Use of Hydrodynamic Flow Focusing for the Generation of Biodegradable Camptothecin-Loaded Polymer Microspheres

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ABSTRACT: The present study was conducted to investigate the use of hydrodynamic flow focusing for the generation of biodegradable polymer microspheres encapsulating the anticancer drug camptothecin. Poly(D,L-lactide-*co*-glycolide) (PLGA) and poly (L-lactide) (PLA) were used as the matrix materials. Camptothecin was dissolved in the disperse phase and microspheres with a mean size between 2 and 3 μm generated using hydrodynamic flow focusing. When up to 1 wt.% of the drug was added to PLA, the drug encapsulation efficiency was 64%. For PLGA, the drug encapsulation efficiency was between 39 and 46%. Drug release from PLA particles was rapid and complete within 6 h, while drug release from PLGA particles showed no burst effect and followed a first order release profile. The encapsulated camptothecin stayed in its active lactone form, as shown by HPLC, and was able to exert cell toxic effects as shown by a cell viability assay. Hydrodynamic flow focusing is a promising tool for the preparation of drug-releasing biodegradable microspheres typically made by solvent evaporation and/or solvent extraction, as indicated by the successful encapsulation of the anticancer drug camptothecin.

Keywords: camptothecin; flow focusing; PLGA; PLA; microspheres

INTRODUCTION

The mass production of uniform and size-defined micro- and nanoparticles is a challenge in many fields, and especially in the pharmaceutical industry. In drug delivery applications, particle size has two important consequences. First, it can determine the final site of particle accumulation within the body[1] which is important not only in intravascular applications, but also in the delivery of inhaled pharmaceuticals, where the size determines how deep into the lungs a drug can be delivered.[2] Second, particle size can affect the release rate and release profile of a drug and thereby its therapeutic efficacy.[3,4] This is important for all drug delivery dosage forms with delayed or slow release, whether they are for

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intravascular, inhalation, oral, or topical applications of any kind. Employing uniformly sized micro- and nanoparticles allows for maximal control over particle biodistribution and precisely regulated drug release. Furthermore, monodisperse microspheres distribute more homogeneously, travel and behave more predictably in a patient's body, agglomerate less, and if biodegradable, degrade and disappear in a predictable way.[5]

One way of producing monodisperse droplets or gas bubbles is to apply hydrodynamic or aerodynamic flow focusing to liquids and gases. Flow focusing is defined as a method in which two or more immiscible liquid or gas streams are coaxially focused within an arrangement of tubings. At one end of the arrangement (downstream), the streams are forced through a small opening or orifice (Fig. 1). In this process, the outer continuous phase has a flow rate several orders of magnitude higher than the inner disperse phase. Thus, the central stream, either being liquid or gaseous, is forced into a thin jet-like stream. After passing through the orifice, the central stream is forced to break up into droplets, due to a rapid change in fluid pressure and the prevailing shear stress of the outer surrounding sheath of the continuous phase. The composition of the continuous phase defines the respective method, either aerodynamic (i.e., using gas) or hydrodynamic flow focusing (i.e., using fluids). To generate particles for use in pharmaceutical drug delivery applications, the droplets often consist of liquid containing dissolved polymers. Once formed, these droplets rapidly undergo the additional step of solvent extraction or solvent evaporation, during which each turns into a particle (or microsphere).

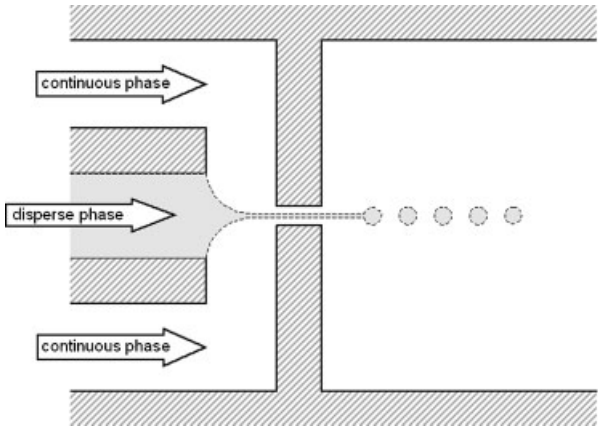

Figure 1. Principle of flow focusing. The disperse phase is injected into a sheath stream of the continuous phase. Both phases are then forced through a small orifice. High shear stress and the prevailing rapid pressure drop at the orifice result in a break-up of the disperse phase into droplets.

