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Poly(ω -pentadecalactone-co-butylene-co-succinate) Nanoparticles as Biodegradable Carriers for Camptothecin Delivery

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Abstract

In this study, we show that degradable particles of a hydrophobic polymer can effectively deliver drugs to tumors after i.v. administration. Free-standing nanoparticles with diameters of 100–300 nm were successfully fabricated from highly hydrophobic, biodegradable poly(ω -pentadecalactone-co-butylene-co-succinate) (PPBS) copolyesters. PPBS copolymers with various compositions (20–80 mol% PDL unit contents) were synthesized via copolymerization of ω -pentadecalactone (PDL), diethyl succinate (DES), and 1,4-butanediol (BD) using *Candida antarctica* lipase B (CALB) as the catalyst. Camptothecin (CPT, 12–22%) was loaded into PPBS nanoparticles with high encapsulation efficiency (up to 96%) using a modified oil-in-water single emulsion technique. The CPT-loaded nanoparticles had a zeta potential of about –10 mV. PPBS particles were non toxic in cell culture. Upon encapsulation, the active lactone form of CPT was remarkably stabilized and no lactone-to-carboxylate structural conversion was observed for CPT-loaded PPBS nanoparticles incubated in both phosphate-buffered saline (PBS, pH = 7.4) and DMEM medium for at least 24 hr. In PBS at 37 °C, CPT-loaded PPBS nanoparticles showed a low burst CPT release (20–30%) within the first 24 hrs followed by a sustained, essentially complete, release of the remaining drug over the subsequent 40 days. Compared to free CPT, CPT-loaded PPBS nanoparticles showed a significant enhancement of cellular uptake, higher cytotoxicity against Lewis lung carcinoma and 9L cell lines *in vitro*, a longer circulation time, and substantially better antitumor efficacy *in vivo*. These results demonstrate the potential of PPBS nanoparticles as long-term stable and effective drug delivery systems in cancer therapy.

1. Introduction

Current chemotherapy is far from satisfactory: drug treatments often have limited effectiveness and patients suffer from serious side effects. Drug delivery devices have been studied extensively over the past few decades, including degradable polymer matrices, polymeric nanoparticles, liposomes, and micelles [1,2]. The use of nano-sized particles in cancer therapy

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is particularly exciting, as these materials can increase drug solubility and stability, as well as improve pharmacological effect by passively delivering chemotherapeutic agents to tumor sites via enhanced permeability and retention (EPR) effect [3–5].

Camptothecin (CPT) is a natural plant alkaloid extracted from *Camptotheca acuminata* (a tree grown in China), which has shown a broad spectrum of antitumor activity against various types of solid tumors [6]. However, effective delivery of CPT to tumor targets is extremely challenging due to its insolubility in water, structural instability, and high toxicity to normal tissue cells. Under physiological conditions, i.e., at pH equal to or above 7, CPT undergoes lactone ring-opening hydrolysis to form the inactive carboxylate form as shown in Scheme 1 [7].

Additionally, human serum albumin in the blood has a high affinity for binding to the carboxylate form of CPT, thus driving the above lactone-carboxylate equilibrium toward the formation of the inactive carboxylate form [8]. As a result, the potency of the drug is reduced substantially when administered to humans. Because of its toxic side effects, CPT, like most other antitumor drugs, needs to be frequently administered with limited doses to achieve desirable drug efficacy. Effective drug delivery methods providing sustained release of controllable amount of drugs over a prolonged period of time would be obviously advantageous for administration of these kinds of drugs [9].

To address these problems in CPT delivery, several different approaches have been taken to improve drug delivery efficiency and reduce adverse drug reactions. Chemical modification of CPT has led to the synthesis of its water-soluble derivatives, such as topotecan and irinotecan [10,11]. CPT-containing liposomes [12–14], amphiphilic diblock copolymer micelles [15, 16], covalently linked polymer-CPT conjugates [17–19], and CPT-encapsulated microspheres [20,21] or nanoparticle carriers [22,23] have also been explored. Microspheres have long been used for drug delivery to tumors: for example, carboplatin-loaded poly(lactide-co-glycolide) (PLGA) microspheres implanted at tumor sites were found to improve the survival of tumor-bearing rats [24]. A significant reduction in toxicity was observed when CPT-loaded poly(ϵ -caprolactone) (PCL) microspheres, instead of free CPT, were used to treat mice injected with B16-F10 melanoma cells [21]. CPT-loaded lipid nanoparticles were capable of releasing the drug into phosphate buffered saline for up to a week [25]. Oxidized cellulose microspheres incorporating CPT released the drug at a significantly faster rate; 50% of the CPT was released within 19–37 hrs [26]. Most recently, antibody-labeled PLGA nanoparticles containing CPT were more effective at killing HCT 116 cells when compared to free CPT [27]. Similarly, poly(lactide)/poly(ethylene glycol-*b*-propylene glycol-*b*-ethylene glycol) nanoparticles loaded with CPT were more effective than free drug in extending the survival time of mice bearing Sarcoma 180 tumors [28].

Previous studies have found that suspended nanoparticles circulate in the blood after i.v. administration [29]. In addition, nanoparticles are readily internalized by cells, due to their small size, and thus are preferred over larger microspheres as drug carriers [30,31]. More recently, nanoparticles and micelles with significant hydrophobicity were found to stabilize CPT and increase its circulatory retention in blood stream [3,16]. To the best of our knowledge, highly hydrophobic, degradable polymers have not thus far been evaluated as carriers for delivering hydrophobic antitumor drugs. Herein we report the results on the antitumor efficiency of poly(ω -pentadecalactone-co-butylene-co-succinate) (PPBS) nanoparticles as carriers for CPT. The PPBS copolyesters with various compositions were synthesized via copolymerization of ω -pentadecalactone (PDL), diethyl succinate (DES), and 1,4-butanediol (BD) employing *Candida antarctica* lipase B (CALB) as the catalyst [32]. The copolymers are substantially hydrophobic due to the PDL units in the polymer chains and are expected to be biodegradable in the presence of lipases and hydrolases. The rationale for selecting PPBS

particles as CPT carriers was on the anticipated strong interactions between the copolyesters and CPT, which would result in good dispersion of the drug in the polymer nanoparticles, leading to effective protection of CPT from hydrolysis; protection of CPT should reduce conversion to the inactive carboxylate form, enhance drug lifetime in the circulation, and provide sustained release of the active drug over a prolonged period. The rate of CPT release from nanoparticles can potentially be controlled by using different drug loadings and/or PPBS copolymers with varied compositions and thus hydrophobicity. This paper describes the synthesis of PPBS copolyesters, fabrication of CPT-loaded nanoparticles from these copolymers, drug release profiles of the particles, and the results on evaluation of both *in vitro* and *in vivo* antitumor efficacy of the nanoparticle formulations.

