Pharmaceutical Research, Vol. 24, No. 8, August 2007 (© 2007)
DOI: 10.1007/s11095-007-9269-5

Research Paper

Ionically Fixed Polymeric Nanoparticles as a Novel Drug Carrier

Sa-Won Lee,[1] Dong-Hoon Chang,[1] Myung-Seop Shim,[1] Bong-Oh Kim,[1] Sun-Ok Kim,[1] and Min-Hyo Seo[1,2]

Received October 19, 2006; accepted February 8, 2007; published online March 23, 2007

Purpose. In this study, we have prepared a novel polymeric drug delivery system comprised of ionically fixed polymeric nanoparticles (IFPN) and investigated their potential as a drug carrier for the passive targeting of water-insoluble anticancer drugs.
Materials and Methods. For this purpose, the physicochemical characteristics of the IFPN were investigated by comparing them with conventional polymeric micelles. IFPN containing paclitaxel were prepared and evaluated for *in vitro* stability and *in vivo* pharmacokinetics.
Results. The IFPN were successfully fabricated using a monomethoxypolyethylene glycol-polylactide (mPEG-PLA) diblock copolymer and a sodium salt of D,L-poly(lactic acid) (D,L-PLACOONa) upon the addition of $CaCl_2$. The transmittance of the IFPN solution was much lower than that of a polymeric micelle solution at the same polymer concentration implicating an increase in the number of appreciable particles. The particle size of the IFPN was approximately 20~30 nm which is in the range of particle sizes that facilitate sterile filtration using a membrane filter. The IFPN also have a regular spherical shape with a narrow size distribution. The zeta potential of the IFPN was almost neutral, similar to that of the polymeric micelles. In contrast, mixed micelles with a combination of mPEG-PLA and D,L-PLACOONa prior to the addition of Ca^{2+} showed a negative charge (−17 mV), possibly due to the carboxyl anion of polylactic acid exposed on the surface of the micelles. The IFPN formulation was highly kinetically stable in aqueous medium compared to the polymeric micelle formulation. The molecular weight of D,L-PLACOONa in the IFPN and the mPEG-PLA/D,L-PLACOONa molar ratio had a great influence upon the kinetic stability of the IFPN. Pharmacokinetic studies showed that the area under the concentration vs time curve (AUC) of IFPN in blood was statistically higher (about two times) when compared with that of Cremophor EL-based formulation (Taxol® equivalent) or polymeric micelle formulation.
Conclusions. The results suggests that the IFPN were retained in the circulation long enough to play a significant role as a drug carrier in the bloodstream, possibly resulting in improved therapeutic efficiency. Therefore, the IFPN are expected to be a promising novel polymeric nanoparticulate system for passive tumor targeting of water-insoluble anticancer drugs including paclitaxel.

KEY WORDS: D,L-PLACOONa; ionically fixed polymeric nanoparticles; long circulating carrier; mPEG-PLA; paclitaxel.

INTRODUCTION

Successful delivery of drugs to the target organ requires stable retention of the drug by the carrier while in circulation. Thus, improved plasma stability of the carrier is required to increase the drug delivery to tumors. Since passive tumor targeting appears to require a long circulation time, the stability of the drug-carrier association needs to be improved over that of rapidly cleared carriers in order to match the prolonged exposure of the carrier to blood components. Recently, there have been great efforts to develop nanoparticulate drug carriers for anticancer agents such as nanospheres (1–3), nanocapsules (4), liposomes (5,6), micelles (7,8) and other nanoparticulates (9). These injectable nanoparticulate carriers have important potential applications such as site specific drug delivery or medical imaging. Conventional carriers, however, cannot generally be used because they are eliminated by the reticulo-endothelial system (RES) within seconds or minutes after intravenous injection. Therefore, various attempts have been made to achieve long blood circulation times by avoiding RES recognition, mainly by chemically attaching (10) or adsorbing (11) appropriate polymers or molecules at the particle surface. In particular, poly(ethylene glycol) (PEG)-coated biodegradable nanospheres are a newly developed type of particle with an increased blood half-life. Several reviews describing PEG-coated, sterically-stabilized nanospheres are available (12–15).

Polymeric nanoparticle systems based on amphiphilic block copolymers can be classified into two categories, namely, micelles and nanospheres according to the hydrophilic:hydrophobic block weight ratios of the amphiphilic block copolymer used (16). Micelles are formed when the molecular weight of the hydrophilic block is larger than that of the hydrophobic block. They are easily fabricated through

[1] Department of Parenteral Delivery Program, Samyang Pharmaceuticals R&D, Daejeon, South Korea.
[2] To whom correspondence should be addressed. (e-mail: seo@samyang.com)

the self-assembly of polymers of low molecular weight (e.g. mPEG2K-PLA1.8K) due to their free solubility in water (17,18). Polymeric micelles (PM) provide many advantages, such as small size, high solubility, simple sterilization and controlled release of drugs. In spite of their distinct advantages, PM systems are not stable enough to provide long-circulation times in blood and are subject to dissociation upon dilution. However, they are much more stable than micelles consisting of small molecular surfactants. On the other hand, nanospheres can be prepared by dialysis or emulsification with polymers of relatively high molecular weight (e.g. mPEG5K-PLGA45K) (19,20). In this case, the molecular weight of the hydrophilic block is smaller than that of the hydrophobic block. Nanospheres from these polymers are very stable and exhibit much longer circulation times in the blood stream. However, their fabrication is not trivial and their relatively large particle size makes sterile filtration difficult.

Among the amphiphilic block copolymers, polyester-block-methoxypolyethylene glycol has been most extensively applied as a self-dispersible drug delivery system. Therefore, several studies have utilized polyester-block-methoxypolyethylene glycol copolymers as micellar carriers of drugs. In these biodegradable diblock copolymers, the polyester block can be poly(L-lactide) (PLLA), poly (D,L-lactide) (PDLLA), polyglycolide (PGA), polycaprolactone (PCL) or their copolymers. The polymer of particular interest for designing a hydrophobic core for block copolymer micelles with a programmed decaying property is poly(lactic acid) or poly (lactide) (PLA) because of its known biocompatible and biodegradable nature as shown by its use in many approved medical devices and controlled release formulations. In addition, methoxypolyethylene glycol as the hydrophilic block is water soluble and biocompatible. Indeed, extensive research has been conducted using polymeric micelles of PEG-PLA block copolymers as drug delivery formulations, especially for cancer chemotherapy. Recently, a paclitaxel-incorporated polymeric micelle formulation using PEG-PLA has been developed by Kim *et al*. (17). Phase II clinical trials of this formulation have been completed in Korea and are on going in the US.