The basic concept of flow focusing and droplet disintegration was studied in the late 19th century by Lord Rayleigh.[6,7] However, it was the progress of the last 2 or 3 decades in microfabrication and the development of new measurement techniques (e.g., scanning force microscopy, electron microscopy) that enabled its advancement into everyday applications such as ink jet printing.[8] Recently, flow focusing methods have been used by Gañan-Calvo to prepare polystyrene microspheres incorporating the fluorescent dyes rhodamine B, nile blue, and fluorescein for use in biomolecule detection assays.[9] In addition to these diagnostic and analytical applications, the potential of flow focusing has been recognized for pharmaceutical drug delivery and medical applications, has been described in several patents, but has yet to be explored in practice.[10–12]

The method of flow focusing has many general advantages including: (1) It is a gentle method of droplet formation, thus allowing for the encapsulation of labile compounds, unlike many of the high-energy physical approaches of generating monodisperse droplets through the breaking up of larger droplets; (2) it does not require surfactants, although their use might support droplet formation; (3) it is generally a simple one-step approach, making additional purification and separation procedures unnecessary; (4) particle size can be adjusted by changing the fluid flow velocity of the two phases; (5) droplet size is not limited by the injector and orifice size—i.e., droplets can be much smaller than the orifice size; (6) the flow focusing process is scalable; and (7) offers the generation of droplets and microspheres at low costs.[13] Examples for successful flow focusing applications include the generation of homogeneously sized water droplets in oil,[14,15] water droplets in gas,[16] as well as single emulsions[9,17] and multiple emulsions[18–20] using nonbiodegradable polymers. None of these publications, however, applied flow focusing to the successful encapsulation of drugs.

Compared to other technologies for the preparation of particles, such as spray drying,[21] emulsion/solvent evaporation,[22–24] supercritical fluid use,[25] and rapid freeze drying/solvent extraction,[26] the flow focusing method lacks an overall high throughput. This disadvantage of the

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technique can be overcome by scaling up of the flow focusing process into arrays of hundreds or even thousands of fluid streams.

Hydrodynamic flow focusing requires the use of immiscible fluids, and thus may require the use of active components that codissolve in the polymer solvent. The DNA topoisomerase I inhibitor camptothecin, a lipophilic anticancer drug with a poor water solubility of 3.8 μg/mL[27], is a suitable candidate for codissolution in the inner disperse phase. Several studies have shown that camptothecin can be incorporated into microspheres using oil-in-water emulsification techniques with subsequent solvent evaporation.[27–29] Furthermore, the action mechanism of camptothecin is dependent on the surrounding pH.[30] While stable at lower pHs (below 5), the active lactone form of camptothecin undergoes hydrolysis at physiological pH to the much less active carboxylate form. The use of PLGA or PLA as matrix material for encapsulation protects the drug from early degradation and provides a low-pH microclimate during polymer degeneration. This was nicely shown by Dora et al.[28], who reported extended drug activity from large (30–40 μm on average) and rather polydisperse slow release microspheres over several days. Tong et al.[29] confirmed these findings, adding that camptothecin loaded microspheres are suitable for drug targeting in cancer therapy, and reduce the risk of side effects and fatal overdose as compared to the administration of the free drug.

The aim of the present paper is to show that hydrodynamic flow focusing can be used to prepare small (around 1–2 μm) biodegradable microspheres encapsulating the anticancer drug camptothecin. Two different polymers, poly (D,L-lactide-*co*-glycolide) (PLGA) and poly(L-lactide) (PLA) were used and compared in terms of particle size, surface properties, encapsulation efficiency, and drug release profiles. In addition, the activity of the released drug was characterized in a cell viability assay.

MATERIALS AND METHODS

Materials

PLGA (85:15, intrinsic viscosity 0.61, MW 24 kDa) was purchased from Durect Corp. (Pelham, AL). PLA (type Resomer® L104; MW 2 kDa) was purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany). Polyvinyl alcohol (PVA; 87–89% hydrolyzed, MW 13–23 kDa), (*S*)-(+)-camptothecin (95% HPLC grade), 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO; ≥99.9%) were purchased from Sigma–Aldrich Ltd. (Oakville, ON, Canada). Dichloromethane was from Fisher Scientific (Ottawa, ON, Canada). All chemicals were of reagent grade if not stated otherwise and used as received.

Design and Fabrication of the Flow Focusing Apparatus

The flow focusing apparatus was designed using ProEngineer software (ProEngineer Wildfire, PTC, Needham, MA) and subsequently fabricated from brass in a local machine shop (UBC, Department of Mechanical Engineering). The 100 μm orifice in a 50 μm thick brass sheet was manufactured using a 5 W argon ion laser beam (Simon Fraser University, Vancouver, Canada). For a more detailed description of the flow focusing apparatus, see "Supporting Information" section.

Disperse Phase Preparation

The polymer concentration (PLA, PLGA) in all microsphere generation experiments was 10 wt.% in dichloromethane. For the drug encapsulation, different batches of polymer/drug solutions were prepared, taking into account the low saturation solubility of camptothecin (370 μg/mL in dichloromethane and 10.07 mg/mL in DMSO). Briefly, for the microspheres with low camptothecin loading, 1.0 mg of the drug was dissolved in 3 mL of dichloromethane with the subsequent addition of 300 mg of PLGA or PLA, resulting in a clear solution with a drug concentration of 0.33 wt.% relative to the respective polymer (denoted PLA 0.33% and PLGA 0.33%). Due to the low solubility of the drug, for experiments with 2× and 3× higher intended drug loads, 1 and 2 mg of camptothecin was first dissolved in 200 μL DMSO supported by 20 min of ultrasonication. The DMSO/camptothecin solution was added to 1300 and 1800 μL of dichloromethane containing 150 mg of PLGA and 200 mg of PLA, respectively, and sonicated for an additional 3 min. The resulting suspensions had a yellowish color with a submicron particle size and were denoted PLA 0.6%, PLGA 0.6%, PLA 1%, and PLGA 1%. Controls without drug were also prepared.

