The Acidic Microclimate in Poly(lactide-*co*-glycolide) Microspheres Stabilizes Camptothecins

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Purpose. The camptothecin (CPT) analogue, 10-hydroxycamptothecin (10-HCPT) has been shown previously to remain in its acid-stable (and active) lactone form when encapsulated in poly(lactide-co-glycolide) (PLGA) microspheres (1). The purpose of this study was to determine the principal mechanism(s) of 10-HCPT stabilization.

Methods. CPTs were encapsulated in PLGA 50:50 microspheres by standard solvent evaporation techniques. Microspheres were eroded in pH 7.4 buffer at 37°C. The ratio of encapsulated lactone to carboxylate was determined by HPLC as a function of time, initial form of drug encapsulated, fraction of co-encapsulated $Mg(OH)_2$, CPT lipophilicity, and drug loading. Two techniques were developed to assess the microclimate pH, including: *i)* measurement of H^+ content of the dissolved microspheres in an 80:20 acetonitrile/H_2O mixture and *ii)* confocal microscopy of an encapsulated pH-sensitive dye, fluorescein.

Results. The encapsulated carboxylate converted rapidly to the lactone after exposure to the release media, indicating the lactone is favored at equilibrium in the microspheres. Upon co-encapsulation of $Mg(OH)_2$, the trend was reversed, i.e., the lactone rapidly converted to the carboxylate form. Measurement of -log(hydronium ion activity) (pa_H^*) of dissolved microspheres with pH-electrode and pH mapping with fluorescein revealed the presence of an acidic microclimate. From the measurements of H^+ and water contents of particles hydrated for 3 days, a microclimate pH was estimated to be in the neighborhood of 1.8. The co-encapsulation of $Mg(OH)_2$ could both increase the pa_H^* reading and neutralize pH in various regions of the microsphere interior. Varying the drug lipophilicity and loading revealed that the precipitation of the lactone could also stabilize CPT.

Conclusions. PLGA microspheres prepared by the standard solvent evaporation techniques develop an acidic microclimate that stabilizes the lactone form of CPTs. This microclimate may be neutralized by co-encapsulating a base such as $Mg(OH)_2$, as suggested by previous work with poly(*ortho* esters) (2).

KEY WORDS: camptothecin; PLGA; microclimate pH; drug stability; pH-sensitive probe.

INTRODUCTION

The advantages of the controlled release (CR) of anticancer agents for both systemic and local/regional therapy are well known (3,4). The successful use of CR strategies depends on the drug stability for extended periods; the lability of anticancer agents often complicates their delivery. Camptothecins (CPTs), potent chemotherapeutic agents examined in this manuscript, undergo rapid hydrolysis under physiological conditions ($t_{1/2}$ ~ 20 min at 37°C, pH = 7.4). This reaction is a reversible pH-sensitive interconversion from the potent lactone form (stable below pH 5) to the poorly active carboxylate form (stable above pH 8) (5,6). Interestingly, when encapsulated in PLGA microspheres 10-hydroxycamptothecin (10-HCPT) was stabilized and delivered for over 10 weeks (1). The purpose of this manuscript was to examine the following important questions: (i) Are all CPTs stable in PLGA microspheres? (ii) What are the mechanisms of their stabilization? (iii) Are these data important for other labile drugs?

To examine the mechanisms of stabilization of CPT, the influence of PLGA microclimate on the kinetics and equilibrium of the lactone-carboxylate interconversion must be considered. The following microenviroment characteristics were examined: (i) microclimate pH, (ii) microsphere water content, and (iii) polymer-drug interactions. Since the pH strongly affects CPT stability, we placed particular emphasis on the measurement of the microenvironmental pH in PLGA microspheres prepared by standard oil-in-water emulsion-solvent evaporation techniques from end-capped PLGA. The degradation of PLGA proceeds with the formation of low molecular weight carboxylic acid oligomers and monomers, which can cause a decrease in microclimate pH. An acidic microclimate and an accelerated degradation inside large specimens of PLGA are well documented (7,8). However, the microenvironmental pH of the microspheres is controversial. For example, the microclimate pH in PLGA microspheres has been reported to be neutral, when determined by NMR (9). Other investigators have postulated an acidic environment from the reactivity of peptides and proteins (10) and small labile drugs in the microspheres (11). In addition to the microclimate pH the effects of solid-state stabilization of CPT and CPT partitioning into the PLGA matrix were addressed.

MATERIALS AND METHODS

Chemicals

10-HCPT (>96% purity) was obtained from Dabur India Limited (New Delhi, India). Topotecan (TPT) was obtained from the Division of Cancer Treatment, National Cancer Institute. 9-Chloro-10,11-methylenedioxycamptotecin (CMDC) was obtained from the laboratories of Drs. M.C. Wall and M.E. Wani from Research Triangle Institute (Research Triangle Park, NC). End-capped PLGA with a 50:50 D,L-lactide to glycolide copolymer ratio of inherent viscosity (i.v.) of 0.15 and 0.20 dl/g was purchased from Birmingham Polymers (Birmingham, AL). Fluorescein free acid was purchased from Molecular Probes (Eugene, OR). Polyvinylalcohol (PVA), 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.