In this study, IFPN containing paclitaxel were prepared using a amphiphilic diblock copolymer (mPEG-PLA) and a salt form of polylactic acid (D,L-PLACOONa) and evaluated *in vitro*/*in vivo* as a possible long-circulating system for the passive targeting of water-insoluble anticancer drugs. Paclitaxel (active ingredient of the Taxol® formulation), which was used in the present study as a prototype drug, is one of the best antineoplastic drugs found in nature. Thus, we have introduced a novel polymeric drug delivery system which demonstrates a longer retention in the systemic circulation. In addition the IFPN formulation uses a relatively simple fabrication method and avoids the use of toxic solubilizing agents such as Cremophors or polysorbates. Overall, this allows for an improved pharmacokinetic profile and better anti-tumoral efficacy of the drug.

MATERIALS AND METHODS

Chemicals and Animals

Paclitaxel (Genexol®) was obtained from Samyang Genex Co. (Seoul, Korea). Monomethoxy polyethylene glycol (mPEG, average molecular weight = 2,000 Da) was supplied by Nippon Oil & Fat Co. (Tokyo, Japan). D,L-lactide was purchased from Boehringer Ingelheim KG (Ingelheim, Germany). Acetonitrile, toluene, dichloromethane and diethyl ether (all HPLC grade) were purchased from Fisher Scientific (NJ, USA). Taxol® was purchased from Bristol-Myers Squibb (NJ, USA) via a local pharmacy in Korea. Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxy biphenyl-2,2′-dicarboxylate (DDB) was obtained from Daehwa Pharm. Co. (Seoul, Korea). All other chemicals were reagent grade and used as received. Purified deionized water was prepared using a Milli-Q plus system from Millipore Co. (MA, USA). Sprague-Dawley (SD) rats were obtained from Charles River Japan Inc.

Synthesis of Polymers

Polymerization of Monomethoxypolyethylene Glycol-polylactide (mPEG-PLA) Block Copolymer

mPEG-PLA diblock copolymers were synthesized by the ring opening polymerization of D,L-lactide in the presence of mPEG homopolymer and a catalyst as described previously (21,22). Briefly, 5.0 g of monomethoxypolyethylene glycol (Mn:2,000 Da) was introduced into a 100 ml two-neck round-bottomed flask, and dried by heating to 100°C under reduced pressure (1 mmHg) for 2 to 3 h. The reaction flask was filled with dry nitrogen, and stannous octoate ($Sn(Oct)_2$) in toluene as a reaction catalyst was injected at 1.0 mol% (10.13 mg, 0.025 mmol) of polyethylene glycol monomethyl ether using a syringe. The reaction mixture was stirred for 30 min, and the pressure was reduced to 1 mmHg at 110°C for 1 h to remove the toluene. Five grams of purified lactide was added to the reaction mixture and the mixture was heated to 130°C for 12 h. The resulting polymer (mPEG-PLA) was dissolved in ethanol, precipitated in diethyl ether, and dried in a vacuum oven for 48 h.

The number average molecular weight (Mn) of the polymers was determined by ^{1}H-NMR. Gel-permeation chromatography (GPC) was also performed to verify the molecular weight of the polymer in comparison to polystyrene standards. No significant difference in the values was observed. The Mn of mPEG block and PLA block was 2,000 (2.0 KD) and 1,765 Da (1.8 KD), respectively, with a polydispersity of 1.0 – 1.2.

Synthesis of Biodegradable Polyesters (PLA-COONa)

The biodegradable polyester, PLA-COONa, was synthesized by direct condensation polymerization of D,L-lactic acid. D,L-polylactic acids with various molecular weights were obtained by varying the reaction temperature, pressure, and time as set forth in Table I. Using a reaction vessel equipped with a stirrer, 100 g of D,L-lactic acid was heated in an oil bath to 80°C for 1 h at a pressure of 25 mmHg to remove excess moisture. After drying, the direct condensation polymerization was performed at a temperature of 150 ~ 160°C under a reduced pressure of 5 ~ 25 mmHg. The resulting product was added to 1 l of distilled water to precipitate the polymer. The precipitated polymer was then added to distilled water to remove the low molecular weight

Table I. Synthesis of Salt Form of D,L-polylactic Acid

Salt Form	Acid Form	Temperature (°C)	Time (h)	Pressure (mmHg)	Mn[a]	Yield(%)[b]
D,L-PLACOONa (0.6 KD)	D,L-PLACOOH (0.6 KD)	150	7	25	646	79
D,L-PLACOONa (1.0 KD)	D,L-PLACOOH (1.0 KD)	160	12	10	1,140	83
D,L-PLACOONa (1.5 KD)	D,L-PLACOOH (1.5 KD)	160	24	10	1,550	84
D,L-PLACOONa (2.0 KD)	D,L-PLACOOH (2.0 KD)	160	24	5	2,100	87

[a] By ^{1}H-NMR
[b] Yield = (Obtained polymer/monomer added) × 100

polymer that was soluble in aqueous solution at pH 4 or less. The precipitated polymer was then added to 1 l of fresh distilled water, and the pH of the aqueous solution was adjusted to 6–8 by the stepwise addition of sodium hydrogen carbonate to dissolve the polymer. The water-insoluble polymer was separated and removed by centrifugation or filtration. A 1 N hydrochloric acid solution was then added dropwise to the polymer solution to precipitate the polymer in the aqueous solution. The precipitated polymer was washed twice with distilled water, isolated and dried under reduced pressure to obtain a highly viscous liquid or white powder (D,L-polylactic acid). The Mn of the polymers was determined by ^{1}H-NMR.

One hundreds grams of the dried D,L-lactic acid was dissolved in 200 ml of acetone. The solution was stirred slowly at room temperature while sodium hydrogen carbonate solution (1 N) was slowly added until a pH of 7 was obtained. Twenty grams of anhydrous magnesium sulfate was added to remove excess moisture. The mixture was filtered using a PTFE membrane filter (1.0 um pore size) and the acetone was evaporated using a solvent evaporator to obtain a white solid. The solid was dissolved in 200 ml of anhydrous acetone and the solution was filtered to remove the insoluble portion using the PTFE membrane filter. Finally, the acetone was evaporated leaving the sodium salt of D,L-polylactic acid as a white solid.