Microsphere Generation

Microspheres were generated using a continuous phase of 2% (w/v) PVA solution that was continuously injected into the flow focusing apparatus by an HPLC pump (Waters 501; Waters Division, Millipore, Milford, MA). The disperse phase was injected using a syringe pump (BS-8000; Braintree Scientific, Inc., Braintree, MA) and gas tight glass syringes. The total flow rate was 8.0 mL/min with a flow rate ratio of the disperse phase to the total flow rate (Q_{DP}/Q_{total}) of 1:1000. The experimental setup was equipped with four-way diagonal switching valves (Upchurch Scientific, Inc., Oak Harbor, WA), enabling the switch from and to different disperse phases without major interruption of the flow. This setup also allowed for the flushing of the disperse phase injector with polymer free solvent before and after each microsphere generation experiment. After establishment of stable flow conditions, the experiments were conducted by submerging the flow focusing apparatus in a beaker containing 100 mL of sterile filtered and impeller stirred (60 rpm) 2% PVA solution. The collection of generated microdroplets was performed for up to 135 min. After the flow focusing procedure, the suspension was stirred for at least 2 h at room temperature to allow for solvent evaporation. Subsequently, the microsphere solution was washed three times (i.e., centrifuged at 900*g* for 20 min and resuspended in distilled water). After imaging, the solution was centrifuged at 2000*g* for 5 min, the supernatant discarded, and the sample air dried.

Microsphere Size Determination and Statistical Analysis

Due to limited available particle quantities for some of the batches, the microsphere size distribution could not be evaluated with automated methods for all samples. In order to be consistent in the size determination method, all microsphere batches were visualized using an inverted microscope (Motic AE31; Motic Instruments, Inc., Richmond, BC, Canada) connected to a high resolution CCD camera (Infinity 3; Lumenera Corp., Ottawa, ON, Canada), followed by image analysis using ImagePro Plus (Media Cybernetics, Inc., Silver Spring, MD). At least 1500 microspheres per individual experiment were classified and counted from three randomly selected images, classified into 1 μm bins (i.e., $0.5\ \mu m \le x_1 < 1.5\ \mu m$, $1.5\ \mu m \le x_2 < 2.5\ \mu m$, etc.), and evaluated according to ISO 9276/1-2 and -4. Since the size distribution did not follow a normal distribution, the results are expressed as the 16th and 84th percentile of the cumulative size distribution representing one standard deviation from the median diameter of the sample. The results are presented together with the coefficient of variation (CV; the ratio of standard deviation to arithmetic mean diameter) as a measure of dispersity.

Scanning Electron Microscopy (SEM)

The surface morphology of drug loaded PLA and PLGA microspheres was examined by SEM (Hitachi S-4700, Tokyo, Japan). After placing a droplet of aqueous microsphere suspension on silicon stubs, the sample was air dried and subsequently coated with 5 nm of gold under reduced pressure (<5 Pa) using a JFC-1600 fine coater (JEOL, Tokyo, Japan). The samples were imaged at 0.8–1.0 keV.

Camptothecin Encapsulation Efficiency

The encapsulation efficiency was determined by dissolving 1 mg of microspheres in 1 mL of dichloromethane and directly measuring the camptothecin concentration spectrophotometrically at its absorption maxima of 364 nm (HP 8452A Diode Array Spectrometer, Hewlett-Packard, Palo Alto, CA). The absorption value was compared to a calibration of various camptothecin concentrations in dichloromethane and the mass of encapsulated camptothecin determined. The encapsulation efficiency was determined in duplicate.

To determine the form (lactone vs. carboxylate) of the drug in the microspheres, 1.3 mg of either PLA or PLGA microspheres was dissolved in 250 μL of dichloromethane. The sealed tubes were allowed to sit for 5 min without shaking and 3.75 mL of acetonitrile/water (60:40 v/v) was added with vortexing. The contents were allowed to settle and the solvents separated into equal 2 mL volumes of a hydrophobic top phase and a hydrophilic bottom phase. Both phases were analyzed for drug content by reverse phase HPLC using a Waters Millennium system with Novapak C18 Waters column and spectrophotometric detection at 368 nm. The mobile phase consisted of acidified water (triethylamine 0.7% v/v acidified to pH 5.5 with glacial acetic acid)/acetonitrile

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(77:23 v/v) running at a flow rate of 1 mL/min. Using these conditions the native lactone form of the drug elutes at 13 min and the degraded carboxylate form at 3.6 min. Standard curves for the lactone and carboxylate drug were obtained using standards made up in 60:40 acetonitrile/water where the water was either at pH 2 (drug fully in the lactone form) or pH 11 (drug fully in the carboxylate form).