2. Materials and methods

2.1. Materials

Diethyl succinate (DES), 1,4-butanediol (BD), ω -pentadecalactone (PDL), and diphenyl ether were obtained in the highest available purity and were used as received (Aldrich Chemical Co). Immobilized CALB (*Candida antarctica* lipase B supported on acrylic resin) or Novozym 435, (*S*)-camptothecin (CPT), poly(vinyl alcohol) (PVA, M_w = 30,000–70,000, 87–89% hydrolyzed), chloroform (HPLC grade), chloroform-*d*, and methanol (98%) were also obtained (Aldrich Chemical Co). The lipase catalyst was dried at 50 °C under 2.0 mmHg for 20 h prior to use.

Lewis lung carcinoma (LLC) cells and 9L glioma cells were provided by the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in DMEM (Gibco) containing 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) penicillin-streptomycin at 37 °C under a 5% CO₂ humidified atmosphere.

2.2. Structural analysis and molecular weight measurements of copolyesters

Polymer compositions and microstructures were analyzed by NMR spectroscopy. ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE 500 spectrometer. The chemical shifts reported were referenced to internal tetramethylsilane (0.00 ppm) or to the solvent resonance at the appropriate frequency. The number and weight average molecular weights (M_n and M_w , respectively) of polymers were measured by gel permeation chromatography (GPC) using a Waters HPLC system equipped with a model 1515 isocratic pump, a 717 plus autosampler, and a 2414 refractive index (RI) detector with Waters Styragel columns HT6E and HT2 in series. Empower II GPC software was used for running the GPC instrument and for calculations. Both the Styragel columns and the RI detector were heated and maintained at 40 °C during sample analysis. Chloroform was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 2 mg/mL and injection volumes of 100 μ L were used. Polymer molecular weights were determined on the basis of a conventional calibration curve generated by narrow polydispersity polystyrene standards (Aldrich Chemical Co).

2.3. Solid state characterization of copolyesters

Differential Scanning Calorimetry (DSC) was used to determine melting points of copolyesters. DSC measurements were performed using a TA DSC-Q100 apparatus, equipped with a Liquid Nitrogen Cooling System (LNCS) accessory. Heating scans were run at 20°C/min heating rate from –90°C to 180°C, in a helium atmosphere. Between heating scans, either constant rate cooling (10°C/min) or quench cooling were applied. Melting temperature (T_m) was taken at the peak maximum of endotherms. Because the PPBS polyesters had a high degree of crystallinity, DSC was not effective in measuring glass transition temperature (T_g) of the polymers. Instead, dynamic mechanical (DMTA) measurements were employed to determine polymer α -relaxation temperature that is associated with the glass transition. DMTA

measurements were carried out on hot pressed rectangular films (40 mm x 8 mm, average thickness = 220 μ m) in tensile mode, at 3°/min and 3 Hz from -150 °C to 10 °C, using a DMTA MkII (Polymer Laboratories Ltd.). Wide angle X-ray diffraction measurements (WAXS) were carried out at room temperature with a PANalytical X'Pert PRO diffractometer equipped with an X'Celerator detector (for ultrafast data collection). A Cu anode was used as X-ray source (K radiation: $\lambda = 0.15418$ nm, 40 kV, 40 mA), and 1/4° divergence slit was used to collect the data in 2 θ range from 2° to 60°. After subtracting the diffractogram of an empty sample holder from the experimental diffraction curve, the amorphous and crystalline contributions in the resulting diffractogram were calculated by a fitting method using the Fityk software. The degree of crystallinity (χ_c) was evaluated as the ratio of the crystalline peak areas to the total area under the scattering curve.

2.4. Synthesis and purification of poly(PDL-co-butylene-co-succinate) (PPBS)

Polymerizations of PDL, DES, and BD were catalyzed by 10 wt-% Novozym 435 (vs. total monomer) in diphenyl ether (200 wt-% vs. total monomer) using a parallel synthesizer connected to a vacuum line with the vacuum (± 0.2 mmHg) controlled by a digital vacuum regulator. The monomer molar ratios of PDL to DES to BD employed for five reactions were 0.20:0.80:0.80, 0.35:0.65:0.65, 0.50:0.50:0.50, 0.65:0.35:0.35, and 0.79:0.21:0.21, respectively. All reactions were performed at 95 °C using a two-stage process: first stage oligomerization under 600 mmHg pressure for 18 hr followed by second stage polymerization under 2.0 mmHg pressure for 48 hr. At the end of the reactions, each product mixture was dissolved in chloroform and the resultant chloroform solution was filtered to remove the enzyme catalyst. After being concentrated under vacuum, the filtrate was added dropwise to methanol while stirring to cause precipitation of a white solid polymer. The five copolymers obtained from the synthesizer were then filtered, washed with methanol three times, and dried under vacuum at 30 °C overnight.

2.5. PPBS nanoparticle fabrication

Blank and CPT-loaded nanoparticles were fabricated using a modified oil-in-water (o/w) single emulsion technique. Briefly, 100 mg of PPBS copolymer and, optionally, 10 mg of CPT were co-dissolved in 2 ml of methylene chloride in a glass tube. The resultant organic solution was added dropwise to 4 ml of 5% PVA aqueous solution while vortexing. The mixture was subsequently sonicated three times (10 seconds each time) at 38% amplitude with a TMX 400 sonic disruptor (Tekmar, Cincinnati, OH) to yield a homogeneous oil-in-water emulsion. This emulsion was immediately poured into 100 ml of aqueous solution containing 0.3% PVA and the whole mixture was magnetically stirred in an open beaker at room temperature for 3 hrs. This process allows nanoparticles to form via gradual evaporation of the methylene chloride solvent. The nanoparticles formed by this method were collected by centrifugation at 11,000g for 15 min, washed three times with deionized water, re-suspended in 5 ml of aqueous solution containing 5% trehalose, and dried on a lyophilizer. The dried particles were stored at -20 °C in airtight containers.