Preparation of Polymeric Micelles and IFPN

The polymeric micelles (PM) were prepared by a solid dispersion technique as described previously (16,17). Briefly, mPEG-PLA (Mn = 2.0–1.8 KD, 100 mg) was dissolved in 1.0 ml of absolute ethanol. After 5 min of stirring, the organic solvent was evaporated on a rotary evaporator under reduced pressure at 60°C to obtain a transparent gel matrix. The resulting gel matrix was dissolved by the addition of 6 ml of distilled water at 60°C to obtain a transparent micellar solution. The solution was filtered through a 0.22 μm filter and lyophilized (Labconco, USA).

The IFPN were prepared by a novel and simple fabrication method as described below. Briefly, mPEG-PLA (Mn = 2.0–1.8 KD; 100 mg) and D,L-PLACOONa (Mn = 0.6, 1.0, 1.5 or 2.0 KD; 17, 30, 41 or 55 mg, respectively) were completely dissolved in 1.0 ml of ethanol to obtain a clear solution. Ethanol was removed on a rotary evaporator under reduced pressure to prepare a polymeric matrix. Six milliliters of distilled water was added to the matrix and the mixture was stirred for 30 min at 60°C to prepare a mixed polymeric micelle solution (mPM). To fabricate the IFPN, an aliquot of a 100 mg/ml solution of calcium chloride was added to the mPM solution, and the mixture was stirred for 20 min at room temperature. The mixture was passed through a 0.22 μm filter prior to lyophilization. The compositions for PM, mPM and IFPN are presented in Table II.

Incorporation of Paclitaxel into PM and IFPN

Paclitaxel and the polymers were accurately weighed and completely dissolved in 1.0 ml of ethanol to obtain a clear solution followed by the same procedure as illustrated

Table II. Ingredients and Comparative Physicochemical Properties of PM, mPM and IFPN

Parameters		Formulations					
		PM		mPM		IFPN	
		Vehicle	Drug Loaded	Vehicle	Drug Loaded	Vehicle	Drug Loaded
Ingredients (mg)	mPEG-PLA (Mn = 2.0–1.8 KD)	100	100	100	100	100	100
	D,L-PLACOONa (Mn = 2.0 KD)	–	–	55	55	55	55
	$CaCl_2$ (mg)	–	–	–	–	3	3
	Paclitaxel (mg)	–	11	–	11	–	11
Transmittance (%)[a]		37.3 ± 0.0	31.6 ± 0.0	51.4 ± 0.0	43.3 ± 0.0	14.6 ± 0.0	11.6 ± 0.0
Particle Size (nm)[a]		22.6 ± 0.3	23.9 ± 1.0	12.0 ± 0.3	14.4 ± 0.2	21.4 ± 0.6	21.3 ± 0.7
Zeta Potential (mV)		−2.0 ± 0.2	−3.6 ± 2.0	−17.0 ± 0.4	−20.0 ± 1.1	−1.3 ± 0.2	−2.4 ± 1.5
Paclitaxel % Remaining in Solution[b]		–	5.3 ± 2.1	–	60.9 ± 3.2	–	96.2 ± 3.3

[a] Polymer concentration was 50 mg/ml
[b] Initial paclitaxel concentration was 1.5 mg/ml. Kinetic stability of the three formulations in aqueous medium at 37°C. Incubation time was 24 h. Amount of Ca^{2+} in formulation was equivalent to the molar concentration of D,L-$PLACOO^-$.

above. Paclitaxel-incoporated PM, mPM and IFPN are denoted as PM-PTX, mPM-PTX and IFPN-PTX, respectively.

Physicochemical Characterization

Transmittance

Optical transmittance of aqueous polymer solutions (50 mg polymer/ml) at 25°C was measured at 300 nm with a UV-VIS spectrometer (V-530, Japan Spectroscopic, Tokyo, Japan). The samples were kept at a constant temperature using a temperature controlled circular system.

Surface Morphology

Morphological examination of the nanoparticles was performed by scanning electron microscopy (SEM) (JSM-6335F, Jeol, Japan). The appropriately diluted sample solution was freeze-dried and coated with platinum using a sputter coater (CRESSINGTON 108, Jeol, Japan) for 240 s in a vacuum at a current intensity of 10 mA.

Particle Size and Zeta Potential

The mean particle size and size distribution of the nanoparticles were measured by dynamic light scattering (DLS). The experiments were carried out using a 10-mW He–Ne laser at 638 nm as a light source. The intensity autocorrelation was measured at a scattering angle of 90° at 25°C. Each freeze-dried sample (200 mg) was diluted to the appropriate concentration using filtered distilled water (4 ml) to avoid multiscattering phenomena and placed into a quartz cuvette. The size analysis of each sample consisted of 50 measurements, and the result was expressed as a mean size ± SD for three separate experiments.

The zeta potential was calculated from the mean electrophoretic mobility values, which were determined by laser Doppler anemometry (LDA). Measurements were performed on the same samples (3 ml) prepared for size analysis. The sample solution was placed in the electrophoretic cell where a potential of ±150 mV was established. The DLS and LDA analysis were performed using a particle size analyzer (ELS-8000, Photal Otsuka Electronics, Japan).

In Vitro Physical Stability Study

IFPN-PTX solution (1.5 mg paclitaxel/ml) was subdivided into approximately 1 ml portions in autosampler vials of 2 ml capacity which were tightly sealed. The experiment was initiated by incubating the vials in a temperature controlled oven at 37°C. At appropriate time intervals, each vial was removed and the solution was filtered using a 0.2 μm membrane filter followed by dilution with acetonitrile. The concentration of paclitaxel remaining in solution was measured using HPLC. Briefly, samples were analyzed by isocratic HPLC on a system consisting of a binary pump, a Curosil PFP column (5 μm, 4.6 × 250 mm, Phenomenex, U.S.A.) with guard column (5 μm, 4.6 × 30 mm, Phenomenex, U.S.A.) using a flow of 1.5 ml/min and UV/Visual detection ($\lambda = 227$ nm) with a detection limit of 10 ng/ml. The mobile phase was a mixture of 55% acetonitrile and 45% deionized water (v/v).

Pharmacokinetic Study

Sprague-Dawley (SD) rats (227~236 g) were used as an animal model. The femoral artery and vein were catheterized with polyethylene tubing. Each rat was allowed to recover from anesthetization before the start of study. Sample solutions were slowly injected into the femoral vein over a period of 20 s at a 5 mg/kg dose of paclitaxel. At time points of 1, 5, 10, 30 min, 1, 2, 4, and 8 hr post injection, 0.4 ml of blood was collected into heparinized polyethylene tubes via the femoral artery and centrifuged at 5,000 rpm for 10 min to obtain plasma. The plasma was stored at −20°C prior to analysis by HPLC.

