Stabilization of 10-Hydroxycamptothecin in Poly(lactide-*co*-glycolide) Microsphere Delivery Vehicles

Anna Shenderova,[1] Thomas G. Burke[1,2] and Steven P. Schwendeman[1,3]

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Purpose. The purpose of this study was to investigate the potential of poly(lactide-*co*-glycolide) (PLGA) microspheres to stabilize and deliver the analogue of camptothecin, 10-hydroxycamptothecin (10-HCPT).

Methods. 10-HCPT was encapsulated in PLGA 50:50 microspheres by using an oil-in-water emulsion-solvent evaporation method. The influence of encapsulation conditions (i.e., polymer molecular weight (M_w), polymer concentration, and carrier solvent composition) on the release of 10-HCPT from microspheres at 37°C under perfect sink conditions was examined. Analysis of the drug stability in the microspheres was performed by two methods: *i*) by extraction of 10-HCPT from microspheres and *ii*) by sampling release media before lactone—carboxylate conversion could take place.

Results. Microspheres made of low M_w polymer (inherent viscosity 0.15 dl/g) exhibited more continuous drug release than those prepared from polymers of higher M_w (i.v. = 0.58 and 1.07 dl/g). In addition, a high polymer concentration and the presence of cosolvent in the carrier solution to dissolve 10-HCPT were both necessary in the microsphere preparation in order to eliminate a large initial burst of the released 10-HCPT. An optimal microsphere formulation released 10-HCPT slowly and continuously for over two months with a relatively small initial burst of the released drug. Both analytical methods used to assess the stability of 10-HCPT revealed that the unreleased camptothecin analogue in the microspheres remained in its active lactone form (>95%) over the entire 2-month duration of study.

Conclusions. PLGA carriers such as those described here may be clinically useful to stabilize and deliver camptothecins for the treatment of cancer.

KEY WORDS: camptothecin; PLGA microspheres; controlled release; cancer chemotherapy.

INTRODUCTION

Camptothecin and related analogues are a promising family of anticancer agents with an unique mechanism of action, targeting the nuclear enzyme topoisomerase I (1). These agents possess the ability to inhibit the growth of a wide range of tumors. Therefore, camptothecin and its more potent analogues have recently undergone extensive clinical evaluation worldwide (1–4). Structure-activity investigations elucidated that the preservation of the lactone ring of camptothecins is crucial for their anti-tumor activity (5). However, the delivery of the active form is quite challenging, since the lactone exists in a pH-dependent equilibrium with an open carboxylate form (Fig. 1). The sodium salt of the carboxylate was found to be significantly less potent than the lactone against *in vivo* tumor models (6) and highly toxic in earlier clinical trials (1). At physiological pH more than 80% of the drug exists as the carboxylate at equilibrium, whereas at pH below 5 essentially all drug is in lactone form (7). For 10-hydroxycamptothecin (10-HCPT), the analogue used in the present study, lactone-carboxylate hydrolysis at pH 7.4 occurs with a $t_{1/2}$ of 21 min and the fraction of lactone at equilibrium is ~16% (8).

A variety of dosage regimens for camptothecin and its more potent analogues were tested in clinical trials to improve the delivery of the active lactone. In spite of differing protocols among analogues, the common delivery requirement was a continuous administration over extended periods of time. For example, camptothecins were administered as a 72–120 h continuous i. v. infusion every 2–4 weeks, a 30 min infusion daily × 5 every 2–4 weeks, and as long as a 21-day continuous i.v. infusion given every 4 weeks (1–4). Thus, the development of a controlled-release formulation of camptothecins that could continuously deliver the active form of the drug appears to be an attractive goal to pursue. Drug delivery approaches to date aimed at promoting active lactone levels have included the use of liposomes (9) and poly(ethylene glycol)—drug conjugates (10).

In the present study, poly(lactide-*co*-glycolide) (PLGA) microspheres were selected for development as camptothecin slow-release vehicles. PLGA microspheres have been used for the sustained delivery of peptides as well as small water-soluble and lipophilic compounds due to their biocompatability and biodegradability (11–13). In addition, PLGA microspheres can be administered by injection with minimal discomfort.

PLGA is composed of lactic and glycolic acids linked together by ester bonds. The polymer degradation proceeds with the formation of free carboxylic end groups. Several current reports have indicated the presence of low microenvironmental pH in the large specimens of PLGA (14). The presence of an acidic pH inside PLGA has been proposed for microspheres, but has not been definitively confirmed to date. Nevertheless, this hypothesized property may favor the stabilization of camptothecins in their active lactone form. Herein, the first purpose of this study was to access the potential of PLGA to stabilize camptothecins. The second objective was to prepare

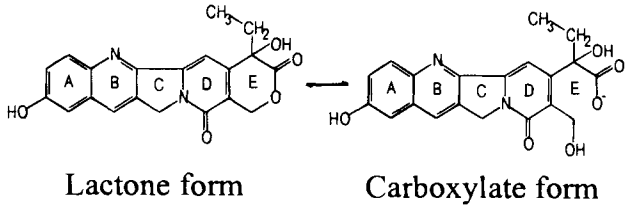

Fig. 1. The structure of 10-hydroxycamptothecin (10-HCPT). In an aqueous environment 10-HCPT undergoes reversible pH-sensitive hydrolysis with opening of the lactone E-ring to form the carboxylate isoform.

[1] Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, The Ohio State University, 500 West 12th Ave., Columbus, Ohio 43210.

[2] Department of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, and The Markey Cancer Center, University of Kentucky, Lexington, Kentucky 40506.

[3] To whom correspondence should be addressed. (e-mail: schwende@dendrite.pharmacy.ohio-state.edu)

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microsphere formulations of 10-HCPT, the camptothecin analogue, which can release the drug in sustained manner for several months.