Preparation of the Microspheres

The conditions used for microsphere preparation are summarized in Table I. For the preparation by the oil-in-water

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Table I. Summary of Microsphere Preparation Conditions

Protocol code	Encapsulated substance	Type of emulsion used in preparation	Inner water phase	Dispersed oil phase	Continuous oil or water phase	$Mg(OH)_2$ theoretical loading	Drug or probe loading
A	10-HCPT carboxylate	w/o/w	50 μl of 25 mg/ml of drug in borate buffer (pH = 9)	450 μl of CH_2Cl_2 with 533 mg PLGA	100 ml of PVA 0.3% (w/v) in borate buffer (pH = 9)	N.A.	0.22%
B	10-HCPT lactone	o/w	N.A.[a]	275 μl of CH_2Cl_2 with 320 mg PLGA + 25 μl of DMF drug solution at 30 mg/ml	30 ml of aqueous PVA 0.3% (w/v)	N.A.	0.18%
C	10-HCPT lactone and $Mg(OH)_2$	o/o	N.A.	0.5 ml of ACN with 300 mg PLGA[b] and 0, 0.75, 7.5 and 75 mg $Mg(OH)_2$ + 25 μl of 30 mg/ml drug in DMF	50 ml of cottonseed oil with 5% (v/v) Span® 85	0, 0.2, 2, 20%	~0.2–0.25%
D	$Mg(OH)_2$	o/w	N.A.	300 μl of CH_2Cl_2 with 320 mg PLGA + 0, 3.2, 10 and 21 mg of $Mg(OH)_2$	30 ml of aqueous PVA 0.3% (w/v) and $Mg(OH)_2$ 0.04% (w/v)	0, 1, 3, 6%	N.A.
E	pH-sensitive probe, florescein	o/w	N.A.	225 μl of CH_2Cl_2 with 320 mg PLGA + 75 μl of probe in DMF at 10 mg/ml + 0, 3.2, 10 mg of $Mg(OH)_2$	30 ml of aqueous PVA 0.3% (w/v) and $Mg(OH)_2$ 0.04% (w/v)	0, 1, 3%	~0.05–0.1%
F	Lactones of 10-HCPT, TPT or CMDC and $Mg(OH)_2$	o/o	N. A.	200 μl of ACN with 120 mg PLGA + 10 μl of 30 mg/ml drugs in DMF	50 ml of cottonseed oil with 5% (v/v) Span® 85	20%	~0.2%
G	10-HCPT lactone and $Mg(OH)_2$	o/w	N.A.	225 μl of CH_2Cl_2 with 320 mg PLGA + 75 μl of drug in DMF at 60, 30, 15, 3, 1.5, 0.3 and 0.15 mg/ml + 36 mg of $Mg(OH)_2$	30 ml of aqueous PVA 0.3% (w/v) and $Mg(OH)_2$ 0.04% (w/v)	6%	0.418, 0.254, 0.147, 0.025, 0.011, 0.002, and 0.001%

[a] N.A.: not applicable.
[b] For protocol C, PLGA with i.v. = 0.2 dl/g was used, in all other cases i.v. = 0.15 dl/g.

emulsion solvent-evaporation method the emulsion was formed by vortexing 20 s the dispersed oil phase with an equal volume of the continuous phase, 1% PVA (*aq*). Then, the emulsion was poured into an aqueous hardening bath water phase and stirred for 3 hours at room temperature and atmospheric pressure. The composition and volume of oil and water phases are given in Table I. The hardened microspheres were washed 3 times with double distilled and deionized water by centrifugation at 3000 r.p.m. (1090× g) for 10 minutes at 0°C. After the final spin, the microspheres were resuspended in water, flash frozen with liquid N_2, and lyophilized with a Labconco Freezone 6 system for 2 days. For the microsphere preparation by the water-in-oil-in-water emulsion-solvent evaporation method, the inner water phase was homogenized with the oil phase at 5000 r.p.m. by a Cyclone/Tempest IQ^2 homogenizer (The Virtis Company, NY) for 20 s to form the first water-in-oil emulsion. Further preparation steps were identical to those described for the oil-in-water emulsion method.

In the oil-in-oil emulsion method the continuous phase was stirred at 800 r.p.m. with an overhead stirrer and the dispersed phase was added drop-wise. The emulsion was stirred for an hour before 50 ml of petroleum ether (b.p. 50–110°C) was added to extract the ACN from the polymer. After an additional 15 min of stirring the hardened microspheres were washed with 250 ml of petroleum ether, collected by filtration, flash-frozen and lyophilized. The microspheres were stored desiccated at −20°C until further use.

Characterization of Microspheres: Loading, Degradation Conditions, and Water Uptake

After microsphere preparation, the loading of CPTs and fluorescein were determined. Microspheres were dissolved in dimethyl sulfoxide (DMSO) and the concentrations of fluorescein and CPTs were measured by fluorescent spectroscopy and HPLC, respectively. A Perkin Elmer luminescence spectrometer

(Perkin Elmer, CT) with the excitation and emission wavelengths set to 440 nm and 512 nm, respectively, was used to determine probe loading. The HPLC conditions for the measuring of CPTs loading are given below.