Plasma concentrations of paclitaxel were determined by reverse-phase HPLC with UV detection (17). Briefly, 100 μl samples of plasma were mixed with 10 ml of ethyl acetate containing 100 μL of 7.5 mg/ml of dimethyl-4,49-dimethoxy 5,6,59,69-dimethylene dioxy biphenyl-2,29-dicarboxylate as an internal standard. The samples were then centrifuged at 2500 rpm for 5 min. Next, the organic layer was transferred to a clean tube and evaporated to dryness using a pressured gas blowing concentrator (Model MGS-2100, EYELA, Tokyo, Japan) at 40°C. The extraction residue was reconstituted in 100 μl of 40% acetonitrile solution, and 75 μl aliquots were injected onto the HPLC system.

The HPLC system consisted of a HP1100 series (Agilent Technologies, Palo Alto, USA): G1322A online degasser, G1312A binary pump, G1313A autosampler, G1316A thermostated column compartment and G1315A diode-array detector. Data were acquired and processed using HP Chemstation (LC Rev. A.06.03 [509]) chromatography manager software from Agilent Technologies.

Chromatographic separations were achieved using a 218MR54 column (4.6 × 250 mm, C_{18}, Vydac, USA) at 25°C. The mobile phase consisted of deionized water and HPLC grade acetonitrile. The following gradient was used to achieve separation: 24% acetonitrile in deionized water for 5 min, linear gradient to 58% acetonitrile over 16 min, linear gradient to 70% acetonitrile over 2 min, linear gradient to 34% acetonitrile over 4 min, and 34% acetonitrile for 5 min at a flow rate of 1.0 ml/min. UV detection was utilized at 227 nm.

Pharmacokinetic Analysis

The elimination rate constant (λz) was estimated by linear regression of the blood concentrations in the log-linear terminal phase. In order to estimate the initial blood concentration (C_0) immediately after iv injection, the linear regression of the log-linear initial state going through the first two time points was extrapolated to time zero. The estimated C_0 was then used in conjunction with the actual measured plasma concentrations to determine the area under the blood concentration-time curve (AUC). The AUC_{inf} was calculated using the combined log-linear trapezoidal rule for data from time of dosing to the last measured concentration, plus the quotient of the last measured concentration divided by λz. Noncompartmental pharmacokinetic methods were used to calculate mean residence time (MRT, by dividing $AUMC_{inf}$ by AUC_{inf}), clearance (CL, by dividing dose by AUC_{inf}), and volume of distribution (V_d, by dividing CL by λz; and V_{dss}, by multiplying CL by MRT).

Statistical Analysis

All values in the text and figures are mean values ± SD. A Student's *t*-test was used to test for significance between unpaired groups. Statistical significance was set at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Fabrication Mechanism of IFPN

Current approaches for the preparation of polymeric nanospheres using amphiphilic block copolymers are mostly based on complex and time-consuming processes such as emulsification (12) or nanoprecipitation (19) which present difficulties for large scale production. In addition, the resulting nanospheres have a large particle size and wide size distribution making sterile filtration difficult.

Recently, we found that derivatives of the monovalent metal salt of poly(D,L-lactic acid) alone can form clear micellar aqueous solutions. When a divalent inorganic metal salt ($CaCl_2$, $ZnCl_2$ and $MgCl_2$) solution is added to the micellar solution, the divalent metal salt of the polymer is precipitated to form a milky solution due to its insolubility in aqueous solution (Fig. 1a). However, the mixture of a polylactic acid monovalent salt and an amphiphilic block copolymer also form mixed micelles in aqueous solution. In this case, after the addition of a divalent metal salt, no precipitate is observed and a stable metal ion-fixed polymeric nanoparticle can be obtained (Fig. 1b). These findings allowed us to fabricate polymeric nanoparticles in a facile manner by simply adding divalent metal cations to the mixed micelles. In this study, $CaCl_2$ was used as a source for the divalent metal cation. Ca^{2+} is one of the inorganic minerals required in relatively large quantities for the normal functioning of the body while the others are needed in trace amounts. So, Ca^{2+} is regarded as safer in terms of toxicity than the other cations. Other divalent cations like Zn^{2+} and Mg^{2+} also exert the same effect because the carboxyl anions of polylactic acid can also interact with the divalent cations to form a water insoluble salt (data not shown).

More specifically, sodium salts of D,L-PLACOOH were soluble in water and could form micelles in water like an ionic surfactant, however, the calcium salt was insoluble. Therefore, it was expected that mPEG-PLA and D,L-PLACOONa could form mixed polymeric micelles, and upon the addition of $CaCl_2$, polymeric micelles with a much more rigid hydrophobic core, a kind of polymeric nanoparticle, could be fabricated due to the electrostatic interaction between D,L-$PLACOO^-$ and Ca^{2+} forming a complex of (D,L-$PLACOO^-)_2Ca^{2+}$ as shown in Fig. 1. These polymeric micelles were named ionically fixed polymeric nanoparticles (IFPN).

Physicochemical Characterization

The physicochemical characteristics of the ionically fixed polymeric nanoparticles (IFPN) such as transmittance, size, size distribution, zeta potential and morphological properties were evaluated in comparison with conventional polymeric micelles (PM) or mixed polymeric micelles (mPM), where PM and mPM represents polymeric micelles with mPEG-PLA only and mixed polymeric micelles with mPEG-PLA and D,L-PLACOONa, respectively. IFPN represents polymeric nanoparticles formed through fixing the mPM with Ca^{2+}. The results for the *in vitro* physicochemical evaluation are summarized in Table II.

As shown in Table II, the mean transmittance of the IFPN was the lowest among the micellar solutions suggesting that it contained more appreciable particles than the others. This result indicates that the addition of Ca^{2+} to mPM fabricated more stable nanoparticles. Transmittance values for PM, mPM and IFPN were 37.3 ± 0.0, 51.4 ± 0.0, and 14.6 ± 0.0%, respectively, at 50 mg polymer/ml. Drug loading decreased the transmittance slightly in all formulations suggesting that the hydrophobic interaction of the drug and the hydrophobic block of the polymers could form more rigid micelles or nanoparticles.

The particle size of the IFPN was 21.4 ± 0.6 nm which is in the range of particle sizes that facilitate sterile filtration using a membrane filter. The mean particle size of the IFPN was very similar to that of the PM (22.6 ± 0.3 nm), while it was nearly double the size of the mPM (12.0 ± 0.3 nm) (Table II). The difference in mean particle size may indicate that the mPM contains more smaller sized micelles of D,L-PLACOONa (2.0 KD) or mixed micelles of mPEG-PLA and D,L-PLACOONa. Drug loading increased the mean particle sizes of the PM and the mPM slightly (no significant difference); however, no change in the particle size of the IFPN was observed in the presence of the drug (Table II). The results from DLS and SEM analysis showed that the IFPN had a regular spherical shape with a narrow size distribution (Fig. 2). In addition, no apparent aggregation was detected. An increase in molecular weight of D,L-PLACOONa from 1.0 to 2.0 KD increased the size of IFPN appreciably but no significant effect of D,L-PLACOONa/mPEG-PLA molar ratio on the particle size was observed (data not shown).