In Vitro Release Studies

In vitro release studies were conducted with a total amount of 48 μg of camptothecin per 12 mL PBS (pH 7.4, 37°C) for each microsphere batch, which assured sink conditions during the drug's slow release over several days. The samples were placed in 15 mL cone tubes (Becton Dickinson, Sparks, MD) and rotated 8 rpm at 37°C (LabQuake Shaker). At time intervals of 0, 2, 4, 6, 10, 24, 34, 48, 72, 96, 120, 144, and 193 h for both polymers, and additionally at 244 and 336 h for the PLGA microspheres, the samples were centrifuged (1000*g*, 20 min), 2 mL of the supernatant withdrawn and replaced by the same amount of fresh PBS. The supernatant samples were frozen and quantified spectrophotometrically at camptothecin's absorption maxima of 368 nm in PBS by comparison to calibration curves of camptothecin after completion of the *in vitro* study. The calibration curve $y = 0.0644x$ had an R^2 value of 0.99925.

Anticancer Activity of Camptothecin Loaded Microspheres in Lung Cancer Cells

An MTT-test was conducted using lung epithelial cancer cells (NCI-H226) in order to test for anticancer activity of *in vitro* released drug. The cells were cultured in 75 cm^2 T-flasks (BD Bioscience, Bedford, MA) in RPMI-1640 media supplemented with 10% FBS and 292 μg/mL L-glutamine at 37°C, 95% humidity and 5% CO_2.

The MTT-test was performed in such a way that each well initially contained the same total drug amount supplied by the respective microsphere batches. Thus, if release of camptothecin is dependent on preparation method or initial drug load, it will be reflected in differences in the assay's viability data. Specifically, 96-well plates were used to seed and culture 10000 tumor cells per well in 100 μL of RPMI-1640 media for 24 h, followed by adding an amount of microspheres which contained a total of 10 ng of camptothecin per well. For the control microspheres (i.e., those without the drug), the same amount of microspheres was taken as was used for the low (0.33% camptothecin) concentration (i.e., 4.7 and 7.7 μg of the 0.33% PLA and PLGA microspheres, respectively). After an incubation of 72 h, the cells were treated with 20 μL of a 0.5% MTT solution. After another 3 h of incubation, the supernatant was removed, 100 μL of DMSO added, and the well plates incubated for 1 h at 37°C on a thermomixer R (Eppendorf, Westbury, NY) in order to lyse the cells and dissolve the MTT crystals. The resulting absorbance difference was evaluated at 540 nm using a Labsystems Multiscan Ascent plate reader (Labsystems, Helsinki, Finland). The results are expressed as cell viability (survival) in reference to an untreated control and with error bars representing the standard deviation ($n = 6$).

RESULTS

Microsphere Properties

The preparation of biodegradable microspheres encapsulating camptothecin with the flow focusing setup allowed us to produce up to 80 mg of microspheres within 2 h under the described conditions. The size and size distribution of the generated PLA microspheres was independent of drug content (Tab. 1 and Fig. 2). Also, the use of DMSO in order to introduce the higher drug amounts into the disperse phase did not seem to alter the particle size. Dispersity for all batches remained constant with a CV of around 15%. For PLA-microspheres, the encapsulation efficiency was constant at $64.3 \pm 0.7\%$ for the tested drug loads of 0.33–1.00%.

The mean diameter of PLGA microspheres was approximately 500 nm larger than that of the PLA microspheres (Tab. 2 and Fig. 2). Using DMSO for the preparation of the higher concentrated microspheres with the higher drug loadings did not influence particle size significantly, but increased dispersity from a CV of 0.16 to 0.27. The encapsulation efficiency of camptothecin increased from $38.6 \pm 1.0\%$ in PLGA microspheres containing 0.3% camptothecin ($n = 2$) to $45.7 \pm 3.5\%$ in PLGA microspheres containing 1.00% camptothecin ($n = 2$).

Only a small amount of the encapsulated camptothecin was in the inactive carboxylate

Table 1. Statistics Summary of PLA Particle Batches With Encapsulated Camptothecin

Batch	Camptothecin Load (%)[a]	Encapsulation Efficiency (%)	d_{16} (μm)	d_{50} (μm)	d_{84} (μm)	Mean (μm ± SD)	CV	n
PLA control	0	0	1.10	1.96	4.56	3.29 ± 0.64	0.19	1511
PLA 0.33%	0.215	64.6 ± 3.0	0.98	2.02	4.19	3.09 ± 0.46	0.15	3029
PLA 0.66%	0.423	63.5 ± 0.1	1.12	2.24	4.78	3.41 ± 0.48	0.14	1515
PLA 1.00%	0.647	64.7 ± 0.3	0.74	1.79	3.69	2.76 ± 0.45	0.16	1514

d, diameter; SD, sample standard deviation; CV, coefficient of variation; n, number of particles evaluated.
[a]Corresponds to encapsulated camptothecin/mass of microspheres.

form. For the PLA microspheres, 96.6% consisted of the active lactone form, while for the PLGA microspheres, 93.7% consisted of the active lactone form.