For polymer degradation, 1–15 mg of microspheres were placed in microcentrifuge tubes and 1.5 ml of 0.05 M HEPES buffer containing 0.02% (w/w) Tween® 80 was added. HEPES was used to prevent precipitation of the buffer ions with Mg^{2+}. Tween® 80 was used to decrease the interfacial tension between PLGA and aqueous media. The microspheres were hydrated in the incubator under agitation at 37°C, the buffer was changed frequently (every 3–4 days) in order to maintain external pH.

The water uptake was determined gravimetrically. Dry microspheres (15 mg) were placed in microcentrifuge tubes of known weight and hydrated for 3, 7, and 14 days. After hydration the microspheres were spun down at 1000 r.p.m, the supernatant was removed. The weights of hydrated microspheres with microcentrifuge tubes were measured. Water uptake was calculated from a ratio of H_2O mass (the weight difference of hydrated and dry microspheres) over the initial mass of dry microspheres. No correction for interparticle water was made.

Analysis of the Composition of Camptothecins in PLGA Microspheres

Microspheres were collected by centrifugation and then dissolved with DMSO. The carboxylate to lactone ratio was determined by high performance liquid chromatography (HPLC). This HPLC method allows separation of the two forms of the drugs within a single chromatographic run. Additional control experiments indicated that no significant drug interconversion occurs during microsphere dissolution and separation. 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 (Middleton, WI), and a Hewlett Packard 3395 integrator (Palo Alto, CA). The excitation filter was 305–395 nm, the emission filters were 430–470 nm for 10-HCPT and TPT and 450–500 nm for CMDC. For separation a Waters Nova-Pack C_{18} 3.9 × 150 mm reverse phase column was used. The mobile phase was composed of acetonitrile (ACN) and aqueous buffer (0.1 M KH_2PO_4, 0.5 mM tetrabutylammonium dihydrogen phosphate and 0.4 mM triethyl amine pH = 6.0). The volume ratios of ACN to buffer were 2:8 for 10-HCPT, 14:86 for TPT, and 32:68 for CMDC, and the flow rate was 1 ml/min in each case.

Confocal Microscopy

Microspheres containing fluorescein and 0, 1, and 3% $Mg(OH)_2$ were incubated in HEPES buffer with 0.02% Tween® 80 (pH = 7.4) containing 0.1 mg/ml fluorescein for 3 days. The initial loading and its concentration in the microspheres after 3 days of hydration were determined by fluorescence spectroscopy, as described above. Microspheres were placed on a glass slide in the release buffer and fixed with a cover slip. Images were obtained using a BIO-RAD 600 confocal laser scanning system equipped with argon-krypton laser. The excitation wavelength was 488 nm, the dichroic reflector was 510 nm, and the emission filter was 515 nm. A 20× objective was used for magnification. To investigate the pH-dependence of fluorescein intensity, the images of probe solutions at 0.001, 0.01, and 0.1 mg/ml concentrations and pH = 2, 3, 4, 5, 6, 7, and 8 were taken. The universal buffer used to control the pH was composed of 0.04 M H_3PO_4, 0.04 M HAc, 0.04 M H_3BO_3 and the pH was adjusted with 0.2 M NaOH (12). The black and white images were transformed into colors with Adobe Photoshop software (Adobe Systems Incorporated, CA). The indexed color mode was selected and the level of gray from black to white was transformed into colors from violet to red with the spectrum color table.

Organic Phase pH Measurements

Microspheres (15 mg) with 0–6% (w/w) $Mg(OH)_2$ were placed in each microcentrifuge tube and hydrated in HEPES buffer. At various times of degradation (0–14 days), microspheres were centrifuged (for 3 min at 1000 r.p.m.) and the supernatant was removed. Microspheres were dissolved in 0.8 ml of ACN by vigorous vortex and centrifuged for 5 min at 5000 r.p.m. to remove undissolved $Mg(OH)_2$. 0.7 ml of the supernatant was placed into a new tube and 175 μl of double distilled water was added with subsequent mixing. The pH was taken immediately after water addition with a Corning Semi-Micro Combination glass pH-electrode (VWR Scientific, PA) attached to a Beckman Φ 40 pH-meter (Beckman Instruments Inc., CA).

The pH meter was equilibrated with aqueous standards pH = 4 and 7 (VWR Scientific), and conventional KCl filling solution for glass electrode (VWR Scientific) was used. The glass electrode with aqueous filling behaves satisfactory in organic solvent-water mixtures up to 90 wt % of organic solvents and the pH-meter is capable of giving reproducible e.m.f. values (13).

The actual proton activity in the organic solution mixture (a_H^*) is correlated to the pH-meter reading (pH) by $pa_H^* = pH + \delta$, where δ is a combined correction factor arising from the residual liquid junction potential, the change in hydrogen activity coefficient and other factors connected to the use of partially aqueous media for pH measurement (14). The value of δ is a function of solvent composition. For an ACN:H_2O 80:20 (v/v) mixture δ = 0.95 (15). To test the effect of microsphere concentration in ACN:H_2O mixtures on pa_H^* measurement, the pa_H^* was determined for microspheres with and without $Mg(OH)_2$ at microsphere concentrations of 3, 7.5, 15, 30 and 45 mg/ml initially after microsphere preparation and after 3 and 7 days of incubation in the degradation media.

