these therapeutic particles by the TMT technique (Hamoudeh et al., 2007). To determine the magnetite amount in MPs, we performed thermogravimetric analysis (TGA) and measured the weight loss at 700°C (Figure 3). The weight losses in heated PLGA, paclitaxel, 37% (wt/wt) paclitaxel-loaded MPs, and 37% (wt/wt) paclitaxel-loaded magnetic MPs were 99.8, 80.3, 94.1, and 80.02%, respectively. The residual weight difference between paclitaxel-loaded magnetic MPs and paclitaxel-loaded MPs (here about 14%), being prepared at the same conditions apart from the magnetite addition, can be reasonably an indicative of the success of magnetite encapsulation within MPs. Along with the TGA results, inductively coupled plasma atomic emission spectrometry (ICP-AES) method results showed a magnetite loading of 15% (wt/wt). In accordance with previously published results by our group (Hamoudeh et al., 2006, 2007), these magnetic particles exhibit a superparamagnetic behavior and the obtained magnetite loading is well sufficient to yield MRI relaxivity properties of a T_2 contrast agent.

FTIR and NMR

Figure 4 demonstrates the FTIR transmittance spectra of PLGA, paclitaxel, their physical mixture, and the paclitaxel-loaded MPs. From the PLGA spectrum, the peaks of C–O–C stretching (1,080 cm^{-1}), C–H stretching in methyl groups (1,450 cm^{-1}), carbonyl (1,750 cm^{-1}), –CH, $–CH_2$, $–CH_3$ stretching vibrations (2,850–3,000 cm^{-1}), and OH stretching (3,300–3,500 cm^{-1}) are observed, in agreement with published data elsewhere (Loo, Ooi, & Boey, 2004; Mu & Feng, 2002). The FTIR spectrum of paclitaxel showed many characteristic peaks of paclitaxel including C–C stretching (709 cm^{-1}), –CH3 stretching (1,370 cm^{-1}), –CH2 scissoring (1,451 cm^{-1}), and C=O amide stretching (1,646 cm^{-1}) (Dhanikula & Panchagnula, 2004; Lee et al., 2001; Liu et al., 2004; Ouameur, Malonga, Neault, Diamantoglou, & Tajmir-Riahi, 2004). Among these paclitaxel peaks, those peaks at 709 cm^{-1} and 1,646 cm^{-1}, being not found in PLGA, were clearly distinct in loaded MPs confirming the success of paclitaxel incorporation. Interestingly, the spectrum of paclitaxel-loaded MPs appeared as the sum of both substances (their physical mixture).

On the contrary, the ^1H NMR spectra of PLGA showed characteristic signals at 5.2, 4.8, and 1.5 ppm, which are assigned to the methine hydrogen of the DL-lactide units, methylene hydrogen of the glycolide units, and methyl hydrogen of the DL-lactide units, respectively, in agreement with reported data in literature (Friess & Schlapp, 2006; Hrkach, Peracchia, Domb, Lotan, & Langer, 1997; Qiao, Chen, Ma, & Liu, 2005). The ^{13}C NMR spectra of PLGA showed characteristic peaks at 16.99 ppm (CH_3, DL-lactide units), 61.035 (CH_2, glycolide