MATERIALS AND METHODS

Chemicals

10-HCPT (>96% purity) was obtained from Dabur India Limited (New Delhi, India). PLGA with inherent viscosities of 0.15, 0.58 and 1.07 dl/g (PLGA0.15, PLGA0.58 and PLGA1.07) and copolymer ratio of D,L-lactide to glycolide 50:50 were purchased form Birmingham Polymers (Birmingham, AL). Polyvinylalcohol, 80% hydrolyzed and 9–10 kDa molecular weight was obtained from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade or purer and purchased from commercial suppliers.

HPLC Analysis of 10-HCPT

Carboxylate and lactone levels were determined by high performance liquid chromatography (HPLC), which allows separation of the two forms of the drug within a single chromatographic run. The HPLC system consisted of the following: a Waters 510 pump (Milford, MA), a Reodyne injector fitted with a 100 μl sample loop (Woburn, MA), a Gilson 121 fluorescence detector with 305–395 nm excitation and 430–470 nm emission filters (Middleton, WI), and a Hewlett Packard 3395 integrator (Palo Alto, CA). The separation was carried out using a Waters Nova-Pack C_{18} 3.9 × 150 mm reverse phase column. The mobile phase, composed of 20% by volume acetonitrile and 80% aqueous solution (0.1 M KH_2PO_4, 0.5 mM tetrabutylammonium dihydrogen phosphate, and 0.4 mM triethyl amine pH = 6.0), was delivered with a flow rate of 1 ml/min. Retention times of the carboxylate and lactone were 2.0 and 4.4 min, respectively. A calibration curve was used for the conversion of total lactone peak area to concentration. Total lactone area was calculated as the lactone area + carboxylate area*K, where K is the response factor defined as the ratio of molar fluorescence intensities of the lactone to carboxylate form.

Preparation of the Microspheres

A standard oil-in-water emulsion-solvent evaporation method was used for preparation of the microspheres (15). Briefly, the polymer was dissolved in methylene chloride at concentrations of 200, 400 and 1067 mg/ml for PLGA1.07, PLGA0.58 and PLGA0.15, respectively. Polymer concentrations were adjusted to these values in order to obtain similar viscosities of the carrier solutions and, respectively, a similar size range for the microspheres. Polymer concentrations from 533 to 1067 mg/ml were used in preparations with PLGA0.15 for the optimization of loading, encapsulation efficiency and drug release characteristics of the formulation. 10-HCPT was either dissolved in dimethylformamide (DMF) or suspended in the polymer solution to a concentration of 6.67 mg/ml in the carrier solution, where the carrier solution composition was varied from 0:1, 1:3, and 1:4 v/v ratios of DMF to methylene chloride. Polymer and drug solutions were mixed together to a total volume of ~0.3 ml and then emulsified with 0.6 ml of aqueous PVA solution (1% w/v) by brief vortexing (20 s). Then, the emulsion was poured in 30 ml of aqueous PVA solution (0.3% w/v) and kept stirring for 3 hours at room temperature and atmospheric pressure. After evaporation of the organic solvent the hardened microspheres were washed 3 times with double distilled water by centrifugation at 3000 rpm (1090 × g) for 10 minutes. After the final spin, the microspheres were resuspended in water, flash frozen with liquid N_2, and lyophilized with Labconco Freezone 6 system for 2 days. The final product was stored desiccated at −20°C until further use.

Determination of Drug Loading and Encapsulation Efficiency

10-HCPT was extracted from the PLGA microspheres with dimethyl sulfoxide (DMSO). Microspheres were immersed in excess DMSO and vigorously vortexed. The undissolved polymer was spun down by brief centrifugation. An aliquot of the supernatant containing 10-HCPT was removed and properly diluted prior to HPLC analysis. Drug loading and encapsulation efficiency were calculated from the ratios of the mass of drug in the microspheres:the mass of the microspheres, and the mass of the drug in the microspheres:the mass of the total drug used in preparation, respectively.

Determination of Microsphere Size Distribution

Greater than one hundred particles for each preparation were sized by sight under a Zeiss Axiolab light microscope equipped with a 10× objective and a sizing scale bar.

Evaluation of *in Vitro* Drug Release

Drug release from microspheres was carried out in PBS (137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1mM KH_2PO_4, pH = 7.4) containing Tween 80 (0.02% v/w) at 37°C under prefect sink conditions. Microspheres were placed in dialysis bags and buffer was replaced at frequent time intervals initially to characterize the burst of drug from particles, and then at less frequent intervals to examine the long-term release behavior. Concentrations of both active lactone and carboxylate forms of 10-HCPT in the release media were determined by the HPLC assay.

Examination of the Stability of 10-HCPT in PLGA Microspheres

The extent to which 10-HCPT remained stable in the microspheres was evaluated by two methods: 1) by extracting the drug from microspheres and 2) by capturing the 10-HCPT in the release media before significant lactone ↔ carboxylate conversion could take place. First, microspheres were removed from the release media at various times during release incubation and cleaned with water by successive centrifugation in order to eliminate the released drug. Then, to examine drug content by the method 1, 10-HCPT was extracted from the microspheres with DMSO as described previously (*see* Determination of Drug Loading and Encapsulation Efficiency), and its composition was analyzed by HPLC. To evaluate drug composition by method 2, the washed microspheres were first exposed to PBS pH = 6.5 (same composition as given above, pH adjusted by 1N HCl) for 10 minutes at 37°C and then released 10-HCPT was immediately analyzed before significant lactone ↔ carboxylate conversion could take place. The

pH = 6.5 was chosen because the lactone ↔ carboxylate conversion rate is minimal at this pH (e.g., $t_{1/2}$ = 89 and $t_{1/2}$ = 82 min for forward and reverse directions, respectively). Control experiments were conducted to assure that no significant conversion takes place within 10 minutes of exposure to PBS pH = 6.5. It was found that <8% of drug converts in this time interval. By using the second method, it was possible to determine not only what form of 10-HCPT is present in the microspheres, but also which form actually is released from the microspheres.