RESULTS AND DISCUSSION

The Lactone Form Is Favored in the PLGA Microenvironment at Equilibrium

The sources of CPT stabilization may be separated into two categories: those that prevent interconversion kinetically, e.g., insufficient molecular mobility of the drug in the polymer to react, and those that favor the formation of the lactone at equilibrium. To distinguish between the sources, both forms of 10-HCPT were encapsulated (Table I, protocols A and B) and the kinetics of drug hydrolysis inside microspheres was examined upon particle hydration. The encapsulated carboxylate rapidly converted back to the lactone (>94% after one day), whereas the lactone did not react in the microspheres (Fig.

1). The reverse reaction occurs rapidly and completely in the polymer, therefore the lactone-carboxylate interconversion is not prevented kinetically. Thus, the lactone is favored by PLGA at equilibrium.

We have identified several possible sources that favor the lactone at equilibrium. First, CPTs exist in the lactone form at a pH < 5 and a low microenvironmental pH in PLGA microspheres is expected during hydrolysis of the polymer ester bonds. Second, a preferential partitioning of the lactone into a polymer phase relative to the carboxylate could increase the lactone content in microspheres at equilibrium. Third, due to the relatively low aqueous solubility of the lactone and low water content in the microspheres, 10-HCPT could precipitate in the lactone form, which would also increase the overall lactone content recovered from the microspheres. Several experiments were performed to test each potential equilibrium sources of stabilization.

Co-Encapsulated $Mg(OH)_2$ Causes 10-HCPT Conversion

If an acidic microclimate exists in the PLGA microspheres, we would expect the addition of base would cause some conversion of 10-HCPT to its carboxylate. $Mg(OH)_2$ has been used successfully to increase the microclimate pH of poly(*ortho* esters) to inhibit polymer degradation (2). Thus, to test this hypothesis, 10-HCPT was co-encapsulated with $Mg(OH)_2$ at 0, 0.2, 2, and 20% (w/w) loading and the lactone content in the microspheres was determined during the exposure to aqueous media. An o/o anhydrous emulsion-solvent evaporation method was used for the encapsulation to prevent lactone conversion during encapsulation (Table I, protocol C). As expected, the more base that was added to the microspheres, the greater the extent of 10-HCPT conversion (Fig. 2). For 20% $Mg(OH)_2$ loading the maximum lactone conversion occurred by 1 hour of the hydration (90% converted), and by 2 hours the lactone fraction of the microspheres was steady at 40% for more than one week. The lactone became favored after 3 weeks. Negligible 10-HCPT conversion was observed for $Mg(OH)_2$ levels at 0 and 0.2%. Without further study of the drug release kinetics, the kinetics of retention of $Mg(OH)_2$, and the kinetics of polymer degradation, it is impossible to fully understand the kinetics of drug conversion inside PLGA microspheres. The overall trend, however is consistent with the neutralization of an acidic microclimate caused by the presence of $Mg(OH)_2$.

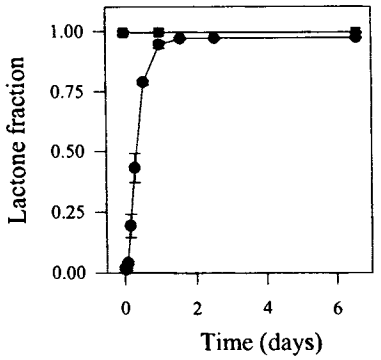

Fig. 1. The lactone is favored in the PLGA microclimate at equilibrium. Microspheres containing 10-HCPT were incubated in the PBS with 0.02% Tween® 80 (pH = 7.4) at 37°C. 10-HCPT was initially encapsulated in lactone (■) and carboxylate (●) forms.

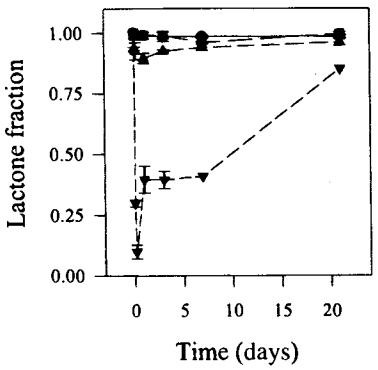

Fig. 2. The effect of co-encapsulated $Mg(OH)_2$ at 0 (●), 0.2 (■), 2 (▲), and 20% (▼) on the 10-HCPT stability in PLGA microspheres. 10-HCPT was initially in the lactone form (100%) and the kinetics of lactone-carboxylate interconversion were determined during microsphere degradation in HEPES containing 0.02% Tween® 80 pH = 7.4 at 37°C.

Effect of $Mg(OH)_2$ on the Water Uptake of Microspheres

Before further evaluating the microclimate pH, the water uptake was determined for the microspheres containing 0–6% $Mg(OH)_2$ (Table I, protocol D). The water uptake increased upon encapsulation of $Mg(OH)_2$, from 14% in blank microspheres after 3 days to 115% in the microspheres containing 6% $Mg(OH)_2$ (Fig. 3). This increase was likely due to the ionization of carboxylic acid groups of PLGA upon the neutralization of microenvironment with base. Similar effects were observed for PLGA films with encapsulated $Mg(OH)_2$ (16).