2.6. Nanoparticle characterization and measurement of CPT contents in nanoparticles

The surface morphology and size of PPBS nanoparticles were analyzed using a XL30 ESEM scanning electron microscope (FEI Company). Particle samples were mounted on an aluminum stub using carbon adhesive tape and sputter-coated with a mixture of gold and palladium (60:40) in an argon atmosphere under low pressure using a Dynavac Mini Coater. The image-analysis application program, ImageJ, (developed by Wayne Rasband, NIH) was used to measure particle diameters, calculate average particle sizes, and determine particle size distributions. The sizes of nanoparticles in aqueous medium were also determined by dynamic

light scattering. The surface zeta-potential values of particles were measured with a Zeta-Potential Analyzer (Brookhaven Instruments Corp).

The amount of CPT drug encapsulated in PPBS nanoparticles was determined by measuring the intrinsic fluorescence of CPT using a SpectraMax spectrofluorometer (Molecular Devices). In a typical example, CPT-loaded nanoparticles (3 mg) were dissolved in dimethyl sulfoxide (DMSO, 1 ml). A small amount of the DMSO solution (10 μ l) was mixed with PBS (1.0 ml), sodium dodecyl sulfate (SDS, 10 μ l), and 1 N hydrochloric acid (10 μ l). The resultant mixture had a pH value of 3. The fluorescence intensity of the extracted CPT was measured at 428 nm emission wavelength (370 nm excitation wavelength).

2.7. Determination of in vitro drug release profiles of CPT-encapsulated PPBS nanoparticles

The rates of CPT release from PPBS nanoparticles were evaluated in PBS solution (pH = 7.4) at 37 °C using membrane dialysis. Briefly, 3 mg of CPT-loaded nanoparticles were added to 2 ml of PBS and the mixture was sonicated for 20 sec to disperse the particles. The nanoparticle suspension was then placed in a Pierce dialysis tube with molecular weight cutoff at 10,000 daltons, and the tube was subsequently immersed in fresh PBS (20 ml) and incubated at 37 °C on a rotary shaker set at a speed of 100 rpm/min. The liquid medium was completely replaced with fresh PBS at various preset intervals. The amount of CPT released was measured according to the method described in section 2.6.

2.8. Stability of CPT free drug and CPT encapsulated in PPBS nanoparticles

To investigate the stability of free CPT and CPT-loaded PPBS nanoparticles under physiological conditions, free CPT and CPT-loaded PPBS nanoparticles were incubated in PBS (pH 7.4, 37°C) or DMEM (pH 7.4, 37°C) with 10% FBS. For free CPT, at different time intervals (0.5, 1, 2, 4, 6, 24 hr), 20 μ l aliquots were withdrawn and immediately analyzed by HPLC to determine the ratio of lactone to carboxylate forms of CPT. For the CPT-loaded PPBS nanoparticles, 3 mg of nanoparticles were suspended in PBS or DMEM and put into a dialysis tube, which was subsequently immersed and incubated in the same medium following the method as described in section 2.7. Then 200 μ l nanoparticle suspension were withdrawn at various time intervals and centrifuged at 16,000 rpm/min. The obtained nanoparticles were lyophilized, dissolved in DMSO and appropriately diluted with PBS (pH 7.4), then immediately analyzed by HPLC to measure the ratio of lactone to carboxylate forms of CPT.

Analytical procedures using HPLC reported in literature were adopted to determine the lactone to carboxylate form ratio of CPT [33]. The HPLC instrument consisted of a Shimadzu SIL-10A system (Kyoto, Japan) with a Ascentis C18 separation column (15 cm $\times$ 4.6 mm, 5 μ m). Mixed solvent, 50 μ M phosphate buffer (pH 6.0)-acetonitrile-tetrahydrofuran (THF) (80:20:2, v/v), was used as the mobile phase that was maintained at 30 °C and pumped at a flow rate of 1.0 ml/min. The detection was performed using a fluorescence detector (Shimadzu RF-10A, Kyoto, Japan) with an excitation wavelength of 370 nm and emission wavelength of 450 nm. The CPT standards in carboxylate, lactone, and mixed carboxylate/lactone forms were prepared by dilution of a CPT stock solution (1 mg/ml in DMSO) to PBS with appropriate pH (10.0, 3.0, or 7.4, respectively).

2.9. In vitro cytotoxicity studies

The cytotoxicity of free CPT and CPT-loaded nanoparticles was tested against LLC and 9L cells using an MTS assay (Promega, WI). The cells were seeded in 96-well flat-bottomed microplates (BD Falcon) at a density of 4,000 cells per well. After allowing the cells to adhere overnight, the culture medium was removed and 100 μ L of the medium containing an appropriate amount of drug (0.001–100 μ M) was added to each well. Free CPT was dissolved in 1:9 (v/v) DMSO/DMEM and CPT-loaded nanoparticles were suspended in DMEM. The

cells were exposed to various concentrations of CPT or CPT-loaded nanoparticles at 37 °C for 24–72 hr. At the end of the incubation period, drug-containing medium was removed and the cells were thoroughly rinsed three times with cold PBS. Then, 100 µL of fresh medium and 20 µL of the MTS reagent were added to each well, and the microplates were incubated at 37 °C in darkness for 2 hr. After the incubation, the plates were placed on a rotational shaker for 10–15 minutes and allowed to cool to room temperature. MTS absorbance, which is related to the number of metabolically-active cells, was measured using a microplate reader (Molecular Devices).