Confocal Microscopy

10-HCPT, like many of the camptothecins, is highly fluorescent. Therefore, it was possible to analyze the drug distribution in PLGA microspheres by confocal microscopy. For the microscopic analyses microspheres were resuspended in water and placed on a glass slide. Images were then obtained using a BIO-RAD 600 confocal laser scanning system equipped with an argon-krypton laser. The excitation and emission wavelengths were 488 and 515 nm, respectively. A 63× objective was used for magnification. Median cross-sections of the microspheres were examined.

Scanning Electron Microscopy

Images of the microspheres were obtained by using a Philips XL-30 Field Emission Gun Scanning Electron Microscope. Samples were gold coated prior to the analysis. An accelerating potential of 5 keV was used and the images were obtained with a scintillating secondary electron detector.

RESULTS

Effect of Encapsulation Conditions on the Incorporation of 10-HCPT in PLGA Microspheres

Three microsphere parameters were examined to assess the quality of 10-HCPT microencapsulation: encapsulation efficiency (and drug loading), microsphere size distribution, and microsphere morphology.

The influence of the encapsulation conditions (i.e., polymer M_w, polymer concentration in the carrier solution, and carrier solution composition) on the encapsulation outcome (i.e., drug loading, encapsulation efficiency, and the size distribution of the microspheres) are summarized in Table 1. Higher drug loading was achieved in the formulation when PLGA of higher M_w was used. The loading of 2% was obtained for PLGA1.07, whereas for PLGA0.15 only 0.4% was recorded. This trend corresponded to the decreased amount of polymer that was required at higher M_w to attain similar polymer solution viscosity across the M_w range studied. The encapsulation efficiency decreased with a decrease in polymer concentration in the carrier solution, from a value of 94% when the PLGA0.15 concentration was 1067 mg/ml, to 53% for 533 mg/ml concentration. The drug loading remained roughly constant (~0.6–0.75) in this case, since the decreased amount of polymer in the formulation compensated largely for the decline in encapsulation efficiency. With an increase in DMF content within the carrier solution both the encapsulation efficiency and the drug loading dropped. In the case where no cosolvent was used in the microsphere preparation, a high standard deviation was obtained in measurements of loading and encapsulation efficiency, indicative of a nonhomogeneous distribution of drug particles in the microspheres.

The size of the low M_w PLGA microspheres (mean = 82 μm) was larger than the size of the PLGA0.58 and PLGA1.07 preparations (mean = 27 and 36 μm, respectively) when prepared using the same carrier solvent composition. This was likely due to variability in the control of concentration-viscosity variations with the use of different M_w PLGA and possible variability in the extent of particle shrinkage during in-liquid hardening. For the PLGA0.15 preparations the particle size was decreased with the reduction of polymer concentration in the 1:3 DMF:CH_2Cl_2 (v/v) carrier solvent. Mean particle size declined from 53 μm for the 1067 mg/ml microspheres to 36 μm for 800 mg/ml and 28 μm for 533 mg/ml PLGA0.15 concentrations, consistent with the well-known polymer concentration effect (i.e., increased polymer concentration causes increased solution viscosity, which causes larger droplets in the oil-in-water emulsion at constant mixing rate) (15).

The surface of the microspheres was characterized by scanning electron microscopy. The images of high, medium, and low molecular weight microspheres are presented in Fig. 2. All preparations were spherical in shape. The micrographs also revealed the presence of drug crystals on the surface of PLGA1.07 (A) and PLGA0.58 (B) microspheres, whereas the surface of the low molecular weight PLGA microspheres was smooth with no evidence of drug crystallization (C).

Table I. Characterization of Microspheres Prepared Under Various Microencapsulation Conditions

Polymer type	Carrier solvent composition (DMF:CH_2Cl_2 v/v)	Polymer concentration (mg/ml)	Loading (%)[a,c]	Encapsulation efficiency (%)[b,c]	Size of the microspheres (μm)[d]
PLGA1.07	1:2	200	2.0 ± 0.3	43 ± 5	27 ± 13
PLGA0.58	1:2	400	1.2 ± 0.2	45 ± 1	36 ± 18
PLGA0.15	1:2	1067	0.41 ± 0.05	67 ± 8	82 ± 41
PLGA0.15	0:1	1067	0.6 ± 0.2	94 ± 32	34 ± 23
PLGA0.15	1:3	1067	0.59 ± 0.04	95 ± 7	53 ± 20
PLGA0.15	1:3	800	0.8 ± 0.2	85 ± 15	36 ± 19
PLGA0.15	1:3	533	0.68 ± 0.01	53 ± 1	28 ± 14

[a] Loading (%) = mass of the encapsulated drug/mass of the microspheres.
[b] Encapsulation efficiency (%) = mass of the encapsulated drug/mass of the total drug used in preparation.
[c] N = 3 ± SD.
[d] N ≥ 100 ± SD.