Determination of H^+ Content in Dissolved Microspheres by pH Electrode in Mixed Solvents

Recognizing that the $Mg(OH)_2$ may influence more than pH (e.g., polymer water uptake), we designed experiments for additional evidence of an acidic microclimate. The first method was the measurement of hydronium ion concentration after

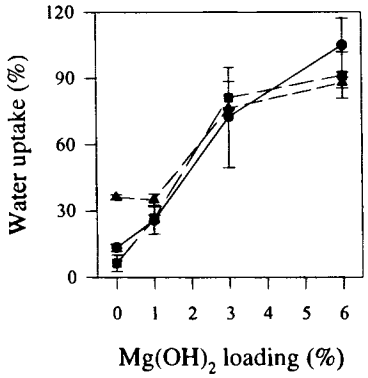

Fig. 3. Water uptake of the microspheres as a function of $Mg(OH)_2$ loading. Microspheres were hydrated in HEPES buffer containing 0.02% Tween® 80 (pH = 7.4) at 37°C for 3 days (●), 7 days (■), and 2 weeks (▲). The water uptake was calculated as mass of H_2O/initial dry mass of polymer.

microsphere dissolution in an ACN/H_2O mixture. The pa_H^* was measured as a function of microsphere erosion time, $Mg(OH)_2$ loading, and amount of particles dissolved. If an acidic microclimate was present, then a low pa_H^* reading should be detected upon microsphere dissolution. In contrast, if acidic species were either absent or neutralized by buffering species from the erosion medium or basic excipients, then the pa_H^* should be neutral. Consistent with the acidic microclimate hypothesis, a low pa_H^* was detected, which increased upon co-encapsulation of $Mg(OH)_2$ (Fig. 4). In the absence of $Mg(OH)_2$ the initial pa_H^* was low at 3.3 (Fig 4A). The pa_H^* increased to 4.6 by 3 days, dropped to 4.2 by 7 days, and then to 2.8 after 4 weeks. The initial increase in pa_H^* is consistent with the known loss of oligomers and monomers upon hydration, while the further decrease in pa_H^* may have been caused by polymer degradation. An initial increase followed by a subsequent decrease in polymer molecular weight and glass transition temperature was observed for PLGA microspheres by Park (17).

As base was added the pa_H^* increased gradually with the increase in $Mg(OH)_2$ loading (Fig. 4B). In a similar fashion as for blank microspheres, the pa_H^* of microspheres containing base increased by 3 days of hydration, and for 1% $Mg(OH)_2$ loading, the pa_H^* decreased after 2 weeks upon polymer degradation. In contrast, for microspheres containing 3 and 6% of $Mg(OH)_2$, the pa_H^* did not change between 3 and 14 days. This suggests that at a loading higher than 3%, the polymer degradation may be slowed down significantly, as was noted for PLGA films containing $Mg(OH)_2$ (16), and the base release occurs slowly due to its low aqueous solubility.

The dependency of hydronium ion concentration on the amount of microspheres dissolved in ACN:H_2O mixture was measured to determine the $[H^+]$ liberated per mg of microspheres (Fig. 4C). After 3 days of microsphere incubation in aqueous media the hydronium ion liberated was $\sim 2.1 \times 10^{-9}$ mole/mg of microspheres and the amount of sorbed water was ~0.14 mg/mg of microspheres (Fig. 3). Therefore, without any correction for changes of the dissociation of the carboxylic acid moieties providing the protons (i.e., between the microsphere microclimate and the ACN/H_2O mixture), the measurement gives a microclimate pH value of ~1.8. We remark that we do expect a change in the dissociation constants, K_a, of the population of carboxylic acid species (RCOOH) between the microclimate and the ACN/H_2O mixture, since the K_a of carboxylic acids decreases in aprotic solvents such as ACN compared to water (14). Thus, the K_a in the hydrated microspheres should be greater than the K_a in the ACN/H_2O mixture, which leads us to postulate that the microclimate pH in this particles was not much greater than 1.8. This value is consistent with pH measured by a glass electrode for large PLGA specimens (pH < 2) (8).