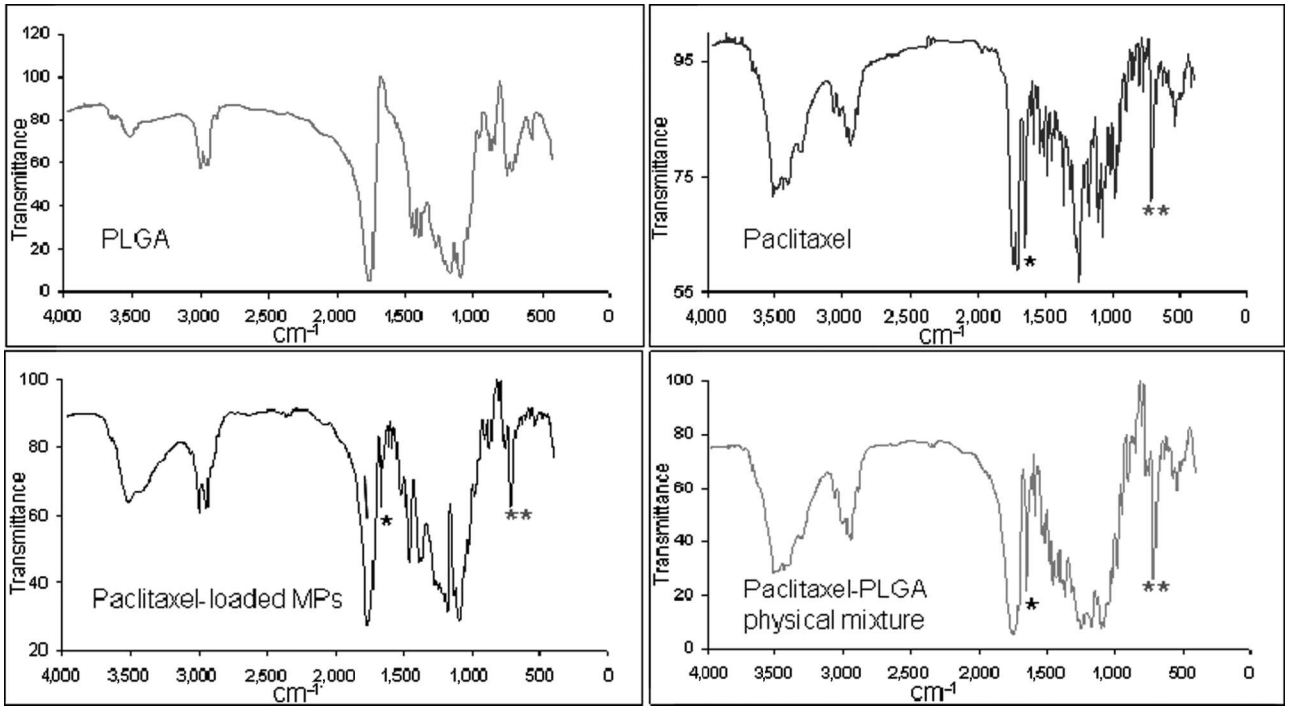

FIGURE 4. Fourier transformed infrared (FTIR) transmittance spectra of poly(D,L-lactic-co-glycolic) acid (PLGA), paclitaxel, a physical mixture, and paclitaxel-loaded MPs. (Notice the paclitaxel peaks present in MPs; ** at 709 cm^{-1} and * at 1,646 cm^{-1}).

units), 69.34 ppm (CH, DL-lactide), 166.69 (COO, glycolide units), and 169.695 ppm (COO, DL-lactide units) in accordance with the results of Dorta, Munguía, and Llabres (1993) and Hausberger and Deluca (1995). All these PLGA characteristic peaks were again noticed in the NMR loaded-MPs spectra. The ^{1}H and ^{13}C NMR spectra of neat paclitaxel showed its various characteristic peaks as described by Falzone, Benesi, and Lecomte (1992), Williams et al. (1993), and Chen, Ranade, and Xie (2005). No significant shifts of PLGA or paclitaxel happened in the paclitaxel-loaded MPs NMR spectra, which coincides with the FTIR results and indicates that perhaps only physical or nonbonding interactions might have been occurred during emulsification and subsequent particle solidification. This appears to be in agreement with the results of Chen et al. (2005), who did not notice significant interactions between paclitaxel and poly(styrene-isobutylene-styrene) in formulations containing both substances.

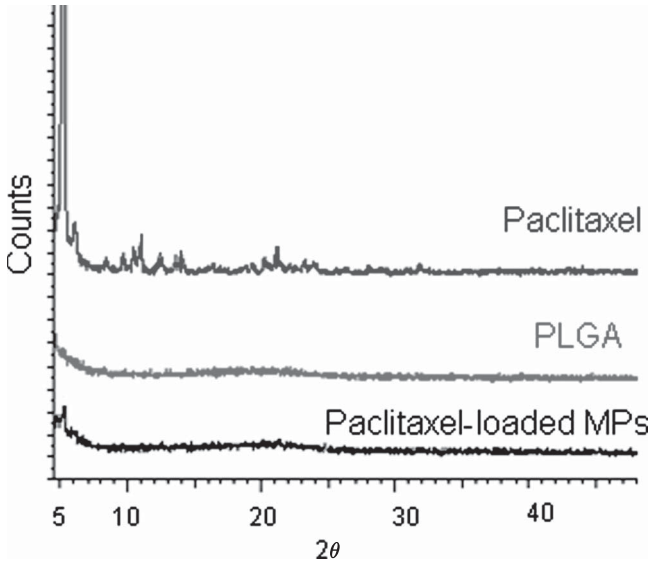

FIGURE 5. X-ray diffraction (XRD) spectra of poly(D,L-lactic-co-glycolic) acid (PLGA), paclitaxel, and paclitaxel-loaded microparticles.