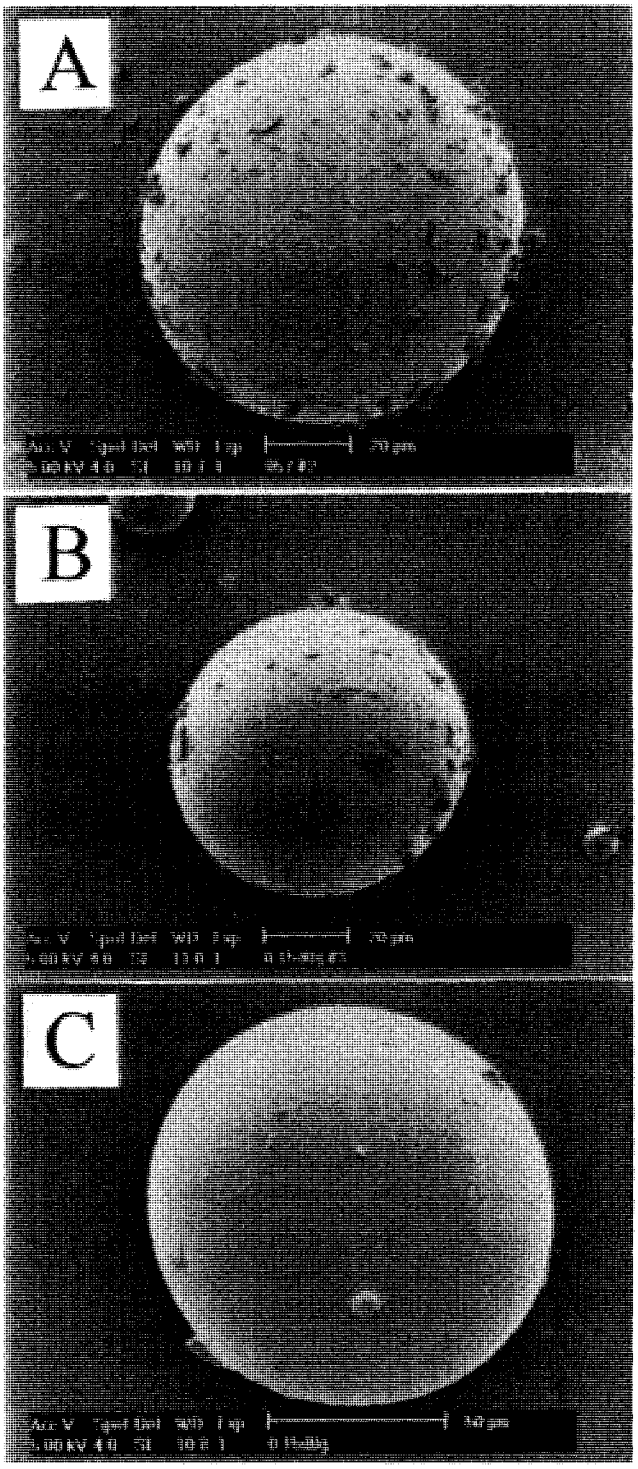

Fig. 2. SEM images obtained from different molecular weight preparations after fabrication. Polymers were: (A) PLGA1.07, (B) PLGA0.58, and (C) PLGA0.15.

The drug distribution inside the microspheres was analyzed by confocal microscopy. Figures 3 A, C and E represent median cross-sections of the microspheres made from PLGA1.07, PLGA0.58, and PLGA0.15, respectively, immediately after their preparation. The bright regions on the micrograph designate the location of drug. The drug was distributed more evenly throughout the PLGA0.15 particles (E) than in the higher molecular weight preparations (A and C), where a definite phase separation between drug and polymer was observed. In all cases, drug-rich spots appeared spherical in shape, indicating that a phase separation between polymer-rich and drug-rich phases occurred in the microsphere during initial microencapsulation and/or hardening of the particles.

Effect of Polymer Molecular Weight on 10-HCPT Release Profile

The molecular weight of the polymer has a strong influence on both the formation of microspheres (e.g., by its effect on polymer solution viscosity) as well as the drug release behavior of the formed particles (e.g., due to its relationship with polymer glass transition temperature and, therefore, polymer permeability and lag time to polymer weight loss). To examine the effect of M_w, 10-HCPT was encapsulated in PLGA microspheres using high (PLGA1.07), medium (PLGA0.58), and low (PLGA0.15) M_w polymers. Drug release kinetics from these formulations are given in Fig. 4. A large initial burst of the released drug was observed for both the PLGA1.07 and PLGA0.58 preparations (up to 45% of the drug was released within one day). In comparison with other formulations, PLGA0.15 exhibited a relatively small burst of initially released 10-HCPT (13%) followed by a linear and continuous drug release for the entire duration of study.

Confocal micrographs of PLGA1.07, PLGA0.58 and PLGA0.15 microspheres after one month exposure to the release media are presented in Figures 3 B, D, and F, respectively. The shape of the PLGA0.15 and PLGA0.58 preparations (D and E) was altered significantly after incubation, whereas the form of the PLGA1.07 microspheres remained relatively unchanged. In particular, PLGA0.15 microspheres did not retain any of their original smooth spherical form, although these particles degraded quite homogeneously with formation of numerous pin-hole pores throughout the interior. In contrast, PLGA1.07 and PLGA0.58 microspheres exhibited a higher extent of the degradation in the interior with formation of large pores inside the microsphere body rather than at the surface. This effect was especially noticeable for the PLGA0.58 preparation, where the core of the microspheres degraded significantly and a dense drug-rich polymer film at the surface of microspheres was formed.

Effect of Polymer Concentration on 10-HCPT Release

Because the low molecular weight preparation exhibited a more continuous and steady release pattern than the PLGA0.58 and PLGA1.07 formulations, PLGA0.15 was used further to investigate the influence of other encapsulation parameters (i.e., polymer concentration and carrier solvent composition) on drug release behavior. Polymer concentrations in the carrier solution of 533, 800, and 1067 mg/ml were used in preparation of the microspheres. The drug release behavior of those preparations was analyzed and the results are presented in Fig. 5. The initial drug burst increased dramatically with the decrease in polymer concentration. In particular, 48% of the encapsulated 10-HCPT was released from the 800 mg/ml preparation within the first day of exposure to the aqueous media, whereas only 16% was released from the 1067 mg/ml preparation.