Camptothecin Release

From the PLA microspheres, all of the encapsulated camptothecin was released *in vitro* within the first 6 h (Fig. 3). The use of DMSO in the preparation of the polymer/camptothecin solution did not influence the release profile of the higher concentrated microspheres, nor was there any dependence on drug loading (Tabs. 1 and 2). A one-way analysis of variance (ANOVA) with equal cell sizes was used to compare the release profiles of microspheres containing concentrations of 0.33, 0.66, and 1.00% of camptothecin. The ANOVA was repeated for each of the 12 time points (Fig. 3) for the PLA and then again for the PLGA microspheres resulting in a total of 24 statistical tests. No significant differences were found between the different concentrations ($p > 0.05$, F-test).

However, drug release from the PLGA microspheres was much slower than from the PLA microspheres, showed no burst effect and followed a first order release profile for the first week. Within 14 days, an average *in vitro* release of 25% of the drug was reached.

All the microspheres produced by flow focusing were of spherical shape with smooth surface morphology, as shown by SEM (Fig. 4A and D). After 24 h the surface of the PLA microspheres appeared slightly rougher than the PLGA micro-

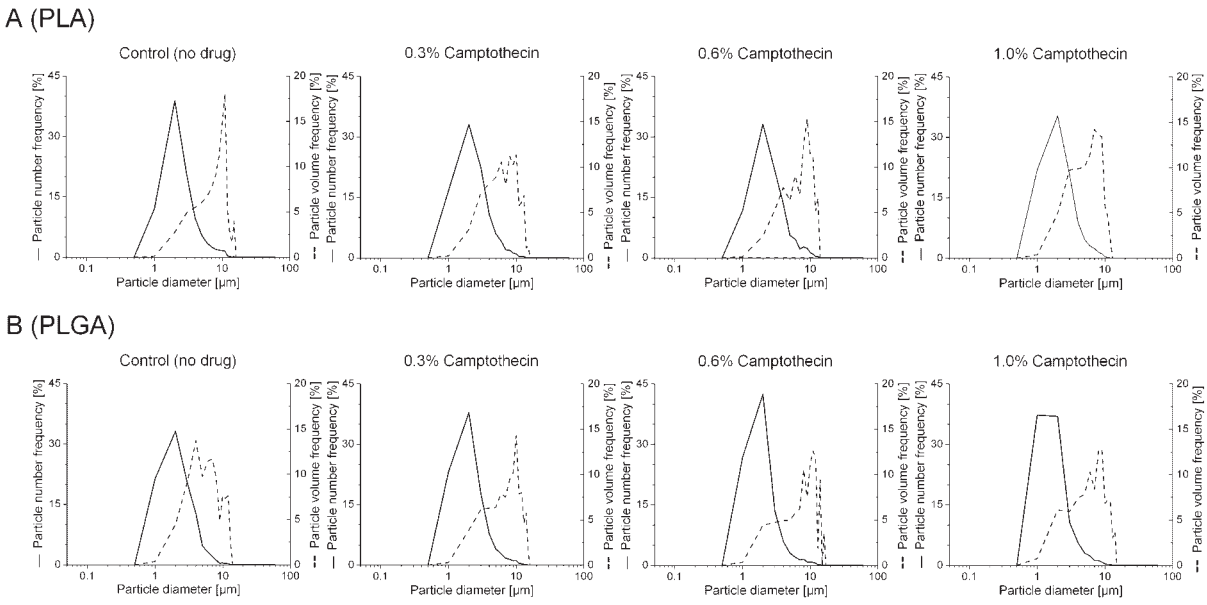

Figure 2. Size distribution of the different PLA (A) and PLGA (B) particle batches with initial camptothecin loading regarding the polymer weight of 0 (control), 0.33, 0.66, and 1.00%. The graphs show particle number frequency distribution (solid line) and particle volume frequency distribution (broken line) of at least 1500 evaluated particles.

Table 2. Statistics Summary of PLGA Particle Batches With Encapsulated Camptothecin

Batch	Camptothecin Load (%)[a]	Encapsulation Efficiency (%)	d_{16} (μm)	d_{50} (μm)	d_{84} (μm)	Mean (μm ± S.D.)	CV	n
PLGA control	0	0	0.66	1.74	3.59	2.68 ± 0.43	0.16	3034
PLGA 0.33%	0.129	38.6 ± 1.0	0.70	1.69	3.67	2.79 ± 0.48	0.17	3031
PLGA 0.66%	0.270	40.5 ± 7.4	0.61	1.54	3.22	2.61 ± 0.70	0.27	1516
PLGA 1.00%	0.457	45.7 ± 3.5	0.44	1.34	2.93	2.31 ± 0.63	0.27	1516

d, diameter; SD, sample standard deviation; CV, coefficient of variation; n, number of particles evaluated.
[a]Corresponds to encapsulated camptothecin/mass of microspheres.

spheres (Fig. 4B and E). The degradation of the PLA microspheres appeared to proceed faster, resulting in rough surfaces with holes, as compared to the smooth surfaces of PLGA microspheres (compare Fig. 4C after 193 h of incubation to Fig. 4F after 336 h of incubation).