2.10. Cellular uptake measurements

Flow cytometry was used to study the drug-associated intracellular fluorescence of monolayer cultures. One ml of suspended cells at a density of 5×10^5 cells/ml was seeded in each well of 12-well tissue culture plates (BD Falcon) and allowed to attach overnight. The cells were then exposed to various concentrations (0–100 µM) of free CPT or CPT-loaded nanoparticles for 2 hr. For the nanoparticles, the drug concentration was calculated using the total amount of CPT in the particles. After incubation, the drug-containing medium was removed and the cells were thoroughly rinsed twice with cold PBS. Subsequently, the treated cells were harvested using trypsin (0.05%), transferred to centrifuge tubes, and centrifuged for 5 min at 1,500 rpm/min. Upon removal of the supernatant, the cells were re-suspended in 0.5 ml of FACS buffer, transferred to roundbottom polystyrene test tubes (BD Falcon, 12×75 mm), and kept in the dark at 4 °C until analysis. Analytical flow cytometry was performed using a BD LSR-II instrument (Becton Dickinson, San Jose, CA) and data were collected and processed using BD FACSDiva software (Becton Dickinson). Signals for forward and side light scatter (excitation at 355 nm, fluorescence emission at 450/50 nm) were collected from 20,000 cells.

The ratio of carboxylate/lactone forms of CPT in the cells were also quantitated by HPLC. LLC cells (1×10^6) were incubated in DMEM containing 50 µM CPT which was added using either free CPT or CPT-loaded nanoparticles. At different time intervals (0.5, 2, and 4 hr), the cells were washed three times with ice-cold PBS (1 ml each time), harvested using trypsin (0.05%), and then centrifuged at 1,500 rpm/min for 5 min at 4 °C. Upon removal of the supernatant, the cells were suspended in 200 µl of PBS (pH 7.4) and disrupted by vigorous sonication. The resulting, sonicated mixture was frozen with liquid N₂, followed by rapid thawing. This process was repeated for three times. Subsequently, 200 µl of DMSO was added to the cell lysate solution to dissolve CPT and/or CPT nanoparticles. This cell lysate solution was then centrifuged at 16,000 rpm/min for 5 min at 4 °C and the supernatant was analyzed by HPLC according to the method described in Section 2.8 to determine the ratio of CPT lactone/carboxylate forms in the solution.

Confocal laser scanning microscopy was used to visualize the internalization of CPT-loaded PPBS nanoparticles with cultured LLC cells. LLC cells were grown to 50% confluent. After incubated for 2 hr with free CPT or CPT nanoparticles, the cells were washed and fixed, and the cell cytoskeletons were labeled with Texas-Red phalloidin. Images were obtained with a Leica TCS SP5 Spectral Confocal Microscope using a 63× objective.

2.11. In vivo antitumor activity evaluation

All animal care and studies were approved by Yale's Institutional Animal Care and Use Committee (IACUC). The antitumor activity of CPT-loaded nanoparticles was evaluated in mice bearing subcutaneous tumors of Lewis lung carcinoma (LLC). LLC cells (1×10^6 cells, 0.1 ml) were transplanted into C57BL/6 male mice (6 week old, Charles River Laboratories) subcutaneously, and drug treatments were started after 7 days, a time when the average tumor volume reached approximately 100 mm³. Forty mice were divided into five groups with eight animals in each group. The average size and size variation of the tumors in all groups were

comparable. CPT in different formulations was injected into the tail vein once or three times on day 7, day 11, and day 16 after tumor cell implantation. Free CPT was formulated as a PBS solution containing DMSO (10%, v/v) and Tween-80 (5%, v/v); CPT-loaded nanoparticles were suspended in PBS (pH 7.4). Mice were divided into five groups with each group receiving formulations as follows: (i) PBS (pH 7.4); (ii) blank 50% PDL-PPBS nanoparticles; (iii) free CPT at 10 mg/kg dose; (iv) CPT-encapsulated 50% PDL-PPBS nanoparticles as a single injection at 10 mg CPT/kg; (v) CPT-encapsulated 50% PDL-PPBS nanoparticles in multiple doses: 10 mg CPT/kg $\times$ 1 followed by 5 mg CPT/kg $\times$ 2. The tumor volumes and body weights of the mice were measured and recorded. Tumor volume was calculated as follows: volume = $1/2LW^2$, where L is the long diameter and W is the short diameter of a tumor. Animals were euthanized when tumor size exceeded 2000 mm³, body weight loss exceeded 20%, or other signals of sickness, such as breathing problems, failure to eat and drink, lethargy or abnormal posture, were observed.

2.12. Studies on CPT biodistribution in tumor-bearing mice

Six week old male C57BL/6 mice, with body weight between 18 and 22 g (Charles River Laboratories), were used to measure CPT biodistribution. Mice were allowed free access to food and water throughout the duration of the experiments and were kept in a 12 h alternating light cycle. At 1 week after subcutaneous transplantation of 1×10^6 LLC cells, when tumor size reached approximately 100 mm³, tumor-bearing mice were injected via a lateral tail vein with free CPT solution or CPT-loaded nanoparticles at a dose of 10 mg/kg of CPT. At 2 hr, 24 hr, and 48 hr after injection, mice were sacrificed via carbon dioxide inhalation and 0.5–1 ml whole blood was collected via cardiac puncture. Plasma was obtained by centrifugation of the blood at 3000 rpm for 5 min. One hundred microliters of plasma was added to 0.1 ml of 0.1% acetic acid followed by mixing. Methylene chloride (0.2 ml) was added to the resultant mixture, stirred vigorously for 1 min using a vortex mixer, and then centrifuged at 12,000 rpm for 5 min. The organic phase was collected for fluorescence measurement. The heart, liver, spleen, lung, kidney and tumor were excised, weighed, and frozen at -20°C . The tissues were homogenized in acidified DMSO. The fluorescence of the extracted CPT in DMSO was measured as previously described. The recovery ratios of CPT were 93%, 96%, 95%, 98%, 95%, and 96% for heart, liver, spleen, lung, kidney and tumor, respectively. For the plasma, the extraction efficiency was 72%. These recovery ratios were used for data correction.

2.13. Statistical analysis

Statistical tests were performed with a two-sided Student's T-test. A P -value of 0.05 or less was considered to be statistically significant.