XRD and DSC Results

XRD spectra of PLGA, paclitaxel, and paclitaxel-loaded MPs are shown in Figure 5. Paclitaxel exhibited several intense peaks at $2\theta = 5.6°$, 9.1°, 10.4°, 12.7°, and 21.1° in resemblance with published data (Huang, Chen, & Lee, 2007; Liu et al., 2004). However, these peaks were not recorded in the XRD patterns of paclitaxel-loaded MPs. Therefore, the diffractogram of the paclitaxel-loaded MPs indicated that the drug would be either molecularly dispersed in the polymers or distributed in an amorphous state in agreement with reported data elsewhere (Huang et al., 2007).

On the contrary, DSC is a very useful technique in the investigation of thermal properties of MPs providing both qualitative and quantitative data about the physicochemical

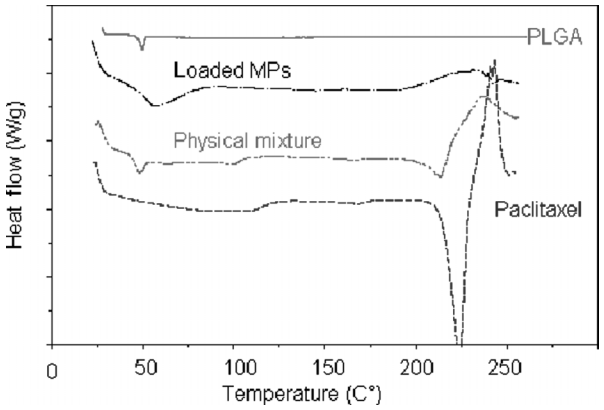

FIGURE 6. Differential scanning calorimetry (DSC) thermogram of paclitaxel, poly(D,L-lactic-co-glycolic) acid (PLGA), a physical mixture, and loaded microparticles (MPs).

state of the drug inside the polymer matrix (Dubernet, 1995). Figure 6 shows the DSC thermogram of paclitaxel, PLGA, a physical mixture, and loaded MPs. The pure paclitaxel showed an endothermic peak of melting at 223°C in accordance with Zhang and Feng (2006a). Although this paclitaxel melting peak remained in the paclitaxel–PLGA physical mixture, it disappeared in the loaded MPs. Indeed, different works reported the same findings that have been ascribed to an encapsulated drug in amorphous or disordered-crystalline phase of a molecular dispersion or a solid solution state in the polymer matrix after particle preparation (Gupte & Ciftci, 2004; Mu & Feng, 2001; Xie, Marijnissen, & Wang, 2006; Zhang & Feng, 2006a).

In Vitro Release

Figure 7 demonstrates the cumulative paclitaxel release pattern from MPs over 30 days. The drug release showed a biphasic pattern with a relatively fast release rate followed by a slow rate, which is in resemblance with reported data concerning

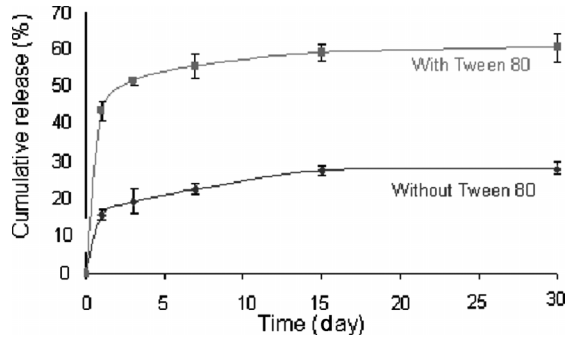

FIGURE 7. Cumulative paclitaxel release pattern from microparticles over 30 days.

paclitaxel-loaded PLGA nanoparticles (Feng & Huang, 2001; Fonseca, Simões, & Gaspar, 2002) and MPs (30–120 μm) (Jackson et al., 2007). Indeed, the obtained first burst effect in our study was less noticeable than in nanoparticles (Feng & Huang, 2001; Fonseca, Simões, & Gaspar, 2002) but higher than the one given by the clearly larger MPs (Jackson et al., 2007). This can be explained, in turn, by the fact that the smaller the particle diameter, the larger the particle-specific surface being in contact with the release medium and the easier becomes the paclitaxel hydrophobic molecule diffusion to this medium. The kinetic data showed a fast release rate in the first 3 days (around 19% of incorporated drug) followed by a slow continuous release afterwards during the 27 following days. The initial fast drug release can be ascribed again to drug molecules located on or near the particles surface, whereas the slow continuous release could be caused by diffusion of paclitaxel molecules inside the MPs. According to Westedt et al. (2007), the initial burst effect has been attributed to the fact that when hydrophobic paclitaxel (log P = 3.5) containing DCM phase is added to the PVA-containing aqueous phase, the paclitaxel might accumulate in the hydrophobic domains of the stabilizer molecules. Hence, the MPs solidify in the aqueous phase and the surfactant molecules become attached to the hydrophobic MPs surface, the drug is not able to diffuse back into the solid core of the nanoparticles (Muller, Maeder, & Gohla, 2000).