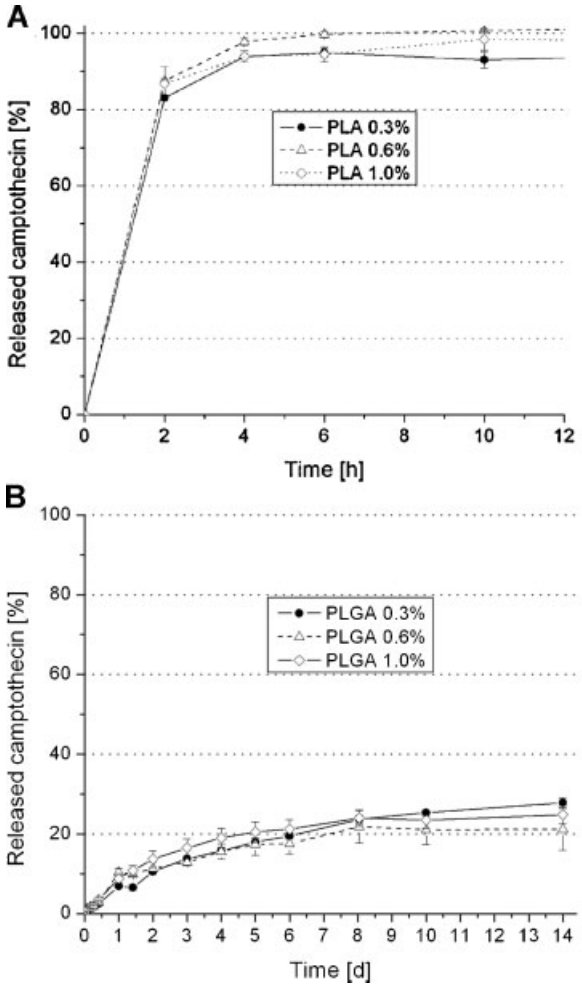

Figure 3. Comparison of the *in vitro* release (PBS, 37°C) of camptothecin from (A) PLA and (B) PLGA microspheres. The encapsulated drug is released within the first 6 h from the PLA microspheres, whereas the release from the PLGA microspheres is much slower and shows a release of only 25% of the encapsulated drug within 14 days.

Anticancer Activity of Encapsulated Camptothecin

Camptothecin which was completely released from the PLA microspheres within the first 6 h reduced cell viability to an average of 42.8% of that of the untreated control cells (Fig. 5). From the PLGA microspheres, only about a tenth of the drug was released during the duration of the MTT assay. It reduced the cell viability to an average of 63.9% (Fig. 5). For each identical drug load, the anticancer activities of the PLA and PLGA microsphere batches were found to differ significantly ($p < 0.05$, independent t-test).

DISCUSSION

The present study demonstrated that hydrodynamic flow focusing is an appropriate method for the encapsulation of drugs into PLA and PLGA microspheres, as shown by the encapsulation of the anticancer drug camptothecin. Particularly noteworthy was that microspheres could be produced in the size of a few micrometers in diameter without the use of high speed mechanical homogenizers (e.g., polytron). Flow focusing therefore might be a promising technology to produce small microspheres, potentially continuously adjustable down to a hundred nanometers, for intravascular drug delivery applications.

Microsphere Properties

The principal promise of the flow focusing technology is its potential in the production of uniform droplets leading to monosized microspheres. Looking at our currently reported CVs between 0.14 and 0.27, this aim has yet to be

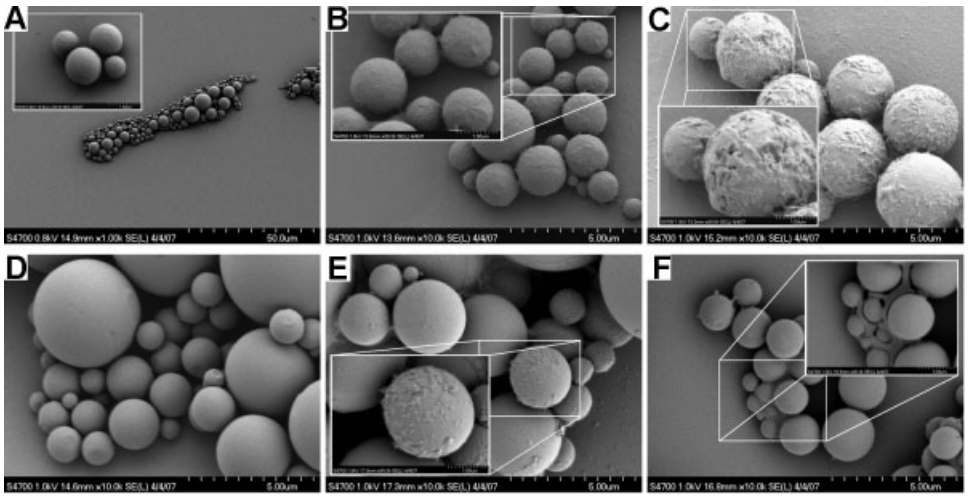

Figure 4. Scanning electron microscopy images of (A–C) PLA and (D and E) PLGA microspheres loaded with nominally 1.00 wt.% of camptothecin. The figure shows 1000-, 10000-, and 30000-fold magnifications of the microspheres prior to *in vitro* release studies (i.e., 0 h; A and D), after 24 h of incubation (PBS, 37°C; B and E), and after 193 h (C) and 336 h (F) of incubation.