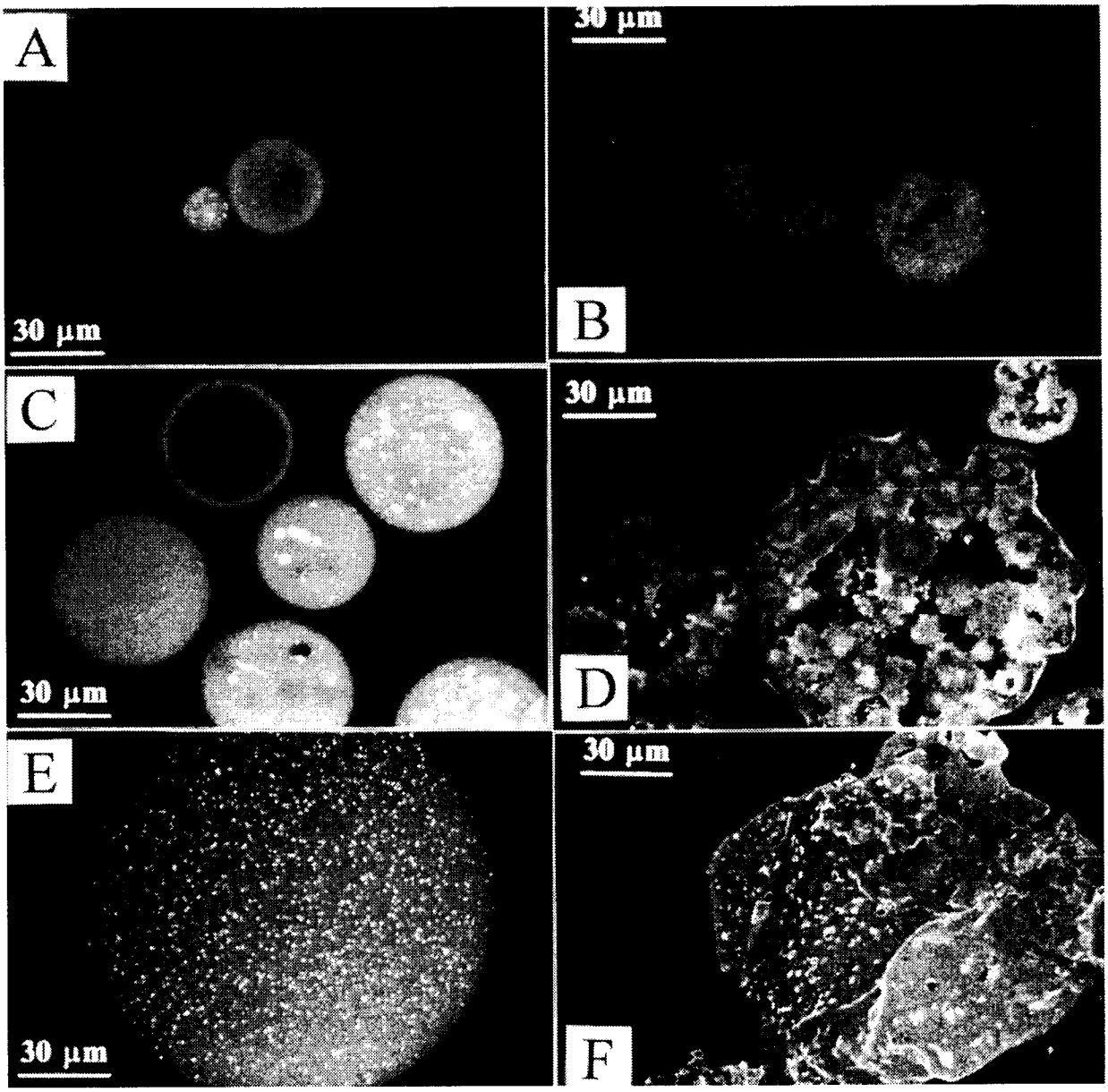

Fig. 3. Confocal microscopy images obtained from different molecular weight preparations. Bright spots correspond to the location of drug. Images were from unincubated microspheres of (A) PLGA1.07, (C) PLGA0.58, and (E) PLGA0.15. Images obtained after one month of *in vitro* erosion were from microspheres of (B) PLGA1.07, (D) PLGA0.58, and (F) PLGA0.15.

Effect of Carrier Solvent Composition on 10-HCPT Release

The solubility of 10-HCPT in methylene chloride, a standard solvent used for the oil-in-water emulsion-solvent evaporation method of microsphere preparation, is too low (~0.01 mg/ml) to dissolve enough drug for useful encapsulation. Therefore, the use of a cosolvent to dissolve the drug was necessary to obtain the desired drug loading. DMF was selected as the cosolvent because of its ability to dissolve 10-HCPT (solubility ~ 60 mg/ml) and its miscibility with methylene chloride.

Microspheres were prepared with the use of a carrier solvent composed of DMF and CH_2Cl_2 mixed in various proportions (1:3 and 1:2 v/v). A suspension of ground 10-HCPT crystals in a polymer solution containing no DMF was also used for microsphere preparation. The effect of carrier solvent composition on the 10-HCPT release behavior was examined *in vitro* (Fig. 6). By using DMF in the preparation of the microspheres a more continuous and reproducible 10-HCPT release was achieved compared to dispersing the drug in methylene chloride without the cosolvent. In addition, the initial burst of the drug was larger for the preparation without DMF than for those in which the cosolvent was used.