3. Results and discussion

3.1 Preparation of Pure Poly(PDL-co-butylene-co-succinate) (PPBS) Copolymers

We recently reported the synthesis of aliphatic copolyesters via copolymerization of dialkyl diester with diol and lactone using a lipase catalyst [32]. Scheme 2 illustrates a general reaction, which is performed in two stages, for the synthesis of PPBS from PDL, DES, and BD monomers. Our early work was focused on copolymerizations using 1:1:1 molar ratio of the PDL/DES/BD monomers. The resulting terpolymers contained equal moles of PDL, succinate, and butylene repeat units in the polymer chains, which were nearly randomly distributed with all possible combinations of the repeat units via ester linkages in the polymer backbone. Thus, the synthesized terpolymers can be designated poly(PDL-co-butylene-co-succinate). Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) showed that the terpolymer with a 1:1:1 (PDL to succinate to butylene) unit ratio is a semicrystalline material with a T_m of 64°C and thermal stability up to 300°C .

Now, using similar procedures employed for the copolymerizations of 1:1:1 PDL/DES/BD mixture feed, the synthesis of PPBS copolymers with various unit ratios was achieved by varying the PDL/DES/BD monomer feed ratio. Table 1 summarizes the polymer yield, composition (repeat unit ratio), weight-average molecular weight (M_w), and polydispersity (M_w/M_n), along with solid state properties, for five pure PPBS copolymers synthesized using 0.20:0.80:0.80, 0.35:0.65:0.65, 0.50:0.50:0.50, 0.65:0.35:0.35, and 0.79:0.21:0.21 PDL/DES/BD monomer molar ratios. ^1H and ^{13}C NMR analyses showed that all polymer products were random copolymers with PDL, butylene, and succinate repeat units randomly distributed along the polymer chains. As shown in Table 1, the copolymers were obtained in good yields (92–95%) with M_w ranging from 85,000 to 103,000 and M_w/M_n between 2.3 and 3.8. Furthermore, consistent with the previous results, the copolymer compositions match the corresponding monomer feed ratios remarkably well. All PPBS copolyesters were highly crystalline with both melting point (T_m) and degree of crystallinity (χ_c) dependent on polymer composition. The degree of crystallinity increased with increasing PDL unit content in the polymer chains. The copolymers exhibited isodimorphic behavior with the pseudo-eutectic composition at 35 mol % PDL, where two different types of crystals coexisted in the polymer, resulting in two melting points (56 and 75 °C, Table 1). Because of the presence of PDL segments, PPBS copolymers possess higher hydrophobicity than either PLGA or PLA.

3.2. Physicochemical characteristics of CPT-loaded nanoparticles

PPBS copolymers are well-suited to encapsulation of hydrophobic drugs, due to the hydrophobicity of the PDL segments in the polymer backbone. CPT-loaded PPBS nanoparticles with high drug loading were prepared using a modified single emulsion technique (Table 2). For all the samples fabricated, the drug loading ranged from 12% to 22%. The nanoparticles with high PDL content had the highest drug loading but the lowest yields. Encapsulation efficiency was close to 95% except for the lowest PDL composition (20%). This high encapsulation efficiency was due to the hydrophobicity of PDL-rich chains and the extremely low water solubility of CPT, which was minimally lost to aqueous solution during nanoparticle preparation. All PPBS nanoparticles had a slightly negative surface charge, of about -10 mV. Previous work—primarily performed with liposomes—has demonstrated that particles with nearly neutral surface charge (i.e. particles with zeta potentials between -10 and $+10$ mV) experience decreased uptake by the reticuloendothelial system (RES), and therefore, increased blood circulation times [34,35]. Because of these prior results, we speculated that the slight charge exhibited by our particles may provide enhanced circulation time over other particles (such as those produced from PLGA, which typically are more strongly charged [36]).

All CPT-loaded PPBS nanoparticles were nearly spherical in shape with a smooth surface morphology (Figure 1, A and B). Image analysis of SEMs suggested that the majority (>95%) of the unhydrated PPBS particles had diameters in the range of 100 to 300 nm (Figure 1, C and D). There was no significant difference in morphology, surface roughness, or size found among nanoparticles made from the polymers with different PDL content.

The particle size distribution in aqueous medium was measured by dynamic light-scattering. Lyophilization increased the particle size from ~ 250 nm to $\sim 61,700$ nm (Figure 2), which indicated that the freeze-drying procedure caused nanoparticle aggregation. Freeze-drying the nanoparticles and storing of drug-loaded nanoparticles in the solid state is a proven approach to improve long-term stability of colloidal nanoparticles, but it can also generate stresses that induce aggregation and, in some cases, irreversible fusion of nanoparticles. Sugars such as trehalose, glucose and sucrose can act as lyoprotectants during freeze-drying [37]. In fact, we found that addition of 5% trehalose, 5% glucose, or 20% sucrose to the aqueous particle suspension prior to lyophilization allowed recovery of nanoparticles with sizes similar to those

in the original formulations. Trehalose was the best, leading to the closest recovery of original particle size; a similar result was reported previously [38,39]

3.3. In vitro drug release profile

In vitro release profiles were obtained by measuring the percentage of drug release with respect to the total amount of CPT encapsulated in PPBS nanoparticles (Figure 3). All particle compositions exhibited a biphasic release profile, consisting of a small initial burst followed by a sustained, more continuous release. Approximately 20–30% of the total drug was released within the first 24 hr from PPBS nanoparticles; the rest of the drug was continually released over a period of ~40 days, although release was slow after day 25. No lag time was observed, and almost 100% of the encapsulated drug was released. The CPT release rate decreased slightly with increasing PDL content (except for particles with 35% PDL). For intravenously- or orally-administrated nanoparticles, the particles must retain drug during circulation in plasma, thereby preventing premature drug release before the nanoparticles accumulate in the tumor. Unfortunately, CPT-loaded PLGA nanoparticles or liposomes always exhibit a substantial burst of release during the first few hours. For example, CPT-loaded PLGA microspheres release 50% of CPT within 15 hr [20] and only ~70% of encapsulated CPT is released from PLGA matrices. This effect is more serious with nanoparticles, because of their small diameter and large surface to volume ratio: in some cases, PLGA nanoparticles release 100% of CPT by only 10 hr [27]. Even nanoparticles of poly(ϵ -caprolactone) (PCL), which are considered to be slowly degrading and hydrophobic delivery materials, release almost 100% of loaded drug after 72 hr [21]. In contrast to these other materials, nanoparticles of PPBS copolymers contain large crystalline fractions, which limit the rate of drug permeation and release. In addition, crystal phases in nanoparticles limit water diffusion into the core of the nanoparticles. These physical properties of the PPBS particles slow down CPT diffusion, which results in a decreased burst and slow release. These features make PPBS nanoparticles more suitable than PLGA or other materials for long-term CPT delivery. In addition, the properties of the nanoparticles can be potentially tuned by varying the polymer molecular weight and composition.