As expected, the zeta potential of the IFPN was almost neutral, similar to that of the PM, which suggests that Ca^{2+} reacts with the carboxyl anion of the polylactic acid electrostatically to form nanoparticles with neutral surfaces (Table II). The mPM showed a much more negative surface charge due to the negative charged carboxyl groups on the surface of the particle. Zeta potentials of the PM, mPM and IFPN were −2.0 ± 0.2, −17.0 ± 0.4, and −1.3 ± 0.2 mV, respectively. Drug loading did not affect the zeta potential of the PM and IFPN. However, it slightly shifted the surface charge of mPM to the negative side possibly due to a marginal increase in the negative charged micelles of D,L-PLACOONa on which the carboxyl ion of PLA is exposed.

In Vitro Physical Stability

The percent paclitaxel remaining in the sample solutions for IFPN-PTX, mPM-PTX and PM-PTX after incubation at 37°C for 24 h was 96.2, 60.9 and 5.3%, respectively (Table II). In other words, approximately 4, 40 and 95% of the drug incorporated in IFPN, mPM and PM was released from their vehicles at 37°C after 24 h. This means that the retention of the drug incorporated in the IFPN was the highest among the formulations implicating that the IFPN-PTX was much more kinetically stable in aqueous environment than PM-PTX or

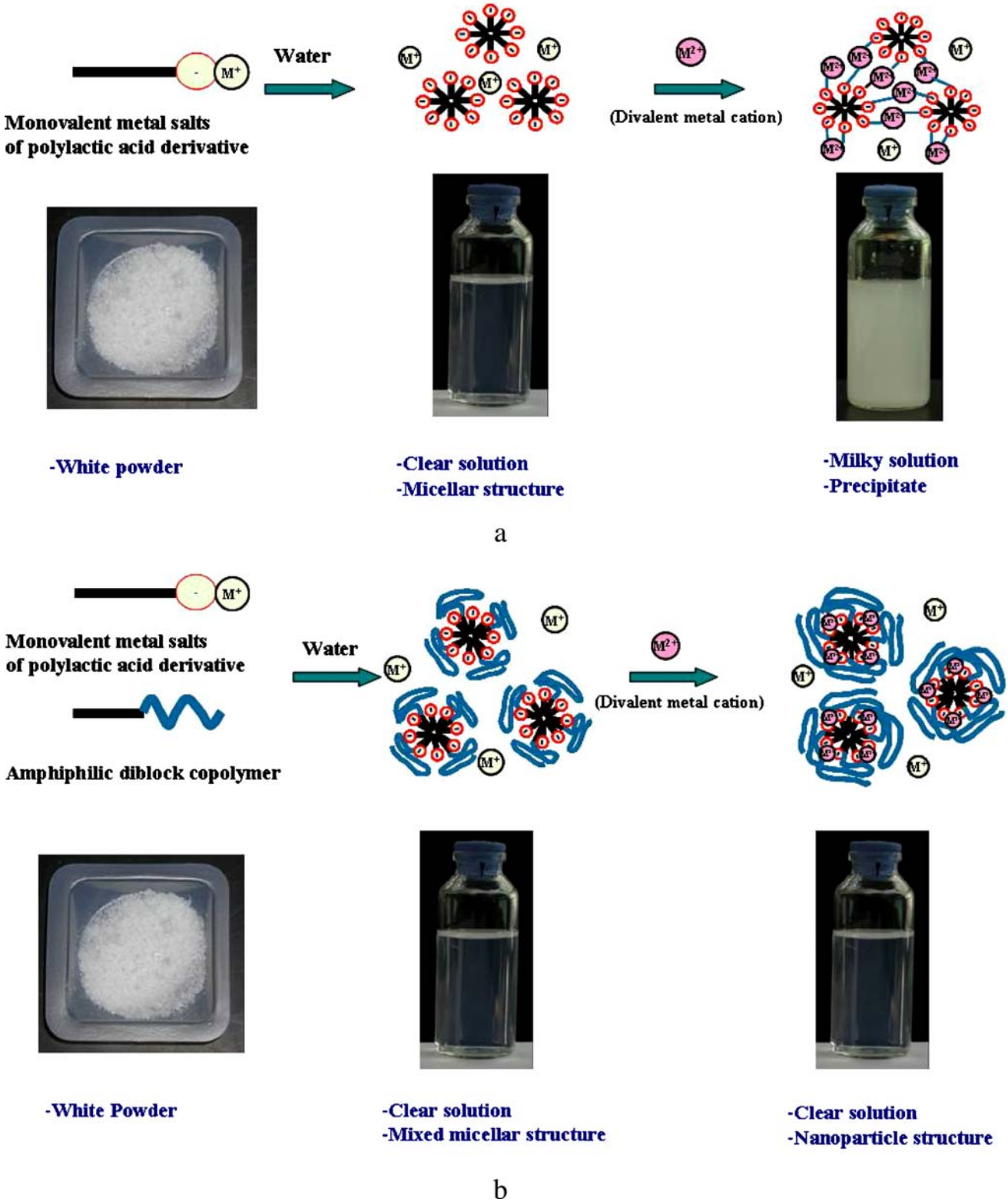

Fig. 1. Novel concept for fabrication of polymeric nanoparticles. (**a**) Property of monovalent metal salts of poly(D,L-lactic acid), (**b**) proposed scheme of the formation of ionically fixed polymeric nanoparticles (IFPN).

mPM-PTX. This is thought to be due to the crosslinking electrostatic interaction between D,L-PLACOO$^-$ and Ca^{2+} which might increase the rigidity of the hydrophobic core. Therefore, release of the drug from the IFPN was hindered by the rigid hydrophobic core of the PLA Ca^{2+} salt intercalated between mPEG-PLA unimers leading to a significant reduction in drug release. This could result in little change in stability when the suspensions are diluted. On