achieved, although the microsphere size distribution is comparable or superior to that produced by established solvent evaporation methods. Tong et al.,[29] for example, used a solvent evaporation method that involved high speed mixing and ultrasonication to make PLGA microspheres containing up to 8.6% camptothecin. The resulting particles had an average size of 1.3 μm with CVs between 23% and 31% for the different batches. Such size distributions seem to be typical for many drug-encapsulating PLGA or PLA microspheres.[31] Much larger particles were made by Ertl et al.,[32] using the oil-in-water emulsion solvent evaporation process and relatively slow stirring at 800 rpm. The produced PLGA particles containing up to 10.1% camptothecin had a mean diameter of 34 μm. Although the CV was not given in their paper, it is possible to calculate a CV of 64% from their description that "two-third of the particle diameters were within a range of 12.5–56.9 μm".[32] Dora et al.[33] made similarly large polycaprolactone microspheres containing 1.2% and 2.4% camptothecin with the same method as Ertl. The reported particles had an average diameter of 32.6 and 40.5 μm and a size range between 0.4 and 120 μm, which translates to a similar CV as Ertl et al.'s particles.

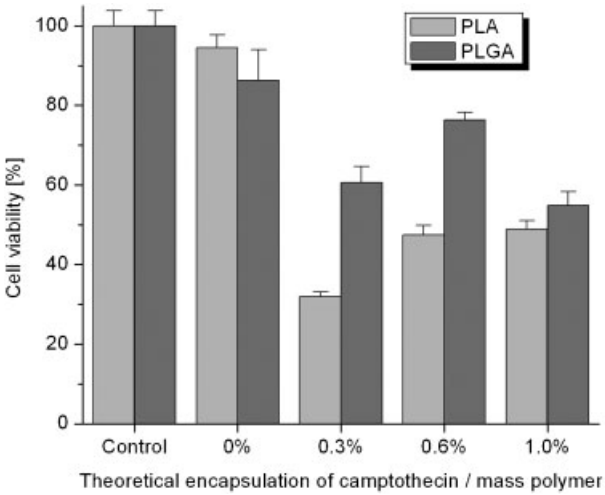

Figure 5. Anticancer activity of drug loaded PLA and PLGA microspheres with a total initial camptothecin amount of 10 ng per well of H266 lung epithelial cancer cells.

The not yet optimal size distributions in our camptothecin microspheres very likely stem from limitations in the production of smooth liquid flows and high necessary pressures, limitations which can be overcome and which are currently being attacked by the construction of a redesigned injection system based on nonpulsing pumps for both disperse and continuous phase.

Drug Encapsulation

The encapsulation efficiencies of 39–64% of drug at the low employed concentrations seem acceptable, considering that the encapsulation of drugs into small microspheres using solvent evaporation

methods is often problematic due to high surface area to volume ratios and the presence of precipitated drug near to the surface. The literature indicates that encapsulation efficiencies of close to 100% are reached when more than 2.4% camptothecin is encapsulated into microspheres.[29,32,33] Below this concentration, encapsulation efficiencies drop, for example, to 78.5% as reported for PLGA microspheres containing 1.2% drug,[32] or 81.5% for polycaprolactone microspheres containing 1.2% drug.[33] For PLA microspheres, a maximal encapsulation efficiency of 38.3% was reached for microspheres containing 1.3% drug.[34]

The differences seen in drug loading between the PLA and PLGA microspheres are not entirely clear, but could have to do with the interaction—or compatibility—of camptothecin with the polymers. These interactions can theoretically be calculated using their solubility parameter. Also referred to as cohesion or Hildebrand parameter, it is defined as the square root of a molecule's cohesive energy density, and contains the three components: van der Waals dispersion forces, dipole–dipole interactions, and hydrogen bonding.[35] The compatibility between a drug and a polymer improves, the closer together their solubility parameters are. Liu et al. have calculated the solubility parameters for PLA and PGA with the group contribution method[36] and determined them to be 23.3 and 28.0 $MPa^{1/2}$, respectively.[37,38] The solubility parameter for the 85:15 PLGA used will thus lie between the two values and would be 24.0 $MPa^{1/2}$ calculated by straight interpolation. The solubility parameter for camptothecin calculated with Hoy's method has recently been published as 23.33 $MPa^{1/2}$.[39] From these data, the Flory-Huggins polymer–solute interaction parameter χ_{SP} is 0.001 between PLA and camptothecin and 0.087 between PLGA and camptothecin.[39] The smaller interaction parameter between camptothecin and PLA might explain the higher achieved drug loading.