3.4. Stability of CPT free drug and CPT encapsulated in PPBS nanoparticles

To investigate the stabilizing effects of PPBS nanoparticles on the lactone structure of CPT under physiological conditions (pH 7.4, 37°C), the lactone and carboxylate forms of CPT were quantitatively analyzed by HPLC. Figure 4 (A) shows typical chromatograms for three separate CPT samples containing a) CPT in the essentially pure carboxylate form, b) CPT in the essentially pure lactone form, and c) CPT with a mixture of carboxylate and lactone forms. CPT structures in the carboxylate and lactone forms were well separated and their retention times were 3.5 and 14.6 min, respectively. The stability of the CPT lactone form at 37 °C in PBS and DMEM is delineated in Figure 4 (B). Free CPT in PBS solution exhibited a rapid hydrolytical conversion of lactone to carboxylate structures, resulting in only 25% content of the lactone form after 4 hr of incubation. The rate of lactone hydrolysis was substantially accelerated when free CPT was incubated in serum-containing DMEM medium: more than 70% of the lactone structure was converted to the carboxylate form within 30 min, and only approximately 10% lactone form remained after incubation for 2 hr. In contrast, essentially no lactone-to-carboxylate structural conversion was observed when CPT encapsulated in PPBS nanoparticles was incubated in both PBS and DMEM media for at least 24 hr. These results demonstrate the effectiveness of drug encapsulation within nanoparticles of the PPBS copolyester in stabilizing the active lactone form of CPT.

3.5. In vitro cytotoxicity

To examine whether PPBS-encapsulated CPT maintains its anticancer activity, we examined its cytotoxicity to two cell lines: Lewis lung carcinoma (LLC) and 9L glioma. Blank nanoparticles (i.e. particles with no CPT) had no cytotoxic effect on either of the two cell lines, even when added at up to 3 mg/ml (data not shown). Treatment of LLC or 9L cells with increasing concentrations of free CPT or CPT nanoparticles resulted in a significant increase in cell killing (Figure 5): cytotoxicity was observed after 24 hr exposure and increased in all cases with longer (72 hr) exposure. Compared to free CPT, CPT nanoparticles induced a significant cytotoxic effect at a lower CPT concentration, particularly at low drug concentration ($< 1 \mu\text{M}$) (Figure 5). For example, the IC_{50} of CPT-loaded 20% PDL-PPBS nanoparticles was 8.1 and 0.57 nM after 24 and 72 hr exposure to LLC cells, respectively; the IC_{50} of CPT-loaded 50% PDL-PPBS nanoparticles was 2.8 and 0.32 nM, respectively; and the IC_{50} of free CPT was 80 and 9.4 nM, respectively (Table 3). Moreover, based on in vitro measured drug release (Figure 3), we noticed that only ~40% of CPT was released from nanoparticles during the first 72 hr, suggesting that nanoparticle preparations are even more potent on a per drug released basis. We believe that two factors account for this enhanced cytotoxic effect: a) nanoparticles increase the aqueous solubility of CPT and maintain the CPT in an active form (as confirmed above) and b) enhancement of drug uptake by endocytosis of drug-loaded nanoparticles (as described in the paragraphs that follow).

3.6. Cellular uptake

We took advantage of the intrinsic fluorescence of CPT and investigated the intracellular delivery of CPT to cancer cells by flow cytometry. LLC cells were treated with different CPT formulations, and uptake of CPT into the cells was measured. LLC cells were exposed to two concentrations (50 and 100 μM) of either CPT or CPT-loaded PPBS nanoparticles for 2 hr (Figure 6). Nanoparticle treatment produced a significantly higher drug accumulation within LLC cells, as evidenced by the increase in fluorescence intensity; this was particularly obvious for the lower concentration, where fluorescence intensity was 2 $\times$ higher with nanoparticles ($p < 0.01$). Even at CPT concentrations of 100 μM , where the difference between free and particle-encapsulated drug was smaller, the difference was still significant ($p < 0.05$). Our observations of nanoparticle uptake by cells is similar to that observed with PLGA nanoparticles, which is a size-, time-, and concentration-dependent endocytic process [30,31, 40]. The smaller particles with higher concentrations showed increasing cellular internalization efficiency [41]. Furthermore, a small fraction of PVA used in the formulation of PPBS nanoparticles remained associated with the nanoparticle surface, which might also increase the cellular uptake efficiency of PPBS nanoparticles [22,42]. More importantly, for these biodegradable PPBS nanoparticles, intracellular drug levels will be maintained by sustaining release of the drug in the cytoplasm [23].

To further verify the effect of nanoparticles on the cellular drug uptake, and to determine the structural form of CPT available within cells, LLC cells were incubated in DMEM with serum containing 50 μM of CPT, which was added as either free CPT or CPT-loaded PPBS nanoparticles. The total concentration of intracellular CPT and the ratio of its carboxylate to lactone forms were measured by HPLC. The total concentration of CPT in LLC cells was substantially higher in the nanoparticle-treated group than in the free CPT-treated group (Figure 7); this finding is consistent with the measurements made by flow cytometry (Figure 6). Furthermore, after 4 hours of incubation, approximately 90% of total intracellular CPT was still in the lactone form for cells treated with CPT-loaded nanoparticles. In comparison, for free CPT-treated cells, the percentage of drug in the lactone form was lower: 66, 88, and 76% after 0.5, 2, and 4 hr of incubation, respectively. Since we know that less than 10% of the lactone form remains after free CPT incubation for 2 hr in serum-containing DMEM medium, this result suggests that, for cells treated with free CPT, there is preferential cellular uptake of

the lactone form versus the carboxylate form of CPT, which is consistent with previous reports [43]. We also note that acidic intracellular conditions might shift the equilibrium between the CPT lactone and carboxylate forms towards the lactone [33]. Confocal microscopic images (Figure 8) confirmed that CPT-loaded PPBS nanoparticles were internalized by cells and distributed in the cell cytoplasm, which supports our interpretation of the quantitative measurements of cellular uptake of nanoparticles.