Stability of 10-HCPT in PLGA Microspheres

Since 10-HCPT, like all camptothecins, undergoes rapid hydrolysis ($T_{1/2}$ = 24 min) to its poorly active carboxylate form, the stability of 10-HCPT in the microspheres after their preparation and during release incubation was the central inter-

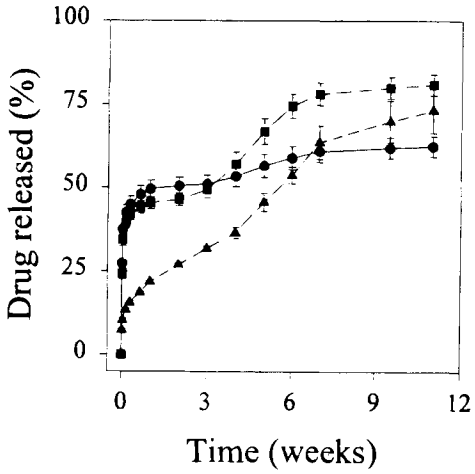

Fig. 4. Effect of polymer molecular weight on 10-HCPT release profile from microspheres. Release was carried out in PBS containing Tween 80 pH = 7.4 at 37°C and polymers (▲) PLGA0.15, (■) PLGA0.58, and (●) PLGA1.07 were used in preparation. Both the 10-HCPT concentration (6.67 mg/ml) in the carrier solution and the carrier solution composition (DMF:CH_2Cl_2 1:2 v/v) were the same in all three preparations. (n = 5, mean ± SEM)

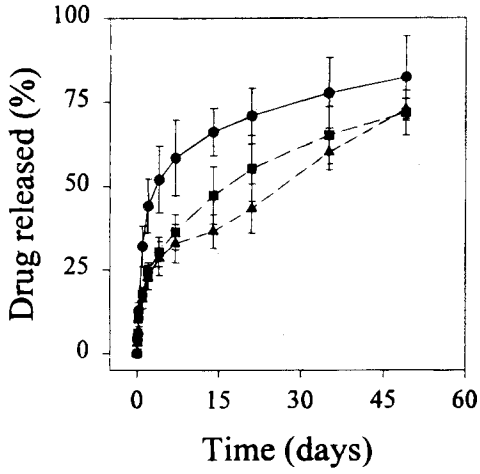

Fig. 6. Effect of carrier solvent composition on 10-HCPT release profile from microspheres made of PLGA0.15. The compositions of carrier solutions were : (●) CH_2Cl_2 (drug suspended), (■) DMF:CH_2Cl_2 1:2 (v/v) (drug dissolved), and (▲) DMF:CH_2Cl_2 1:3 (v/v) (drug dissolved). Both concentrations of the drug (6.67 mg/ml) and the polymer (1067 mg/ml) in the carrier solution were the same in all three preparations. (n = 3, mean ± SEM)

est of this study. The stability of the drug in microspheres was examined initially (soon after preparation) as well as after several time periods of PLGA degradation and drug release (e.g., 1 week, 1 and 2 months). Two methods were used for the assessment of drug stability, as described in the Materials and Methods section.

Drug stability results for the PLGA0.15 (DMF:CH_2Cl_2 1:2 v/v, 1067 mg/ml polymer concentration) formulation are presented in Fig. 7. After preparation the entire drug fraction (100%) remained in its active lactone form in the PLGA microspheres. Furthermore, the predominance of lactone form persisted for the entire duration of the release study (>95% lactone after two months). In marked contrast, in aqueous solutions (pH 7.4, T = 37°C) 10-HCPT is nearly completely (80%) converted to the carboxylate form after only 2 hours. The PLGA0.58 and PLGA1.07 formulations were also found to

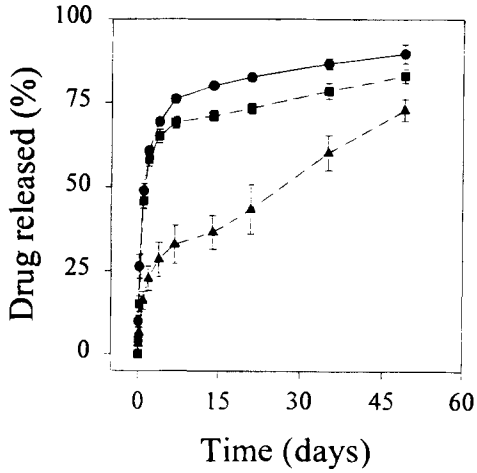

Fig. 5. Effect of polymer concentration in the carrier solvent on 10-HCPT release profiles of microspheres made from PLGA0.15. Release was carried out in PBS containing Tween 80 pH = 7.4 at 37°C. Polymer concentrations used in the preparations were (●) 533 mg/ml, (■) 800 mg/ml, and (▲) 1067 mg/ml. Both the drug concentration in the carrier solution (6.67 mg/ml) and the carrier solvent composition (DMF:CH_2Cl_2 1:3 v/v) were the same in all three preparations. (n = 3, mean ± SEM)