Camptothecin Release

The release profile of camptothecin from the PLGA microspheres is almost identical to the profiles shown by Tong et al.,[29] once their burst effect of about 40% is disregarded. Our experiments showed no burst effect (Fig. 3B). These differences in burst effect release, as well as the rather different encapsulation efficiency of about 40% with our method versus >99% in Tong et al.'s experiments might be due to the different drug concentrations used (2.5–10% vs. 0.33–1.00% of the polymer weight) and the form the drug was in when added to the polymer (suspended vs. dissolved).

The release profile of camptothecin from the PLA microspheres was unexpected, as a complete release took place within the first 6 h (Fig. 3A). The drug was very likely completely surface associated with the PLA. A recently published article described a similar rapid release of camptothecin from DL-PLA nanoparticles.[34] Kunii et al. achieved an encapsulation of 38%, and then described the drug "to be located around the surface and/or to be incorporated incompletely", since 100% of the drug was released within an hour. Their nanoparticles, however, also contained 37 wt.% of a poly(ethylene glycol)—poly(propylene glycol) copolymer, and comparisons might thus not be very meaningful. More work is necessary to clarify these large differences between the camptothecin release seen in PLA and PLGA microspheres.

Based on the literature degradation data for the pure polymers, L-PLA should degrade fourfold slower than the used PLGA.[40] Our observed results, however, at best describe a similar degradation behavior for the two biodegradable polymers. After 24 h the surfaces of the PLA microspheres appeared a bit rougher than those of the PLGA microspheres, but the difference was not enough to have predicted the observed complete drug release (Fig. 4B and E). The fact that complete drug release occurred suggests an interaction, perhaps rearrangement processes, between the drug and the polymer either during the flow focusing process, during the solvent extraction process, or most likely, during the drying which took place before the drug release experiments started. The low drug concentration in the microspheres did not allow for the clarification of these processes, although the morphology of the particles was carefully observed and an attempt was made to analyze interactions between drug and polymer using differential scanning calorimetry (DSC). The DSC experiments, however, did not add information about the interactions between drug and polymer (data not shown).

Anticancer Activity of Encapsulated Camptothecin

The objective of the anticancer activity study was to test for differences in drug release profile from the PLA and PLGA microspheres and to

determine the *in vitro* effects of the drug on tumor cells. Previous studies have shown no effect of PLGA nanoparticles on the viability of different cells.[41] Therefore, the main test parameter was the camptothecin release profile and its effect on the cell viability.

The drug amount of 10 ng of camptothecin per well containing 10000 initially plated cells was chosen according to the work of another research group that generated camptothecin loaded PLGA microspheres by the water in oil emulsification method.[29] Comparable cell viabilities were expected, but higher values were measured, i.e., an average of 63.9% cell survival (Fig. 5) compared to the 9.1% shown by Tong et al. The difference can be attributed to the slower release over the time of the assay, that is, 7–9% within 3 days in the present study compared to the large burst effect and overall 70–90% release within 3 days in the study by Tong et al.[29] Factors leading to these differences include the type of polymer used (PLGA 50:50 vs. 85:15), the form of the camptothecin in the microspheres (large precipitates vs. more homogeneous distribution within the polymer matrix), the different shape of the microspheres (irregular vs. the spherical microspheres made by hydrodynamic flow focusing), and the cell types used (B16 melanoma vs. H226 lung epithelial cancer cells).

Unexpectedly, the average viability of the cells incubated with PLA microspheres was only slightly lower than that of the cells incubated with PLGA microspheres, i.e., 42.8% vs. 63.9%, and this was the case despite complete drug release within the first few hours (Fig. 5). This difference is very likely explained by the fact that camptothecin is not stable in PBS, converting rapidly to the much less active carboxylate form.[29] Meanwhile, the constant supply of the active lactone form of the drug over 3 days in the case of the PLGA microspheres might provide a constant impediment to cell growth, resulting in similarly depressed cell growth curves.

CONCLUSIONS

The present study is the first to illustrate the use of hydrodynamic flow focusing for the preparation of drug-encapsulating biodegradable microspheres. The lipophilic anticancer drug camptothecin was encapsulated into PLA and PLGA microspheres without the use of high-energy mechanical mixing methods, yielding small 2–3 μm in diameter microspheres. Compared to the typically used solvent evaporation/extraction methods, a relatively narrow size distribution with a CV of 0.14 was reached. Monodispersity, one of the foremost promises of the hydrodynamic flow focusing method, was however not achieved. Based on an ongoing parameter study with nondrug loaded biodegradable microspheres, further changes to the experimental setup and a thorough study of additional process parameters will be necessary to improve monodispersity. Such process parameters include (higher) flow rates and (larger) pressure drops across the orifice, altered orifice dimensions and shapes, and the potential use of additives (e.g., surface active agents). In summary, hydrodynamic flow focusing is a valuable method for the generation of drug-containing biodegradable microspheres. It is simple to use, allows the production of different sizes of particles simply by changing the flow ratio of continuous to disperse phase, works in continuous mode, and has the potential to be used for the preparation of uniform particles sized from hundreds of nanometers to tens of micrometers.

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