Furthermore, when Tween 80® was added to the release medium, the released amount was significantly higher with a burst effect reaching about 50% of incorporated drug. Indeed, the presence of Tween 80® renders the MP surface less hydrophobic enabling a cocktail of accelerated events to take place, including MPs wetting, swelling, and erosion. The morphologies of MPs before and after 7 days or 30 days of incubation with or without Tween 80®, respectively, are shown in Figure 8. Although MPs, without medium-added Tween 80®, showed clear signs of surface erosion from day 30, these signs appeared earlier and very clear after only 7 days in the presence of Tween 80®, which can explain again the difference in paclitaxel released amounts in the presence or absence of Tween 80®. Furthermore, this was accompanied with sharper MW decrease in the case of medium-added Tween 80® as the MW value decreased about 55–60% after 30 days of incubation, whereas this decrease was only 9–12% without Tween 80®.

We should keep in mind that upon administration of MPs in vivo, paclitaxel release profile is more similar to that shown without the addition of Tween 80®, and it can be expected that drug would be slowly and continuously released during several weeks. Therefore, talking about more likely physiological conditions without Tween 80® and similar additives, the burst release (here about 19%) is clinically desirable to achieve initial local high drug concentrations in the tumor. After the release of this loading dose, the following sustained drug release rate enables obtaining constant drug levels into the tumor.

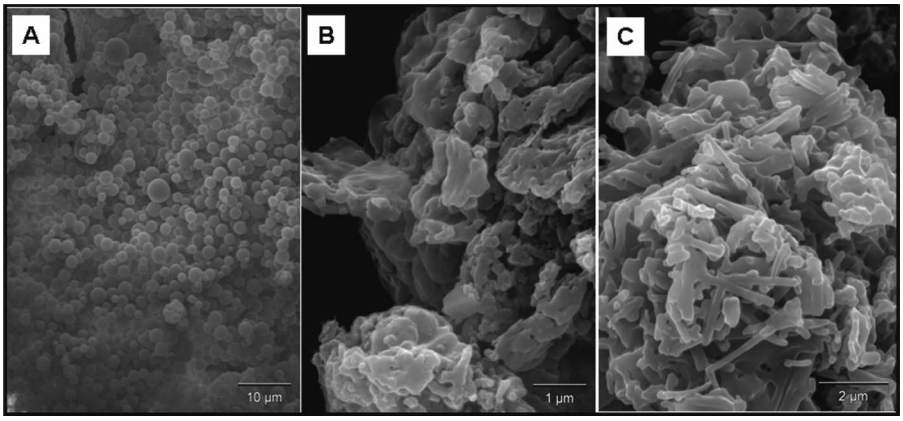

FIGURE 8. The morphologies of microparticles before (A) and after 30 days or 7 days of incubation without (B) or with Tween 80 (C).

However, the addition of Tween 80®, here, remains helpful in providing an appropriate shorter term test in vitro for controlled drug delivery systems like, for example, applying temperatures higher than 37°C (Du et al., 2006; Shameem, Lee, & DeLuca, 1999) to accelerate the release rate and also adding to the PBS buffer some agents not physiologically found in tumoral tissues such as *N*,*N*-diethylnicotinamide (DENA) for the release of paclitaxel from PLGA MPs Elkharraz et al. (2006). These authors found that paclitaxel release could increase obviously although highlighting the fact that this substance (DENA) cannot be found in physiological tumoral tissue and only applied to speed up the release kinetics and study different interfering phenomena in shorter time.

Experiments in Cells

These experiments included the evaluation of MP uptake by Hela cells and MPs' antitumor effect toward MESSA cells, two types of the female reproductive system cancer cells (cervix and uterus cancer, respectively).

Visual evidence of MPs' uptake by the HeLa cells after 24 h was obtained with fluorescence microscopy using MPs labeled with red nile (Figure 9) The MPs and cell nuclei appear red and blue, respectively. In this experiment we applied a relatively very small MP concentration (10 µg/mL), being clearly smaller than those reported in other papers (Evangelos, Gryparis, Evangelia, & Konstantinos, 2007; Jin, Bai, Wu, Tian, & Guo, 2007), to enable better visualization (dilution of MPs in the medium) and to avoid a killing effect due to high concentrations in culture medium. Control experiments without fluorescent MPs' addition showed no red fluorescence within the cells.