3.7. In vivo antitumor efficacy

We tested the *in vivo* antitumor efficacy of CPT nanoparticles by i.v. injection into mice with subcutaneous LLC tumors. CPT-loaded 50% PDL-PPBS nanoparticles were selected for use because of their smaller size distribution (Table 2), better cytotoxic effect (Figure 5), and reasonable cellular uptake efficiency (Figure 6, Figure 7) among the five PPBS nanoparticle preparations. The growth rate of LLC tumors in mice treated with blank PPBS nanoparticles was indistinguishable from that in PBS-treated mice (Figure 9), indicating that PPBS nanoparticles alone have no effect on tumor growth. The dose of CPT (10 mg/kg) was based on our experience with tolerable doses in healthy C57BL/6 mice and is consistent with the literature [15]: in our pilot studies, a single i.v. administration of 30 mg/kg of free CPT caused substantial weight loss (>20%) in ~10% of animals whereas 30 mg/kg of CPT-loaded nanoparticles caused almost no weight loss. The group treated with free CPT injections showed a significantly smaller tumor volume than the PBS control group ($p < 0.01$). In contrast, the tumor volume in CPT/PPBS NP-treated mice was significantly smaller than that of mice treated with either PBS or free CPT ($p < 0.01$). For example, injection of free CPT at 10 mg/kg suppressed tumor size by 62% at day 16, compared with the PBS control group, whereas injection of CPT-loaded PPBS nanoparticles at the same dose decreased tumor volume by 83%. Further reduction in tumor growth was observed with repeat nanoparticle injections. Thus, a 90% tumor volume reduction was achieved by two PPBS nanoparticle injections, initially at 10 mg/kg and subsequently at 5 mg/kg after 4 days.

Table 4 compares antitumor efficiency of various CPT formulations reported in literature. Although there are important differences in the animal models and endpoints that have been used to test CPT delivery systems in the past, which make it difficult to make a definitive statement, our PPBS nanoparticle formulations appear to be comparable to the best previously reported systems (e.g., PLA/PEG-PPG-PEG) that have been developed for CPT delivery.

We also note that CPT-loaded PPBS nanoparticles were better tolerated than free CPT. The group treated with three serial injections of CPT/PPBS nanoparticles showed no obvious body weight loss (data not shown), whereas animals injected with only 1 dose of free CPT lost ~5% body weight. This finding suggests that even higher doses of CPT may be safely delivered via PPBS nanoparticles.

3.8. Biodistribution of CPT in tumor-bearing mice

The biodistribution of CPT in major tissues and tumors was monitored for 48 hr after i.v. injection of either free CPT or CPT nanoparticles to mice bearing subcutaneous LLC tumors (Figure 10). CPT-loaded PPBS nanoparticles exhibited higher accumulation in lung, liver, spleen and tumor than free CPT; additionally, higher drug retention in the mouse body was observed at 2 and 24 hr after injection with the CPT nanoparticles (20.8% and 2.5% of injected dose of CPT, respectively) vs. free CPT (3.45% and 0.3%, respectively). For nanoparticles, an initial localization was found in the lungs, which dropped from 136 $\mu\text{g/g}$ tissue (2 hr) to 19 $\mu\text{g/g}$ tissue (24 hr). Tumor accumulation of CPT in nanoparticles increased with time. Forty-eight hours after injection, CPT nanoparticles showed 3.5 times higher accumulation in the tumor compared to free CPT (Figure 11); because of the size of our particles, we attribute this accumulation in tumor tissue to the well-known EPR effect [44, 45]. The plasma CPT

concentration decreased for both CPT nanoparticles and free CPT during the 48 hrs after injection; there was no significant difference in rate of CPT disappearance from plasma for nanoparticles and free drug. This result is similar to previous work, where rats injected with CPT nanoparticles did not necessarily exhibit enhanced plasma concentrations of CPT [28, 46, 47]. The CPT-loaded PPBS nanoparticles yield good retention in the body, good tumor localization, and prolonged release of CPT, factors that we believe contributed to a superior anti-tumor efficacy.

4. Conclusion

We employed a series of newly synthesized aliphatic copolyesters, PPBS, as carriers for CPT delivery. CPT-encapsulated PPBS nanoparticles with diameters of 100–300 nm and drug loading up to 22% were fabricated. These nanoparticles had low toxicity to cultured cells, a low burst of CPT release within the first three days, and sustained release for the subsequent 40 days. Upon encapsulation, the active lactone form of CPT was remarkably stabilized. Compared to free CPT, CPT-loaded PPBS nanoparticles showed a significant enhancement of cellular uptake, higher cytotoxicity against Lewis lung carcinoma and 9L cell lines *in vitro*, a longer circulation time, and substantially better antitumor efficacy *in vivo*.

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Fig. 1. SEM images and particle size distributions of unhydrated 20% PDL-PPBS NP (A, C) and 50% PDL-PPBS NP (B, D).

Fig. 2. Effect of sugars on mean size stability of particles subjected to lyophilization (□) and resuspension (■). Size distributions were determined by dynamic light scattering (mean $\pm$ SD; $n = 10$).

Fig. 3.

In vitro cumulative release of CPT from different PPBS nanoparticles. Data were given as mean $\pm$ SD (n = 3).

Fig. 4. The stability of free CPT and CPT-loaded PPBS nanoparticles under different conditions. (A) HPLC chromatograms of carboxylate form and lactone form of CPT in different pH buffers. (B) Stability of CPT lactone form in different solutions (pH 7.4, 37°C): (•) Free CPT in PBS; (○) Free CPT in DMEM; (Δ) CPT nanoparticle in PBS; (*) CPT nanoparticle in DMEM.

Fig. 5.
In vitro cytotoxicity of CPT-loaded 20% PDL PPBS nanoparticles (○), 50% PDL PPBS nanoparticles (Δ) and free CPT (•) against (i) LLC cell line after 24 (A) and 72 hr (B); (ii) 9L cell line after 24 (C) and 72 hr (D). Data were given as mean $\pm$ SD (n = 4).