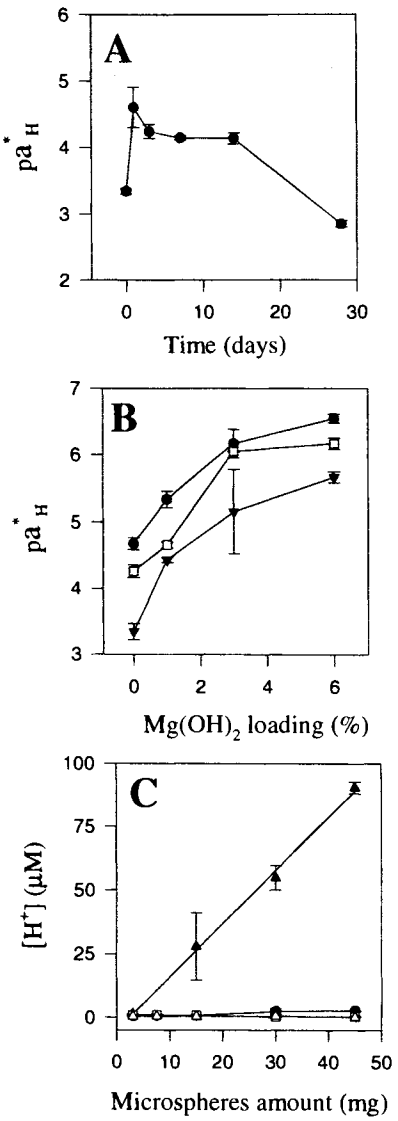

Fig. 4. Determination of the H^+ content in microspheres by pH-electrode after dissolution in a ACN/H_2O mixture. pa_H^* = pH(measured) − 0.95 for 80:20 v/v ACN: H_2O. A. The pa_H^* of the blank microspheres (capped-end PLGA 0.15 dl/g i. v.) as a function of time of incubation in PBS containing 0.02% Tween® 80 (pH = 7.4) at 37°C. B. The pa_H^* from microspheres as a function of $Mg(OH)_2$ loading after 0 (▼), 3 (●), and 14 days (□) of degradation in HEPES containing 0.02% Tween® 80 (pH = 7.4) at 37°C. C. The effect of microsphere concentration in ACN:H_2O 80:20 (v/v) solutions on measured H^+ concentration. In the microspheres without $Mg(OH)_2$ (▲) and with 1% base (●), the $[H^+]$ increased linearly with microsphere concentration, with the slopes of 2.1×10^{-6} M/mg ($r^2 = 0.997$) and 5.6×10^{-8} M/mg ($r^2 = 0.967$), respectively. The $[H^+]$ was constant at $\sim 5 \times 10^{-7}$ M (independent of the microsphere concentration) for 3% (□) and 6% (△) $Mg(OH)_2$ loading.

Encapsulated Fluorescent Probe Indicates An Acidic Microclimate

The second method used to assess the microclimate pH involved the utilization of a pH-sensitive fluorescent probe, fluorescein. The intensity of fluorescein emission decreases with the decrease in pH (18,19). The calibration of change in the image color with pH is given in Fig. 5 for 0.01 mg/ml fluorescein concentration. If the microsphere microclimate were acidic, we would expect to observe no emission from microsphere interior. To test this, we encapsulated fluorescein at ~0.1% (w/w) loading in PLGA microspheres along with 0, 1 and 3% $Mg(OH)_2$ (Table I, protocol E) and incubated the microspheres for 3 days at 37°C in HEPES with 0.02% Tween® 80 (pH = 7.4) containing 0.1 mg/ml of the dye. The fluorescein was added to the release media to obtain a control emission intensity