The antitumor activity of paclitaxel-loaded MPs was further evaluated against the MESSA human uterine sarcoma cells using MTT method (Xie et al., 2007; Zhang & Feng, 2006b). Figure 10 shows the cell viability after 72-h incubation as a function of paclitaxel concentration (nmol) used under the form of Taxol® or paclitaxel-loaded MPs. The drug-loaded MPs were sterilized by γ-radiation for a dose of 30 kGy. We can see from the figure that the loaded MPs demonstrate a cell-killing effect increasing with the incubated MPs (encapsulated drug amount), which is in accordance with other published data elsewhere (Zhang et al., 2005; Zhang & Feng, 2006b). A general and similar dose-depending killing effect was recorded for Taxol® but with more pronounced antitumoral

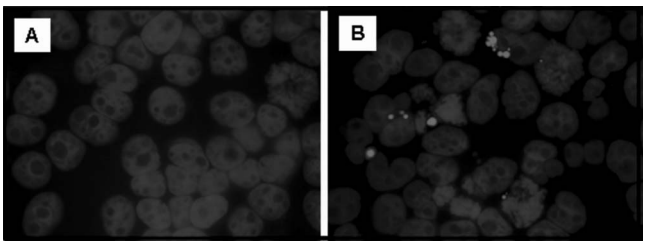

FIGURE 9. Fluorescence study in nontreated HeLa cells (A) and fluorescent microparticle (MP)-treated cells (B). Blue staining represents cell nuclei whereas red fluorescence comes from microparticles (MPs).

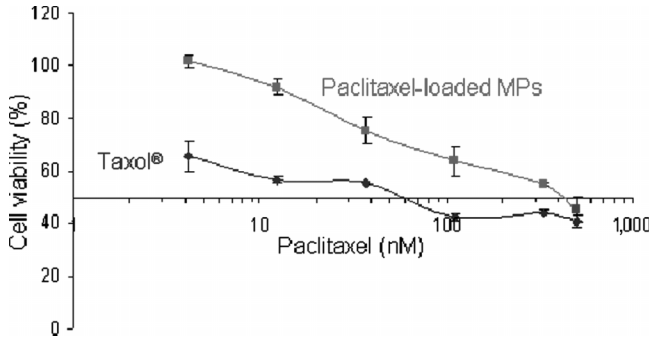

FIGURE 10. Antitumor effect of Taxol® and paclitaxel-loaded microparticles (MPs) at different concentrations.

effect (IC_{50} = 60 nmol), whereas for our MPs, IC_{50} was 400 nmol. However, at the maximum applied dose, here 500 nmol, the cell viability was found relatively the same in both paclitaxel formulations (Taxol®, 40.7% and loaded-MPs, 45.5%). In our opinion, the delayed and lower killing effect induced by MP-encapsulated paclitaxel compared with Taxol® at similar drug concentrations, which comes in accordance with some reported results elsewhere (Xie et al., 2007; Zhang et al., 2005), can be explained by the relative prolonged drug release rate as shown in the in vitro release study (Section "XRD and DSC Results").

CONCLUSION

This study describes the preparation of paclitaxel-loaded PLGA MPs for intratumoral administration with sizes between 0.7 and 5 μm, being adequate for the TMT technique. The paclitaxel loading was between 35 and 38% (wt/wt) with an encapsulation efficacy reaching around 90%. Oleic-acid-coated magnetite was also encapsulated in these paclitaxel-loaded MPs at a magnetite content of 15% (wt/wt) to enable obtaining MPs labeled with an MRI contrast agent to track their distribution after local injection into tumors. XRD and DSC experiments showed that paclitaxel was not encapsulated in its initial crystalline form. The paclitaxel in vitro release pattern showed a biphasic tendency with a burst effect followed by a sustained release (28% released amount after 1 month), which was accompanied with MP erosion and degradation signs as confirmed by SEM micrographs. The MPs' cellular uptake was showed in vitro by fluorescence microscopy. Furthermore, the paclitaxel-loaded MPs showed a dose-dependent antitumor effect on MESSA uterine cancer cells, with an IC_{50} value being close to that of commercial Taxol®. In summary, we think that these paclitaxel-loaded MPs represent a suitable adjuvant as an antiproliferative and radiosensitizer agent for intratumoral administration by the TMT technique.

REFERENCES

Chen, J. Z., Ranade, S. V., & Xie, X. Q. (2005). NMR characterization of paclitaxel/poly (styrene-isobutylene-styrene) formulations. *Int. J. Pharm., 305*, 129–144.