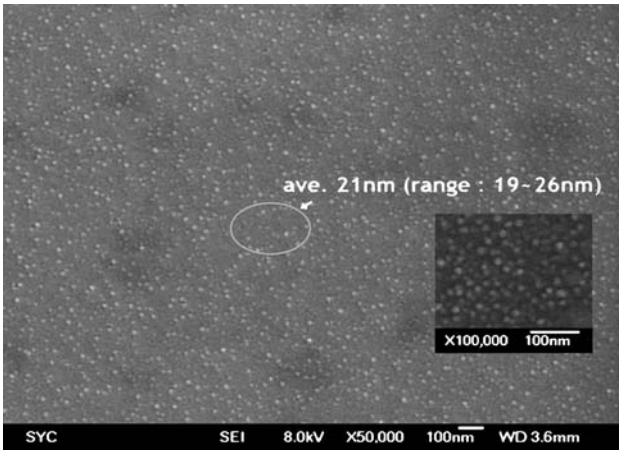

Fig. 2. Scanning electron microphotograph of freeze-dried IFPN. The mPEG-PLA(2.0–1.8 KD)/D,L-PLACOONa (2.0 KD) molar ratio was 1:1.

the other hand, when surfactant micelles or polymeric micelles are diluted to a concentration below their CMC (critical micelle concentration), the micelles become kinetically unstable and are disassembled to unimers followed by fast release of the hydrophobic drug.

In addition, the effect of the molecular weight of D,L-PLACOONa on the stability of IFPN-PTX was investigated. As the molecular weight of D,L-PLACOONa increased from 0.6 to 2.0 KD at a D,L-PLACOONa/mPEG-PLA molar ratio of 1:1, the release of incorporated paclitaxel from the IFPN was delayed (Fig. 3a). When the molecular weight was above 2.0 KD, IFPN-PTX could not be fabricated at a D,L-PLACOONa/mPEG-PLA molar ratio of 1:1 due to its poor solubility in water. Increasing the D,L-PLACOONa/mPEG-PLA molar ratio from 1:2 to 4:2 improved the stability of IFPN-PTX dramatically (Fig. 3b). However, during incubation of the samples, precipitation of the polymers was observed at a PLACOONa/mPEG-PLA molar ratio of 4:2. The precipitated polymer was hypothesized to be due to the excess calcium salts of D,L-PLACOOH unincorporated in the IFPN-PTX. From the above results, it was found that an optimal molecular weight of D,L-PLACOONa and an optimal D,L-PLACOONa/mPEG-PLA molar ratio are required to obtain stable IFPN-PTX.

Pharmacokinetics

It is believed that paclitaxel release from the nanoparticles may be accelerated by enzymes (e.g. esterase) or plasma proteins in serum. However, the nanoparticles are relatively more stable in serum than polymeric micelles. So, the distribution rate of drug in the nanoparticle onto plasma proteins in serum is relatively lower than that of polymeric micelles or surfactant micelles. Thus, the slower distribution of drug onto plasma proteins should lower the distribution rate of drug into tissues from the systemic circulation leading to the longer circulation of the drug.

As previously mentioned, therefore, PM systems fabricated through self-assembly of polymers of low molecular weight (e.g. mPEG2K-PLA1.8K) are not sufficiently kinetically stable to retain hydrophobic drugs (e.g. paclitaxel) in the blood circulation for extended periods of time. Thus, their potential has been limited as a long circulating carrier in spite of their very good biocompatibility (16,20). On the other hand, Cremophor EL (CrEL) is a formulation vehicle used for various poorly-water soluble drugs, including the anticancer agent paclitaxel. However, it is not an inert vehicle, but exerts a range of biological effects, some of which have important clinical implications (23,24). In particular, it alters the biodistribution of paclitaxel as a result of entrapment of the drug in circulating CrEL micelles, thereby improving its pharmacokinetic profile and consequently, contributes to the better pharmacological effect of paclitaxel (23,25). This property of CrEL makes it difficult to further improve upon the current CrEL-based formulation in terms of pharmacokinetic/pharmacodynamic profiles (5,9,17). Thus, we compared the pharmacokinetics of the IFPN formulation

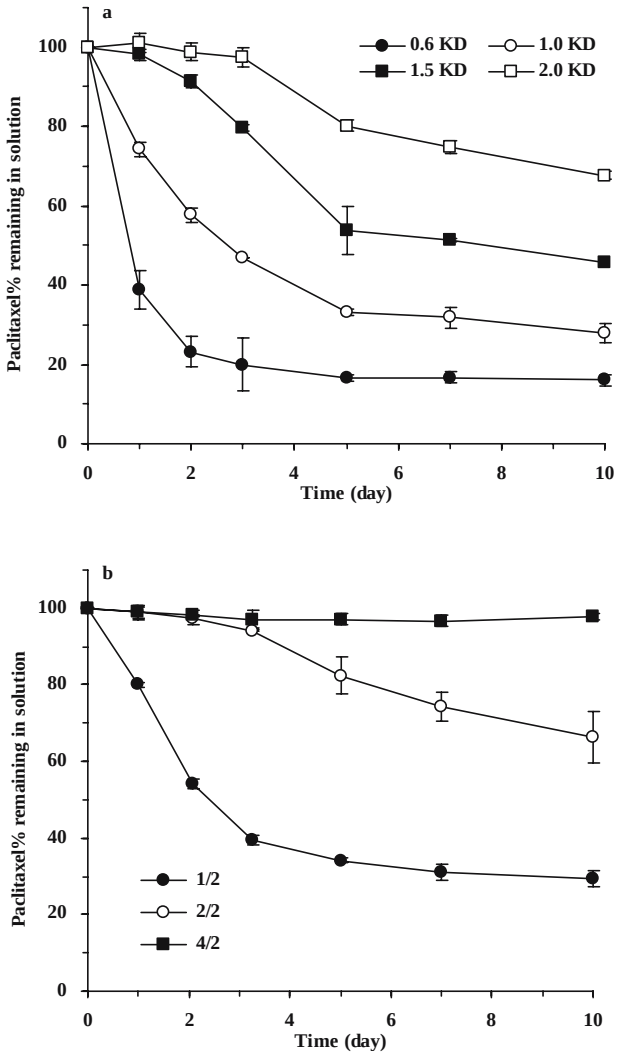

Fig. 3. Kinetic stability of IFPN-PTX in aqueous medium as a function of molecular weight of D,L-PLACOONa (**a**), in which the mPEG-PLA (2.0–1.8 KD)/D,L-PLACOONa/paclitaxel molar ratio was 3:3:1, and D,L-PLACOONa/mPEG-PLA molar ratio (**b**), in which molecular weight of D,L-PLACOONa was 2.0 KD. Initial concentration of paclitaxel was 1.5 mg/ml and the incubation temperature was 37°C. The amount of Ca^{2+} in formulations was equivalent to the molar concentration of D,L-$PLACOO^-$.