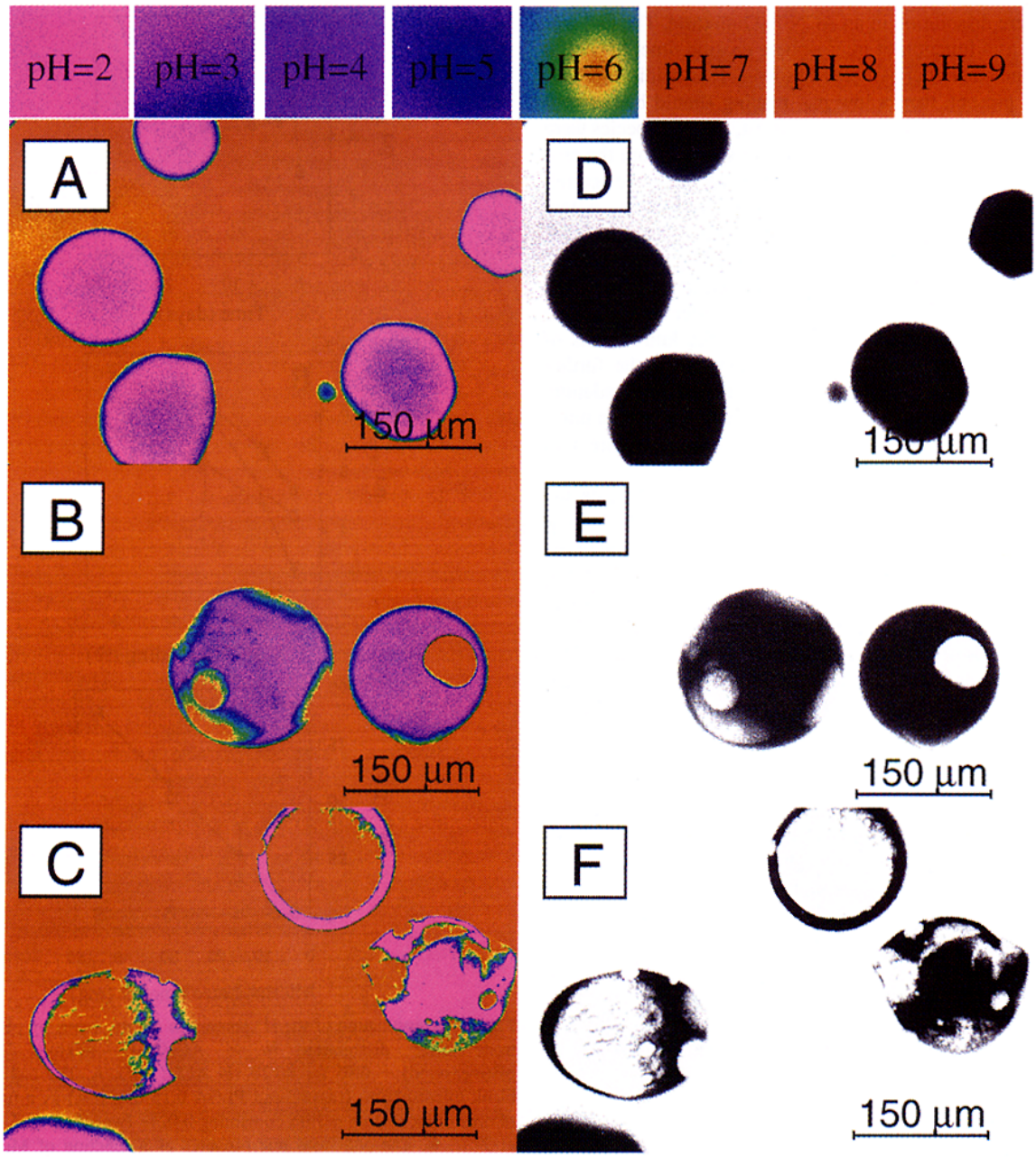

Fig. 5. Confocal microscopy images of the microspheres containing the pH-sensitive fluorescent probe, fluorescein, and 0% (A and D), 1% (B and E), and 3% (C and F) of $Mg(OH)_2$ after 3 days of hydration in HEPES buffer containing 0.02% Tween® 80 (pH = 7.4) and 0.1 mg/ml fluorescein at 37°C. Images D-F were transformed into color images A-C with the use of a spectrum color table in Adobe Photoshop. The color of fluorescein solution images at 0.1 mg/ml concentration of pH from 2 to 9 are given for the comparison.

at pH = 7.4 (fluorescein concentration in the release buffer approximately equals its concentration in the microspheres within a factor of 5). The interior of microspheres was violet, which roughly corresponded to a pH = 2 at 0.1 mg/ml dye concentration, compared with a bright red color of pH = 7.4 outside of the particles (Fig. 5D). As expected from the partial neutralization by $Mg(OH)_2$, the fluorescein emission from the microsphere core was observed when the probe was co-encapsulated with 1 and 3% of $Mg(OH)_2$ (Figs. 5B, C, D, and E). With the increase in base loading to 3%, the acidic microclimate was almost completely neutralized and the red regions begin to dominate over the violet regions in the PLGA matrix (Fig. 5C). It is important to note that the pH does not rise *homogeneously*. Instead, it appears to increase near base particles. It is impossible to determine the exact pH inside the microspheres from the single image by fluorescein mapping technique, since the emission intensity depends both on media pH and probe concentration. However, the observations confirm the presence of acidic microclimate in PLGA microspheres, which can be partially neutralized by the encapsulation of $Mg(OH)_2$.

Stabilization Effect of Drug Precipitation

The water uptake of the blank microspheres during the first two weeks of degradation was ~12–30% (weight water/weight polymer) (Fig. 3). Knowing that the 10-HCPT loading is ~0.2% and the lactone solubility is ~0.002 mg/ml, one can calculate that only ~0.01% of the encapsulated drug is in solution, assuming that all of the microsphere water is available to dissolve the drug. To assess the contribution to overall lactone content from the undissolved drug, the loading and aqueous solubility of the encapsulated CPTs were varied.

In the first experiment, 10-HCPT was encapsulated in PLGA microspheres with an artificially neutralized microclimate (6% $Mg(OH)_2$) at a drug loading range of 0.4 to 0.001% (w/w) (Table I, protocol G). We hypothesized that at a low loading 10-HCPT completely dissolves in the microenvironment and the lactone fraction in the microspheres reflects the microclimate pH according to the equilibrium constant for lactone-carboxylate conversion in the microclimate. At higher drug loading only a small fraction of 10-HCPT is in solution and the rest will exist as the precipitated lactone. Therefore, a sharp increase in overall lactone content of the microspheres with the increase in drug loading is expected, which corresponds to a 10-HCPT transition from the solution to the solid-state.

The expected trend was observed. In Fig. 6A the lactone fraction in microspheres is plotted as a function of drug loading remaining in the particles after 3 and 7 days of hydration. As drug content increased to greater than 0.01% (w/w), roughly 80% of 10-HCPT was recovered in the lactone form, whereas a sharp decrease in lactone content occurred at a loading of ~0.005–0.001%. For the microspheres with 6% encapsulated $Mg(OH)_2$ the water uptake during the first two weeks was ~120%. A rough calculation shows that 10-HCPT will be completely dissolved in the microsphere environment at a loading on the order of $\sim 10^{-3}$%, which is close to the curve minimum in Fig. 6A. Interestingly, this minimum is represented by different initial drug loading on different days, since the drug is released rapidly and we corrected for this by plotting lactone fraction versus the actual drug content in the polymer on the given day. Thus, the minimum will occur for each preparation throughout the release as the remaining 10-HCPT loading reaches the critical value of $\sim 10^{-3}$%. The plateau of lactone content at 80% in the microspheres at high drug loading can be explained by the precipitation of the carboxylate with magnesium ion. This salt formation can be observed easily by the mixing of solutions of 10-HCPT carboxylate and $MgCl_2$.