Dhanikula, A. B., & Panchagnula, R. (1999). Localized paclitaxel delivery. *Int. J. Pharm., 183*, 85–100.

Dhanikula, A. B., & Panchagnula, R. (2004). Development and characterization of biodegradable chitosan films for local delivery of paclitaxel. *AAPS J., 6*(3), Article 27.

Dong, Y., & Feng, S. S. (2005). Poly(d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs. *Biomaterials, 26*, 6068–6076.

Dorta, M., Murguía, O., & Llabres, M. (1993). Effects of polymerization variables on PLGA properties: Molecular weight, composition and chain structure. *Int. J. Pharm., 100*, 9–14.

Du, L., Cheng, J., Chi, Q., Qie, J., Liu, Y., & Mei, X. (2006). Biodegradable PLGA microspheres as a sustained release system for a new luteinizing hormone-releasing hormone. *Chem. Pharm. Bull., 54*, 1259.

Dubernet, C. (1995). Thermoanalysis of microspheres. *Thermochim. Acta, 248*, 259–269.

Elkharraz, K., Faisant, N., Guse, C., Siepmann, F., Arica-Yegin, B., Oger, J. M., Gust, R., Goepferich, A., Benoit, J. P., & Siepmann, J. (2006). Paclitaxel-loaded microparticles and implants for the treatment of brain cancer: Preparation and physicochemical characterization. *Int. J. Pharm., 314*, 127–136.

Evangelos, C., Gryparis, M. H., Evangelia P., & Konstantinos, A. (2007). Anticancer activity of cisplatin-loaded PLGA-mPEG nanoparticles on LNCaP prostate cancer cells. *Eur. J. Pharm. Biopharm., 67*, 1–8.

Falzone, C. J., Benesi, A. J., & Lecomte, J. T. J. (1992). Characterization of taxol in methylene chloride by NMR spectroscopy. *Tetrahedron Lett., 33*, 1169–1172.

Feng, S. S., & Huang, G. (2001). Effects of emulsifiers on the controlled release of paclitaxel (taxol®) from nanospheres of biodegradable polymers. *J. Control. Release, 71*, 53–69.

Fonseca, C., Simões, S., & Gaspar, R. (2002). Paclitaxel-loaded PLGA nanoparticles: Preparation, physicochemical characterization and in vitro antitumoral activity. *J. Control. Release, 83*, 273–286.

Friess, W., & Schlapp, M. (2006). Sterilization of gentamicin containing collagen/PLGA microparticle composites. *Eur. J. Pharm. Biopharm., 63*, 176–187.

Gupte, A., & Ciftci, K. (2004). Formulation and characterization of paclitaxel, 5-FU and paclitaxel+5-FU microspheres. *Int. J. Pharm., 276*, 93–106.

Hamoudeh, M., Al Faraj, A., Canet-Soulas, E., Bessueille, F., Léonard, D., & Fessi, H. (2007). Elaboration of PLLA-based superparamagnetic nanoparticles: Characterization, magnetic behaviour study and in vitro relaxivity evaluation. *Int. J. Pharm., 338*, 248–257.

Hamoudeh, M., & Fessi, H. (2006). Preparation, characterization and surface study of poly-epsilon caprolactone magnetic microparticles. *J. Colloid. Interface Sci., 300*, 584–590.

Hamoudeh, M., Fessi, H., Salim, H., & Barbos, D. (2007a). Holmium-loaded PLLA nanoparticles for intra-tumoral radiotherapy via the TMT technique: Preparation, characterization and stability evaluation after neutron irradiation. *Drug Dev. Ind. Pharm.* In Press.

Hamoudeh, M., Fessi, H., Mehier, H., AlFaraj, A., & Canet-Soulas, E. (2007b). Dirhenium decacarbonyl-loaded PLLA nanoparticles: Influence of neutron irradiation and preliminary in vivo administration by the TMT technique. *Int. J. Pharm., 348*, 125–136.

Hamoudeh, M., Salim, H., Barbos, D., Paunoiu, C., & Fessi, H. (2007c). Preparation and characterization of radioactive dirhenium decacarbonyl-loaded PLLA nanoparticles for radionuclide intra-tumoral therapy. *Eur. J. Pharm. Biopharm.*, doi:10.1016/j.ejpb.2007.04.003.

Hausberger, A. G., & DeLuca, P. P. (1995). Characterization of biodegradable poly(D, L-lactide-co-glycolide) polymers and microspheres. *J. Pharm. Biomed., 13*, 747–760.