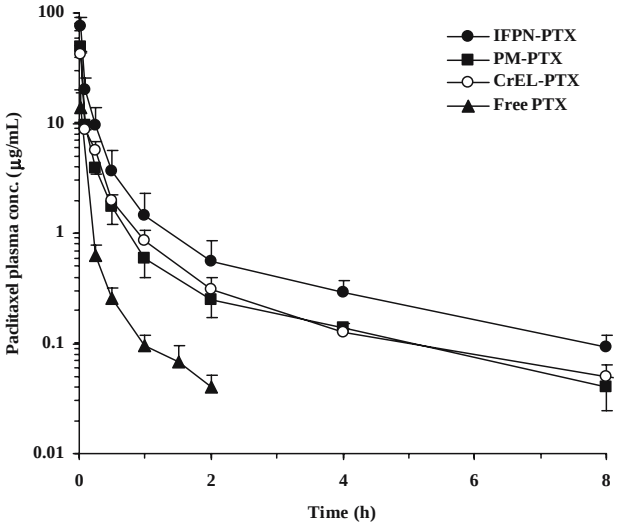

Fig. 4. Plasma concentration-time curves of IFPN-PTX, CrEL-PTX and Free PTX. The mPEG-PLA(2.0–1.8 KD)/D,L-PLACOONa (2.0 KD)/paclitaxel molar ratio for IFPN-PTX was 23:23:1. The amount of Ca^{2+} in the formulation was equivalent to the molar concentration of D,L-$PLACOO^-$. A 5 mg/kg dose of paclitaxel was administered via the femoral vein of SD rats. *Each point* represents a mean ± S.D.

(IFPN-PTX, mPEG-PLA/D,L-PLACOONa (2 KD)/paclitaxel molar ratio = 23:23:1) with those of the PM formulation (PM-PTX, mPEG-PLA/paclitaxel molar ratio of 23:1) and CrEL formulation (CrEL-PTX, Taxol® equivalent) to evaluate the potential of IFPN as a long circulating drug carrier. A Tween 80-based formulation (Free PTX, 50:50 (v/v) ethanol/polyoxyethylene-20-monooleate) was used as a negative control since it has been reported that Tween 80 has a negligible effect on pharmacokinetic profiles of paclitaxel in contrast to CrEL (23,25). The IFPN-PTX's composition, mPEG-PLA/D,L-PLACOONa (2 KD)/paclitaxel molar ratio of 23:23:1, for the pharmacokinetic study was selected from the results of the previous *in vitro* formulation study and a preliminary *in vivo* pharmacokinetic study. The amount of Ca^{2+} in IFPN-PTX formulation was equivalent to the molar concentration of D,L-$PLACOO^-$.

Figure 4 shows the paclitaxel concentration in plasma versus time curves after administration of IFPN-PTX, PM-PTX, CrEL-PTX and Free PTX at the same paclitaxel dose (5 mg paclitaxel per kg animal body weight). The parameters estimated from the pharmacokinetic analysis are shown in Table III. In the case of IFPN-PTX, the AUC was 11.5 μg·h/ml which was 1.9, 1.8 and 8.2 times higher than those for PM-PTX (6.0 μg·h/ml), CrEL-PTX (6.0 μg·h/ml), and Free PTX (1.4 μg·h/ml), respectively (Table III). A statistically significant difference in AUC was observed when the AUC for IFPN-PTX was compared with those of the other formulations. On the other hand, no significant difference between the AUC for PM-PTX and that for CrEL-PTX was observed. Nevertheless, the AUC for PM-PTX and CrEL-PTX were 4.3 and 4.5 times, higher than that of Free PTX, respectively. The terminal half life ($t_{1/2}$) and mean residence time (MRT) for the IFPN formulation were not significantly different from those of the other formulations except for the Free PTX. In contrast, V_d and CL for IFPN-PTX were significantly lower than those for the other formulations implying a longer retention of the drug in the blood circulation with a relatively slow distribution of the drug into excretory organs like the liver and kidney. Therefore, these results illustrate the potential utility of IFPN as a long circulating drug carrier for water insoluble anti-cancer drugs.

CONCLUSIONS

A novel nano-particulate system, IFPN, was successfully fabricated using an amphiphilic diblock copolymer and a salt form of polylactic acid. This approach enables us to prepare sterile filterable nanoparticles without complex manipulations such as emulsification or dialysis. The IFPN was highly stable with long blood circulation times which may allow the IFPN to play a significant role as a drug carrier in the blood stream with improved therapeutic efficiency. From these results, IFPN is expected to be a promising novel polymeric nanoparticulate system for passive tumor targeting of water-insoluble anticancer drugs including paclitaxel.

Table III. Comparative Pharmacokinetic Parameters of Paclitaxel Formulations

Parameters	Formulations			
	IFPN-PTX[a] ($n=4$)	PM-PTX ($n=3$)	CrEL-PTX ($n=4$)	Free PTX ($n=4$)
$t_{1/2}$(h)	2.39 ± 0.19[g]	3.45 ± 1.80	2.56 ± 0.36	0.87 ± 0.22
C_0(μg/ml)	104.5 ± 18.7[b,e,g]	71.7 ± 7.7	60.8 ± 6.0	15.7 ± 6.0
AUC_{last}(μg h/ml)	11.5 ± 3.5[b,d,g]	6.0 ± 0.4	6.3 ± 0.6	1.4 ± 0.4
AUC_∞(μg·h/ml)	11.8 ± 3.5[b,d,g]	6.4 ± 0.9	6.6 ± 0.7	1.5 ± 0.4
MRT (h)	0.80 ± 0.05[g]	0.79 ± 0.17	0.85 ± 0.18	0.19 ± 0.05
V_d (l/kg)	1.56 ± 0.47[b,e,f]	3.75 ± 1.37	2.82 ± 0.35	4.57 ± 1.87
V_{dss} (l/kg)	0.49 ± 0.13[b,d]	1.23 ± 0.74	0.95 ± 0.26	1.18 ± 0.70
CL(l/h·kg)	0.45 ± 0.11[c,e,g]	0.79 ± 0.11	0.77 ± 0.09	3.57 ± 0.84

A 5 mg/kg dose was administered to the femoral vein of SD rats. Each point represents a mean ± SD ($n = 3 \sim 4$)

[a] The mPEG-PLA (2.0–1.8 KD)/D,L-PLACOONa (2.0 KD)/paclitaxel molar ratio for IFPN-PTX was 23:23:1. The amount of Ca^{2+} in the formulation was equivalent to the molar concentration of D,L-$PLACOO^-$.