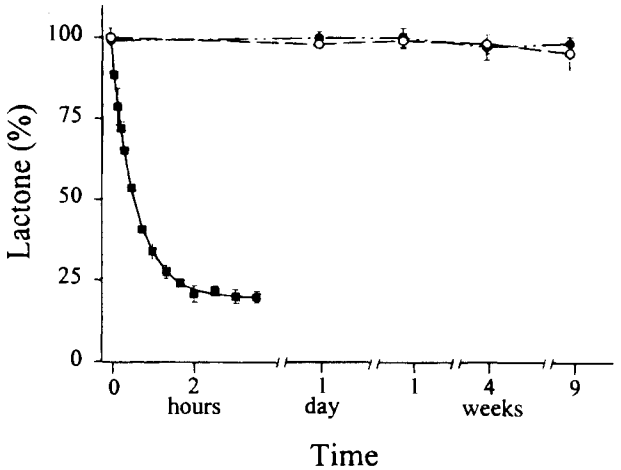

Fig. 7. Stability of 10-HCPT remaining in the microspheres versus rapid hydrolysis in simulated physiological environment. To find kinetic parameters of hydrolysis of the free drug in PBS pH = 7.4 at T = 37°C the equation $f = a + b \exp(-k_1 t)$ was fitted to the fraction of the intact lactone (f) versus time data (■). The fit yielded a $T_{1/2} = \ln(2)/k_1$ of 24 ± 2 min and a lactone fraction at the equilibrium (a) of 19 ± 2 % (n = 3, mean ± SEM). Drug stability inside the microspheres was examined by two methods: ○—10-HCPT was captured in the release media (pH = 6.5, n = 2 ± SEM) ●—microspheres were dissolved in organic solvent (n = 2, mean ± SEM). Microspheres were made from PLGA0.15, the concentrations of the drug and the polymer in the carrier solution were 6.67 and 1067 mg/ml respectively, and the carrier solvent composition was DMF:CH_2Cl_2 1:3 v/v.

stabilize the drug (>94% for the entire 2-month period, data not shown). Both analytical assays revealed high recovery of 10-HCPT lactone, indicating that the encapsulated drug exists in, and is released from, PLGA microspheres as the lactone form.

DISCUSSION

Stability of Camptothecins in PLGA Microspheres

Based on evidence that the delivery of camptothecins in the lactone form is crucial for their anti-tumor activity (6), we postulated that an optimal controlled-release preparation would exclusively release the lactone form of 10-HCPT. We observed that the lactone form was retained within PLGA microspheres for more than 10 weeks (>95% lactone) under a simulated physiological environment (Fig. 7). Thus, PLGA microspheres have an unprecedented ability to stabilize 10-HCPT and, therefore, have the potential to stabilize other analogues within this class of novel chemotherapeutic agents.

Whether or not the aforementioned stabilization has a positive effect on the use of PLGA microspheres to deliver camptothecins *in vivo* will likely depend on the quotient of two characteristic times: *i*) the time required for the drug molecule to be transported from the microsphere to the site of action, and *ii*) the half-life for carboxylate conversion in the drug transport path. This critical issue is currently being examined using stabilized PLGA formulations in animal models of cancer.

Possible Mechanisms for 10-HCPT Stabilization

The structure of the interior of the partially degraded microsphere is quite complex. At least two phases are present: an aqueous phase inside the pores and a gel-like polymer and/or oligomer phase. Therefore, the drug may be dissolved either in water or in polymer, or it also may exist in the form of undissolved crystals. Camptothecins undergo reversible pH-sensitive hydrolysis, where the lactone is favored at pH 5 and below and the carboxylate is favored at pH above 8 (7). The two forms of 10-HCPT differ significantly in their physicochemical properties. The lactone is a lipophilic compound, whereas the carboxylate is more polar with higher aqueous solubility.

The potential mechanisms of stabilization can be separated into two classes: those that prevent *kinetically* lactone-carboxylate conversion by preventing the favorable reaction from taking place, and those that favor the lactone form at *equilibrium*. A number of parameters responsible for the shifting the lactone-carboxylate equilibrium inside the microspheres may be identified. The equilibrium is affected by the effective pH of the medium. Since microspheres are made from polymer composed of two highly soluble acids, a low microenvironmental pH is expected as degradation proceeds. The occurrence of an acidic pH in the interior of large PLGA specimens has been reviewed (14). However, the presence of a low microenviromental pH for PLGA microspheres is currently unknown and may be influenced by the method of preparation and the raw materials in the formulation. If the majority of drug is located in aqueous pores then a low microenviromental pH may explain the observed drug stability. The second equilibrium-related explanation is the possibility of some interactions between the drug and polymer phase. Although the end-groups of PLGA following hydrolysis are polar, the back bone of the polymer is very lipophilic with respect to water. It is conceivable that the active E-ring may be protected from hydrolysis by the increased solubility of lactone form of the drug in the polymer phase. Preferential partitioning of the lactone form into lipid bilayers has been previously reported to have a stabilizing effect for camptothecins (16).

From a kinetic point of view, insufficient water in the microsphere interior could be another reason for 10-HCPT stabilization. The rate of hydrolysis will be slower due to insufficient water activity to carry out the reaction. Also, water can act as a "mobile phase" for the reaction, i.e., increase molecular motions (17). A second kinetic effect may be due to the fact that both low water content and low microenviromental pH would presumably slow down or prevent the dissolution of the lactone crystals. The undissolved drug is likely very stable and the conversion of lactone to carboxylate in the solid state is quite slow, if any, due to low water penetration into the drug crystal. It is more likely that in order for 10-HCPT lactone to be hydrolyzed it must first be solubilized.

Release Kinetics

The large initial burst of released drug from PLGA1.07 and PLGA0.58 microspheres (Fig. 4), and also from PLGA0.15 microspheres when low polymer concentrations were used (Fig. 5), is consistent with the dissolution of 10-HCPT crystals deposited on the surface of the microspheres. For PLGA0.58 and PLGA1.07, the presence of the drug crystals was confirmed by SEM micrographs (Fig. 2). The initial drug burst also correlates well with the mean particle size of the various preparations. Two PLGA0.15 formulations with 1067 mg/ml polymer concentration and carrier solvent compositions of 1:2 and 1:3 ($DMF:CH_2Cl_2$ v/v) exhibited the best release behavior with ~ linear and continuous long-term 10-HCPT release and small initial drug bursts of 19% and 16%, respectively. The mean particle size for these preparations was 82 and 53 μm, respectively, whereas the mean particle size of all other preparations was in a range of 27–36 μm and the initial burst was 32–50%. The large particle size of two preparations displaying favorable release profiles could account partially for their slow and continuous drug release, since larger particles have a smaller overall surface area normal to transport.