He, L., Wang, G. L ., & Zhang, Q. (2003). An alternative paclitaxel microemulsion formulation: Hypersensitivity evaluation and pharmacokinetic profile. *Int. J. Pharm., 250*, 45–50.

Hiltbrand, E., Belenger, J., Binzoni, T., Buchegger, F., Costa, M., & Mehier, H. (2004). A new method of thermoablation with hot water vapour for localized tumours. *Anticancer Res., 24*, 2757–2763.

Hiltbrand, E., Belenger, J., Binzoni, T., Buchegger, F., Fessi, H., Costa, M., Quash, G., Foray, J., & Mehier, H. (2003). Thérapie focalisée par microinjections haute pression à l'aide d'un microtube implantable. *ITBM-RBM., 24*, 136–144.

Hrkach, J. S., Peracchia, M. T., Domb, A., Lotan, N., & Langer, R. (1997). Nanotechnology for biomaterials engineering: Structural characterization of amphiphilic polymeric nanoparticles by 1H NMR spectroscopy. *Biomaterials, 18*, 27–30.

Huang, C. Y., Chen, C. M., & Lee, Y. D. (2007). Synthesis of high loading and encapsulation efficient paclitaxel-loaded poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion. *Int. J. Pharm., 338*, 267–275.

Huang, S. F., Kim, S. J., Lee, A. T., Karashima, T., Bucana, C., Kedar, D., Sweeney, P., Mian, B., Fan, D., Shepherd, D., Fidler, I. J., Dinney, C. P., & Killion, J. J. (2002). Inhibition of growth and metastasis of orthotopic human prostate cancer in athymic mice by combination therapy with pegylated interferon-alpha-2b and docetaxel. *Cancer Res., 62*, 5720–5726.

Jackson, J. K., Hung, T., Letchford, K., & Burt, H. M. (2007). The characterization of paclitaxel-loaded microspheres manufactured from blends of poly(lactic-co-glycolic acid) (PLGA) and low molecular weight diblock copolymers. *Int. J. Pharm.*, doi, 10.1016/j.ijpharm, 022.

Jin, C., Bai, L., Wu, H., Tian, F., & Guo, G. (2007). Radiosensitization of paclitaxel, etanidazole and paclitaxel+etanidazole nanoparticles on hypoxic human tumor cells in vitro. *Biomaterials, 28*, 3724–3730.

Lee, J. H., Gi, U. S., Kim, J. H., Kim, Y., Kim, S. H., Oh, H., & Min, B. (2001). Preparation and characterization of solvent induced dihydrated, anhydrous, and amorphous paclitaxel. *Bull. Korean Chem. Soc., 22*, 925–928.

Lee, M. K., Lim, S. J., & Kim, C. K. (2007). Preparation, characterization and in vitro cytotoxicity of paclitaxel-loaded sterically stabilized solid lipid nanoparticles. *Biomaterials, 28*, 2137–2146.

Liggins, R. T., & Burt, H. M. (2004). Paclitaxel-loaded poly(L-lactic acid) microspheres 3: Blending low and high molecular weight polymers to control morphology and drug release. *Int. J. Pharm., 282*, 61–71.

Liu, Y., Chen, G. S., Chen, Y., Cao, D. X., Geb, Z. Q., & Yuan, Y. G. (2004). Inclusion complexes of paclitaxel and oligo(ethylenediamino) bridged bis(b-cyclodextrin)s: Solubilization and antitumor activity. *Bioorg. Med. Chem., 12*, 5767–5775.

Loo, S. C. J., Ooi, C. P., & Boey, Y. C. F. (2004). Radiation effects on poly(lactide-co-glycolide) (PLGA) and poly(L-lactide) (PLLA). *Polym. Degrad. Stab., 83*, 259–265.

Milas, L., Milas, M. M., & Mason, K. A. (1999). Combination of taxanes with radiation: Preclinical studies. *Semin. Radiat. Oncol., 9*, 12–26.

Mu, L., & Feng, S. S. (2001). Fabrication, characterization and in vitro release of paclitaxel (taxol®) loaded poly (lactic-co-glycolic acid) microspheres prepared by spray drying technique with lipid/cholesterol emulsifiers. *J. Control. Release, 76*, 239–254.

Mu, L., & Feng, S. S. (2002). Vitamin e TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (taxol®). *J. Control. Release, 80*, 129–144.

Muller, R. H., Maeder, K., & Gohla, S. (2000). Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. *Eur. J. Pharm. Biopharm., 50*, 161–177.

Ouameur, A. A., Malonga, H., Neault, J. F., Diamantoglou, S., & Tajmir-Riahi, H. A. (2004). Taxol interaction with DNA and RNA – stability and structural features. *Can. J. Chem., 82*, 1112–1118.