In the second experiment, we encapsulated three CPTs with various aqueous solubilities in PLGA microspheres containing 20% $Mg(OH)_2$. The selected CPTs, TPT, 10-HCPT, and CMDC, exhibit identical pH-dependent conversion kinetics (11), while the aqueous solubilities of their lactones are ~2, 0.002, and 0.0001 mg/ml, respectively. All CPTs were in 100% lactone form after microsphere preparation, since an anhydrous o/o emulsion-solvent evaporation method was used (protocol F, Table I). The response of the encapsulated agents on the neutralized environment is shown in Fig. 6B. The two more water-soluble agents, TPT and 10-HCPT exhibited a fast conversion into the carboxylate form, whereas the least soluble CMDC remained in its lactone form even in very basic microspheres with 20% $Mg(OH)_2$. The results of these experiments emphasize that although a low microclimate pH must favor camptothecin

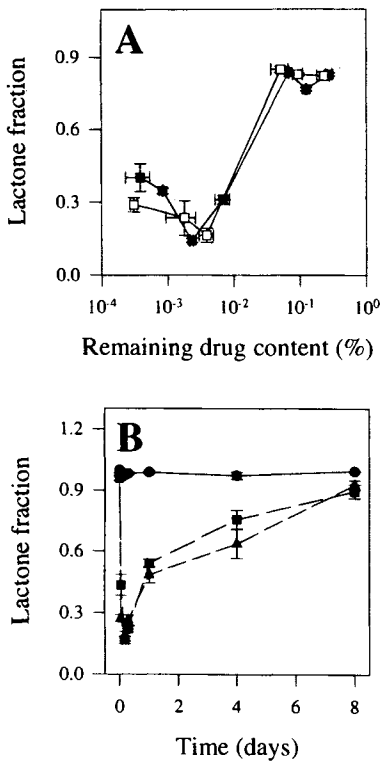

Fig. 6. A. The effect of 10-HCPT loading on its composition in PLGA microspheres containing 6% (w/w) $Mg(OH)_2$ after 3 (■) and 7 (●) days of hydration in HEPES buffer containing 0.02% Tween® 80 (pH = 7.4) at 37°C. B. The effect of camptothecin lipophilicity and solubility on its conversion in PLGA microspheres containing 20% (w/w) $Mg(OH)_2$ upon hydration in HEPES buffer containing 0.02% Tween® 80 (pH = 7.4) at 37°C. Three camptothecins were studied: CMDC (●), 10-HCPT (▲), and TPT (■). Their corresponding lipid partition coefficients and solubility were approximately 400 M^{-1} and 0.0001 mg/ml, 75 M^{-1} and 0.02 mg/ml, 10 M^{-1} and 1 mg/ml, for CMDC, 10-HCPT, and TPT, respectively.

stabilization, the contribution to stability from their low solubility can also be significant, allowing some analogues to remain in the lactone even in a case of a neutralized microenvironment.

The Effect of Drug-Polymer Interactions on Lactone Stability

The preferential partitioning of the unionized lactone form of 10-HCPT in the polymer phase compared to the anionic carboxylate could also increase the lactone content of PLGA microspheres at equilibrium. The results of the kinetics of interconversion following the encapsulation of the three CPTs (Fig. 6B) also can be used to address the partitioning phenomena. TPT, 10-HCPT, and CMDC not only have quite different aqueous solubility but also have very different lipid/water partition coefficients. The equilibrium binding constants for unilamellar vesicles composed of lipid dimyristoylphosphatidylcholine were found to be 400, 75, and 10 M^{-1} for CMDC, 10-HCPT, and TPT, respectively (20). If no partitioning of lactone in the polymer occurs, then all three drugs should exhibit the identical conversion kinetics in the microspheres. Virtually identical conversion kinetics was observed for the two more hydrophilic drugs, TPT and 10-HCPT (Fig. 6B), suggesting no significant

interactions between PLGA and these CPTs with ~10-fold lipophilicity difference. The low aqueous solubility of CMDC explains its exceptional stability in this case, although the interactions between CMDC and PLGA cannot be ruled out. The CMDC stability is analogous to the improved stability of suspensions relative to solutions (21). Overall, we can conclude that no significant drug-polymer interaction occur for more hydrophilic CPTs (e.g., TPT and 10-HCPT) that has any substantial impact on the lactone equilibrium content.

CONCLUSIONS

The acidic microclimate of the PLGA microspheres has been identified to be the main source of stabilization of the 10-HCPT lactone. The use of the analytical techniques to assess microclimate pH, as discussed herein, may be very useful as general tools for determining the extent of the acidic microclimate in formulations of acid-labile substances such as peptides, proteins and other anticancer drugs. Upon co-encapsulation of $Mg(OH)_2$ the microclimate pH is neutralized, although in a heterogeneous fashion. Due to the low intrinsic solubility of the lactone, 10-HCPT is present as a lactone precipitate rather than molecularly dispersed in the microenvironment, which also contributes to drug stability. Finally, $Mg(OH)_2$ can be used potentially as an additive for the formulation of acid-labile pharmaceuticals in PLGA microsphere delivery vehicles.

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