Fig. 6. Uptake of CPT-loaded PPBS nanoparticles and free CPT by LLC cells: (i) flow cytometric histogram profiles of fluorescence intensity for cells treated with 50 μ M (A) and 100 μ M (B) of CPT; (ii) quantitative uptake of CPT-loaded nanoparticles and free CPT in different drug concentrations (C). Data were given as mean $\pm$ SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ compared with free CPT.

Fig. 7.
Concentrations of total CPT, carboxylate form, and lactone form of CPT in LLC cells after incubation with free CPT or CPT-loaded PPBS nanoparticles.

Fig. 8. Confocal microscopic images of LLC cells after 2 hr incubation with (A) free CPT and (B) CPT-loaded 50%-PDL PPBS nanoparticles. Cells and CPT are visualized in the red and blue channels, respectively.

Fig. 9.

Antitumor effects of CPT/50% PDL-PPBS nanoparticles, free CPT, blank PPBS nanoparticles, and PBS on C57BL/6 mice bearing LLC. Data were given as mean $\pm$ SEM ($n = 8$). Compared to the free CPT group, both CPT/PPBS NP treatment groups showed substantially smaller tumor volume ($p < 0.01$).

^a Multiple injections of CPT/PPBS nanoparticles: initial injection at 10 mg/kg followed by two separate injections at 5 mg/kg; arrows indicate the days of drug injections.

Fig. 10.

Biodistribution of CPT at 2 hr (A), 24 hr (B), and 48 hr (C) after i.v. injection of CPT-loaded 50% PDL-PPBS nanoparticles or free CPT into mice bearing LLC. Data were given as mean $\pm$ SD (n=3). * $p < 0.05$ and ** $p < 0.01$ compared with free CPT results.

Fig. 11. CPT concentration change in tumor (A) and plasma (B) at various time points after injection of 50% PDL-PPBS nanoparticles (○) or free CPT (▪). Data were given as mean $\pm$ SD (n = 3). * $p < 0.05$ compared with free CPT.

Scheme 1.
pH-dependent equilibrium of camptothecin.

Scheme 2.

Two-Stage Process for Copolymerization of PDL, DES, and BD.

Table 1

Characterization of the isolated poly(PDL-co-butylene-co-succinate) copolymers.

PDL/DES/BD (Molar Ratio)	Polymer Yield	PDL/Succinate/Butylene Unit Ratio ^a	PDL Unit Content ^b (mol %)	Copolymer		T_g^c (°C)	T_m^d (°C)	χ_c^e (%)
				M_w	M_w/M_n			
0.20:0.80:0.80	94%	0.20:0.80:0.80	20	103000	3.8	-37	92	47
0.35:0.65:0.65	92%	0.35:0.65:0.65	35	101000	3.4	-35	75, 56	52
0.50:0.50:0.50	95%	0.50:0.50:0.50	50	85400	3.5	-33	64	54
0.65:0.35:0.35	94%	0.65:0.35:0.35	65	100000	3.5	-29	72	59
0.79:0.21:0.21	93%	0.79:0.21:0.21	79	91100	2.3	-23	81	64

^aCalculated from the proton NMR spectra.

^bMolar ratio of PDL units vs. total (PDL+succinate) units in the polymer chains.

^cPeak temperature of main relaxation determined by DMTA (dynamic mechanical measurements)

^dMelting point measured by DSC (differential scanning calorimetry).

^eCrystallinity degree from WAXS (wide angle X-ray diffraction measurements).

Table 2

Characteristics of the CPT-loaded PPBS nanoparticles (mean $\pm$ SD; n = 3).

Copolymer PDL content (mol%)	Particle mass Yield (%)	Drug loading (%)	Encapsulation Efficiency (%)	Particle size distribution (nm)	Zeta Potential (mV)
20%	69.7	12 $\pm$ 2	85	178 $\pm$ 48	-15.46 $\pm$ 0.48
35%	50.2	18 $\pm$ 0.4	91	208 $\pm$ 49	-12.05 $\pm$ 0.59
50%	59.8	16 $\pm$ 0.9	96	185 $\pm$ 47	-10.76 $\pm$ 0.63
65%	47.6	20 $\pm$ 0.9	96	215 $\pm$ 60	-13.59 $\pm$ 0.86
79%	42.3	22 $\pm$ 1	93	255 $\pm$ 74	-13.41 $\pm$ 0.45

Table 3IC₅₀ (nM) of PPBS nanoparticles compared to free CPT against LLC and 9L cell lines

	Incubation time	20% PDL	50% PDL	Free CPT
LLC	24 hr	8.1	2.8	80
	72 hr	0.57	0.32	9.4
9L	24 hr	334	165	728
	72 hr	16.2	19.8	482

Table 4

Comparison of tumor growth inhibition after injection of different CPT formulations.

Carrier ^a	Tumor model	Days after tumor inoculation	Total dose (mg/kg)	Routes ^b	Tumor growth inhibition ^c	Ref.
HGC	MDA-MB231	32	10	Single	32%	[3]
PEG-P(Asp(Bz-70))	Colon 26	15	30	Single	23%	
			15	Single	27%	
HAS-DB-L	Colon 26	15	30	Single	18%	[15]
			30	10 × 3	41%	
			10	Single	35%	
PLA/PEG-PPG-PEG	Sarcoma 180	22	15	Single	15%	[12]
			20	10 × 2	Mice dead	
			7.5	2.5 × 3	~ 10%	
EndoTAG@-2	LLC	18	15	2.5 × 6	20%	[47]
			10	Single	17%	
			15	10 + 5	10%	
50% PDL-PPBS	LLC	16	15			[14]
						[this work]

^a **HGC**: hydrophobically modified glycol chitosan;
PEG-P(Asp(Bz-70)): poly (ethylene glycol)-poly (benzyl aspartate-70);
HAS-DB-L: PEG and human serum albumin coated 3,5-bis (dodecyloxy) benzoic acid;
PLA/PEG-PPG-PEG: poly(DL-lactic acid) and poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) copolymer
EndoTAG@-2: DOTAP complexed camptothecin
50% PDL-PPBS: Poly(ω-pentadecalactone-co-butylene-co-succinate) containing 50% PDL units

^b All animals received i.v. injection via a lateral tail vein.

^c Tumor growth inhibition (*T/C* %) was calculated according to the following equation: *T/C* % = (mean tumor volume of treated group)/(mean tumor volume of control group)