Qiao, M., Chen, D., Ma, X., & Liu, Y. (2005). Injectable biodegradable temperature-responsive PLGA–PEG–PLGA copolymers: Synthesis and effect of copolymer composition on the drug release from the copolymer-based hydrogels. *Int. J. Pharm., 294*, 103–112.

Roux, C., Rauber, N., Hiltbrand, E., Belenger, J., Khan, H., Dfouni, N., Michel, N., Knopf, J. F., Foray, J., & Mehier, H. (2006). Experimental study on a large animal model of a new thermoablation technique. *Anticancer Res., 26*, 1–8.

Rowinsky, E. K., Burke, P. J., Karp, J. E., Tucker, R. W., Ettinger, D. S., & Donehower, R. C. (1989). Phase I and pharmacodynamic study of taxol in refractory acute leukemias. *Cancer Res., 49*, 4640–4647.

Sahoo, S. K., Panyam, J., Prabha, S., & Labhasetwar, V. (2002). Residual polyvinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *J. Control. Release, 82*, 105–114.

Seung-Jun, L., Jong-Ryul, J., Sung-Chul, S., Jin-Chul, K., Young-Hwan, C., Kwon-Hyung, L., & Jong-Duk, K. (2005). Magnetic enhancement of iron oxide nanoparticles encapsulated with poly(d,l-latide-co-glycolide). *Colloids Surf. A, 255*, 19–25.

Shameem, M., Lee, H., & DeLuca, P. P. (1999). A short-term (accelerated release) approach to evaluate peptide release from PLGA deport formulations. *AAPS Pharmsci., 1* Article 7.

Sindermann, J. R., Skaletz-Rorowski, A., Bartels, A., Hohage, H., Plenz, G., Schmidt, A., & Breithardt, G. (2002). Paclitaxel and cyclosporine a show supra-additive antiproliferative effects on smooth muscle cells by activation of protein kinase C. *Basic. Res. Cardiol., 97*, 125–131.

Stearns, M. E., & Wang, M. (1992). Taxol blocks processes essential for prostate tumor cell (PC-3, ML) invasion and metastases. *Cancer Res., 52*, 3776–3781.

Steren, A., Sevin, B. U., Perras, J., Angioli, R., Nguyen, H., Guerra, L., Koechli, O., & Averette, H. E. (1993). Taxol sensitizes human ovarian cancer cells to radiation. *Gynecol. Oncol., 48*, 252–258.

Tishler, R. B., Schiff, P. B., Geard, C. R., & Hall, E. J. (1992). Taxol: A novel radiation sensitizer. *Int. J. Radiat. Oncol. Biol. Phys., 22*, 613–617.

Westedt, U., Kalinowski, M., Wittmar, M., Merdan, T., Unger, F., Fuchs, J., & SchällerBakowskyKissel, S. U. T. (2007). Poly(vinyl alcohol)-graft-poly(lactide-co-glycolide) nanoparticles for local delivery of paclitaxel for restenosis treatment. *J. Control. Release, 119*, 41–51.

Williams, H. J., Scott, A. I., Dieden, R. A., Swindell, C. S., Chirlian, L. E., Francl, M. M., Heerding, J. M., & Krauss, N. E. (1993). NMR and molecular modeling study of the conformations of taxol and of its side chain methylester in aqueous and non-aqueous solution. *Tetrahedron, 49*, 6545–6560.

Xie, J., Marijnissen, J. C. M., & Wang, C. H. (2006). Microparticles developed by electrohydrodynamic atomization for the local delivery of anticancer drug to treat C6 glioma in vitro. *Biomaterials, 27*, 3321–3332.

Xie, Z., Guan, H., Chen, X., Lu, C., Chen, L., Hu, X., Shi, Q., & Jing, X. (2007). A novel polymer–paclitaxel conjugate based on amphiphilic triblock copolymer. *J. Control. Release, 117*, 210–216.

Zhang, Z., & Feng, S. S. (2006a). Self-assembled nanoparticles of poly(lactide)–vitamin E TPGS copolymers for oral chemotherapy. *Int. J. Pharm., 324*, 191–198.

Zhang, Z., & Feng, S. S. (2006b). The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)–tocopheryl polyethylene glycol succinate nanoparticles. *Biomaterials, 27*, 4025–4033.

Zhang, X., Li, Y., Chen, X., Wang, X., Xu, X., Liang, Q., Hu, J., & Jing, X. (2005). Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate. *Biomaterials, 26*, 2121–2128.