[b] (vs PM-PTX, $p<0.05$), [c] (vs PM-PTX, $p<0.01$), [d] (vs CrEL-PTX, $p<0.05$), [e] (vs CrEL-PTX, $p<0.01$), [f] (vs Free PTX, $p<0.05$), [g] (vs Free PTX, $p<0.01$)

ACKNOWLEDGEMENTS

We would like to thank Dr. Chad Brown of Samyang Research Corporation for his valuable comments. This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A040020).

REFERENCES

1. L. Mu and S. S. Feng. 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**(1–3): 129–144 (2002).
2. S. Y. Kim and Y. M. Lee. Taxol-loaded block copolymer nanospheres composed of methoxy poly(ethylene glycol) and poly(epsilon-caprolactone) as novel anticancer drug carriers. *Biomaterials* **22**(13): 1697–1704 (2001).
3. H. Suh, B. Jeong, R. Rathi, and S. W. Kim. Regulation of smooth muscle cell proliferation using paclitaxel-loaded poly (ethylene oxide)-poly(lactide/glycolide) nanospheres. *J. Biomed. Mater. Res.* **42**(2): 331–338 (1998).
4. S. Peltier, J. M. Oger, F. Lagarce, W. Couet, and J. P. Benoit. Enhanced oral paclitaxel bioavailability after administration of paclitaxel-loaded lipid nanocapsules. *Pharm. Res.* **23**(6): 1243–1250 (2006).
5. J. A. Zhang, G. Anyarambhatla, L. Ma, S. Ugwu, T. Xuan, T. Sardone, and I. Ahmad. Development and characterization of a novel Cremophor EL free liposome-based paclitaxel (LEP-ETU) formulation. *Eur. J. Pharm. Biopharm.* **59**(1): 177–187 (2005).
6. M. L. Immordino, P. Brusa, S. Arpicco, B. Stella, F. Dosio, and L. Cattel. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing docetaxel. *J. Control. Release* **91**(3): 417–429 (2003).
7. Z. Gao, A. N. Lukyanov, A. R. Chakilam, and V. P. Torchilin. PEG-PE/phosphatidylcholine mixed immunomicelles specifically deliver encapsulated taxol to tumor cells of different origin and promote their efficient killing. *J. Drug Target.* **11**(2): 87–92 (2003).
8. A. Krishnadas, I. Rubinstein, and H. Onyuksel. Sterically stabilized phospholipid mixed micelles: *in vitro* evaluation as a novel carrier for water-insoluble drugs. *Pharm. Res.* **20**(2): 297–302 (2003).
9. W. J. Gradishar. Albumin-bound nanoparticle paclitaxel. *Clin. Adv. Hematol. Oncol.* **3**(5): 348–349 (2005).
10. D. Papahadjopoulos and A. Gabizon. Liposomes designed to avoid the reticuloendothelial system. *Prog. Clin. Biol. Res.* **343**:85–93 (1990).
11. R. H. Muller, S. Maassen, H. Weyhers, and W. Mehnert. Phagocytic uptake and cytotoxicity of solid lipid nanoparticles (SLN) sterically stabilized with poloxamine 908 and poloxamer 407. *J. Drug Target.* **4**(3): 161–170 (1996).
12. R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, and R. H. Muller. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf., B Biointerfaces* **18**(3–4): 301–313 (2000).
13. I. Brigger, J. Morizet, G. Aubert, H. Chacun, M. J. Terrier-Lacombe, P. Couvreur, and G. Vassal. Poly(ethylene glycol)-coated hexadecylcyanoacrylate nanospheres display a combined effect for brain tumor targeting. *J. Pharmacol. Exp. Ther.* **303**(3): 928–936 (2002).
14. I. Brigger, P. Chaminade, V. Marsaud, M. Appel, M. Besnard, R. Gurny, M. Renoir, and P. Couvreur. Tamoxifen encapsulation within polyethylene glycol-coated nanospheres. A new antiestrogen formulation. *Int. J. Pharm.* **214**(1–2): 37–42 (2001).
15. R. Gref, Y. Minamitake, M. T. Peracchia, A. Domb, V. Trubetskoy, V. Torchilin, and R. Langer. Poly(ethylene glycol)-coated nanospheres: potential carriers for intravenous drug administration. *Pharm. Biotechnol.* **10**:167–198 (1997).
16. R. T. Liggins and H. M. Burt. Polyether-polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations. *Adv. Drug Deliv. Rev.* **54**:191–202 (2002).
17. S. C. Kim, D. W. Kim, Y. H. Shim, J. S. Bang, H. S. Oh, S. W. Kim, and M. H. Seo. *In vivo* evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J. Control. Release* **72**:191 (2001).
18. H. M. Burt, X. Zhang, P. Toleikis, L. Embree, and W. L. Hunter. Development of copolymers of poly(D,L-lacitde) and methoxypolyethylene glycol as micellar carriers of paclitaxel. *Coll. Surf., B Biointerfaces* **16**:161 (1999).
19. K. Avgoustakis, A. Beletsi, Z. Panagi, P. Klepetsanis, E. Livaniou, G. Evangelatos, and D. S. Ithakissios. Effect of copolymer composition on the physicochemical characteristics, *in vitro* stability, and biodistribution of PLGA-mPEG nanoparticles. *Int. J. Pharm.* **259**:115–127 (2003).
20. M. T. Peracchia, R. Gref, Y. Minamitake, A. Domb, N. Lotan, and R. Langer. PEG-coated nanospheres from amphiphilic diblock and multiblock copolymers: Investigation of their drug encapsulation and release characteristics. *J. Control. Release* **46**:223–231 (1997).
21. K. J. Zhu, L. Xiangzhou, and Y. Shilin. Preparation, characterization and properties of polylactide(PLA)-poly(ethylene glycol) (PEG) copolymers: a potential drug carrier. *J. Appl. Polym. Sci.* **39**:1–9 (1990).
22. B. Jeong, Y. H. Bae, D. S. Lee, and S. W. Kim. Biodegradable block copolymers as injectable drug delivery systems. *Nature* **388**:860–862 (1997).
23. A. J. ten Tije, J. Verweij, W. J. Loos, and A. Sparreboom. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. *Clin. Pharmacokinet.* **42**(7): 665–685 (2003).
24. R. T. Dorr. Pharmacology and toxicology of Cremophor EL diluent. *Ann. Pharmacother.* **28**:S11–S14 (1994).
25. H. Gelderblom, J. Verweij, K. Nooter, and A. Sparreboom. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur. J. Cancer* **37**(13): 1590–1598 (2001).