The long-term drug release behavior for different molecular weight preparations follows the common pattern for PLGA microspheres (18). It is not surprising that the medium M_w microspheres exhibited the lag time prior to an increase in release rate. For the high M_w preparation this increase was not observed during the entire 11-week release study. Significant time is required for PLGA0.58 and PLGA1.07 to degrade to the point where the effective glass transition temperature drops below 37°C and the transition to rubbery state leads to an increase in drug release (19). Hutchinson and Furr suggest that the M_w at which this transition occurs is ~ 15,000 (18). The transition for PLGA0.58 microspheres occurred after 3 weeks, whereas the PLGA1.07 microspheres (at least at the surface) remained in glassy state for the entire period of the release study. The confocal micrographs taken after one-month of microsphere degradation suggested that the PLGA0.15 and PLGA0.58 preparations are in a rubbery state and the PLGA1.07 formulation remains in a glassy state. In contrast, the low poly-

mer M_w preparation exhibited continuous drug release, which indicates that water could freely penetrate into, and 10-HCPT diffuse out of, the microspheres from very early stages of release.

The accelerated degradation in the interior of large polymer specimens of relatively high M_w PLGA has been well studied (20). It appears to be due to two characteristic effects. First, formation of a hard 'skin' on the specimen surface composed of high molecular weight polymer. The skin prohibits the diffusion of polymer degradation products out of the microspheres. The latter causes the effective decrease in pH and, therefore, the increase in rate of acid-catalyzed hydrolysis of PLGA ester bonds. However, the extent to which this phenomenon is present in microspheres is still unclear. For the PLGA0.58 preparation the presence of a drug-rich film on the microsphere surface after one month of degradation was observed in the confocal micrograph (Fig. 3, D). This may have occurred because of the formation of a hard high M_w film on the microsphere surface analogous to the 'skin'. This film is expected to represent a limiting barrier for the diffusion of encapsulated drug, which causes accumulation of the fluorophore (10-HCPT) at the particle surface. In addition, the interior of PLGA0.58 preparation degraded significantly during the one-month period with formation of large aqueous pores inside microsphere body. Thus, accelerated polymer degradation in the interior of this formulation of microspheres also occurs. For the PLGA0.15 formulation, this effect was not observed, since the polymer M_w is not high enough for the formation of the 'skin' to occur. The interior of PLGA1.07 microspheres was not altered to such an extent as the PLGA0.58, probably due to insufficient degradation time.

Formulation Considerations

Microspheres made of low Mw PLGA with the inherent viscosity of 0.15 dl/g initially possess the ability to degrade and release drug continuously without any lag time. Therefore, further PLGA0.15 formulations were developed with an intent to increase encapsulation efficiency and decrease the initial burst of the released 10-HCPT. To accomplish these objectives, carrier solution composition and polymer concentration used in microsphere fabrication process were optimized. The presence of cosolvent DMF in the carrier solution was necessary to increase 10-HCPT loading. DMF dissolves the drug, which leads to homogeneous distribution of 10-HCPT in the microsphere body and, therefore, to continuous and reproducible release behavior (Fig. 6). However, the high solubility of DMF in water leads to the diffusion of drug solution out of the oil droplets during the microsphere hardening process. Thus, the encapsulation efficiency decreases with an increase in DMF content. For two PLGA0.15 formulations, which differed only in carrier solution composition, the encapsulation efficiency was found to be 95% for 1:3 and 67% for 1:2 DMF:CH_2Cl_2 (v/v) (Table 1). Therefore, there is a compromise between high DMF content to increase the loading and a low DMF content to maximize the encapsulation efficiency. The use of high polymer concentration appears to improve the encapsulation efficiency. In addition, this leads to the increase in mean particle size of the microspheres and, consequently, to the diminishing of initial drug burst and slow long-term release. Based on this study the optimal formulation was obtained with the use of PLGA0.15, a polymer concentration of 1067 mg/ml and a 1:3 DMF:CH_2Cl_2 (v/v) carrier solvent composition.

Potential Applications of PLGA Microspheres to Deliver Camptothecins

A variety of clinical applications of PLGA microspheres containing camptothecins arises from their ability to slowly release the active form of the drug. Such formulations could potentially be used for systemic drug delivery to mimic a low-dose 21-day continuous i.v. infusion. This regimen is effective and well-tolerated for the delivery of topothecan, a potent camptothecin analogue, that has been recently FDA approved (4).

PLGA microspheres containing chemotherapeutic agents are extensively used for the treatment of non-resectable hepatic carcinoma by using chemoembolization (21–23). Due to their high potency and unique mechanism of action, the use of encapsulated camptothecins could effectively compete with, or compliment, the administration of cisplatin, doxorubicin, and other drugs used for this purpose.

Other possibilities of local/regional administration of PLGA microspheres containing camptothecins are: *i*) direct injection into tumor, or *ii*) implantation during surgery (24). Examples of few tumors accessible by first route are those found in the prostate, skin, and oral cavity. In contrast, a number of tumors are treated by excision and the complete removal of cancer cells is not always achievable. Therefore, local application of the microspheres slowly releasing drug for an extended period after surgery may increase the probability of complete regression of residual cancer cells (25,26).

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