

PLGA micro/nanosphere synthesis by droplet microfluidic solvent evaporation and extraction approaches

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In this paper, we present two approaches for the synthesis of poly(lactide-*co*-glycolide) (PLGA) micro/nanospheres using non-toxic organic solvents in droplet-based microfluidic platforms. Solvent evaporation and solvent extraction methods were employed to enable the controlled generation of monodisperse PLGA particles that range from 70 nanometres to 30 microns in diameter.

Determination of particle size was carried out with dynamic light scattering (DLS) and image analysis to show less than 2% variation in particle size. Sizes of the PLGA microspheres were controlled by the PLGA concentration in solvent and by the relative flow rates of oil and aqueous phases in the system. A penetration imaging assay was performed to determine the depth of diffusion of a model drug molecule fluorescein, out of the PLGA nanoparticles into corneal tissue. With the ability to prepare high quality, monodisperse, biodegradable particles, our methods have great potential to benefit drug delivery applications.

Introduction

Over the years, the throughput for drug synthesis and testing has increased exponentially, leading to the discovery of a large number of novel and effective therapeutic compounds. Initially, many of these drugs could be administered *via* absorption through the gastrointestinal tract, but as more compounds, proteins, and other bioactive molecules are discovered, it is found that a large number of these compounds do not have the characteristics of an ideal drug; *i.e.* one that is stable, soluble and has a low molecular weight. In many cases, these drug compounds are complex, large, insoluble, and unstable.¹ Thus, for these drugs to reach the target site and maintain sufficient therapeutic activity, a reliable delivery system is required.

Micro/nanoparticles have been explored extensively as drug delivery systems for small drug molecules as well as macromolecules such as nucleic acids, proteins, and hormones.² The microencapsulation of drugs and vaccines is a popular method of delivery due to its ability to protect the encased cargo, improve solubility in the bloodstream, and reduce immunological side effects to the patient.³

Approved by the US Food and Drug Administration (FDA) for therapeutics, poly(lactic-*co*-glycolic acid) (PLGA) is one of the most frequently used biomaterials for drug delivery and therapeutic encapsulation.⁴ PLGA is a synthetic copolymer composed of glycolic acid and lactic acid. It is known for its biocompatibility and biodegradability since it breaks down into

harmless monomer units of acids normally found in the human body. This characteristic allows multiple dosages of this polymer without worry of harmful side effects to the patient. The rate of degradation is also well characterized and can be predicted by the composition of the polymer. The degradation rate depends on the relative amounts of glycolic and lactic units that comprise the polymer. In general, the greater the ratio of glycolic units, the faster the degradation rate. This property provides a simple method of controlling the polymer particle's cargo release. PLGA spheres have been used as delivery vehicles for bioactive compounds such as drugs,⁵ peptides,⁶ proteins^{7,8} DNA,^{9–11} viruses,¹² and bacterial antigens.¹³ These polymer particles are widely sought for use as delivery vehicles for biomedical and pharmaceutical applications.

However, formation of PLGA particles by conventional bulk methods result in particles that are unstable, have large size distributions, allow only limited encapsulation control, and can be harmful to the substance being encapsulated. One example is the spray-drying method which requires high temperature operation, that can damage the polymer structure and the cargo within. The coacervation method which forms emulsions in bulk solution that are evaporated to form particles, creates non-uniform particles and presents challenges in removing solvent residues.¹⁴ Electrospray^{15,16} and acoustic ejector^{17,18} methods have also been developed for the formation of droplets and particles, however, these methods are limited to liquid-in-air systems, and are unable to incorporate additional reagent sources. Solvent evaporation and extraction methods do not require high operating temperatures, and nano/micro-sized particles can be effectively achieved. Droplet microfluidic technology provides a route to generate monodisperse and uniform droplet emulsions, using a variety of different materials and reagents.¹⁹ Droplet-based microfluidic systems have been investigated and developed rapidly in the last few years, to enable monodisperse droplet and particle generation in the micron size range.^{20–23} However to date, a microfluidic solvent method for the

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formation of particles in the nanometre size range has not been reported. We use an alternating droplet formation and fusion^{24,25} method to generate PLGA nanoparticles. In addition, we present the formation of biodegradable PLGA particles in the micron size range using a safer, non-toxic solvent, dimethyl carbonate.²⁶ Other organic solvents commonly used for particle and liposome formation such as benzene, toluene, and methyl chloride are known to be toxic.^{27,28}

Here we describe our methods for droplet microfluidic synthesis of monodisperse PLGA particles of controllable size with low toxicity solvents, and demonstrate the encapsulation of a drug model, fluorescein, and its penetration through animal tissue.

Experiment set-up

In this study, solvent evaporation and solvent extraction approaches have been applied to generate micro to nano-sized PLGA spheres using a microfluidic platform. Unlike chemical- or UV-catalyzed polymerization, which involves the introduction of an external catalyst, the solvent evaporation and extraction methods depend on the removal of the solvent. We developed a solvent evaporation approach that consistently forms monodisperse micron sized PLGA particles using a flow-focusing geometry.²⁹ This approach consists of three main steps: first, the polymer is dissolved in a soluble volatile organic solvent. In this case, PLGA was dissolved in organic solvent dimethyl carbonate (DMC). DMC was chosen because it dissolves PLGA, is immiscible with water, and does not swell PDMS. In addition, it evaporates rapidly, and is biodegradable and less toxic than other conventional organic solvents. Next, monodisperse polymer droplets are generated by shear-induced emulsification in an immiscible liquid phase (DI water with an aqueous surfactant), using a flow-focusing geometry. Lastly, the solvent is evaporated

away and the particles are collected. This method results in particles in the micron-size range.

We employ the solvent extraction approach in order to generate particles in the hundreds of nanometre size range and smaller. Under pathological conditions, the endothelium displays an increase in permeability. Particles in the nanometre size range may be employed in the passive targeting of tumors, which have leaky blood vessels with openings of 200 nm or less. The particles must be large enough such that few are passed through the kidneys, yet small enough to diffuse into tumor tissue.³⁰ The mechanism of nanoparticle generation by microfluidic solvent extraction consists of three main steps: first, PLGA is dissolved in a water-soluble solvent and emulsified by an immiscible liquid phase. We use dimethyl sulfoxide (DMSO) as the solvent, and mineral oil as the continuous phase. Then extraction of the solvent from the PLGA droplets is accomplished by fusion of the PLGA–DMSO droplet with a water droplet. Due to the high solubility of PLGA in DMSO and its low solubility in water, PLGA is precipitated out of solution, resulting in the production of nanoparticles. Selection of the water-soluble solvent is key, since the use of a strongly water-soluble liquid is necessary to properly precipitate out and form the PLGA particles. We chose DMSO because it dissolves PLGA well, but has a higher affinity for water than that of PLGA. After solvent removal, the nanoparticles are allowed to solidify and be collected. We perform the extraction process using an alternative droplet generation and fusion design as shown in Fig. 1a.

Microchannel design and fabrication

Solvent evaporation device

A 50 μm thick layer of SU-8 50 was spun onto a silicon wafer, and patterned using conventional photolithography. The

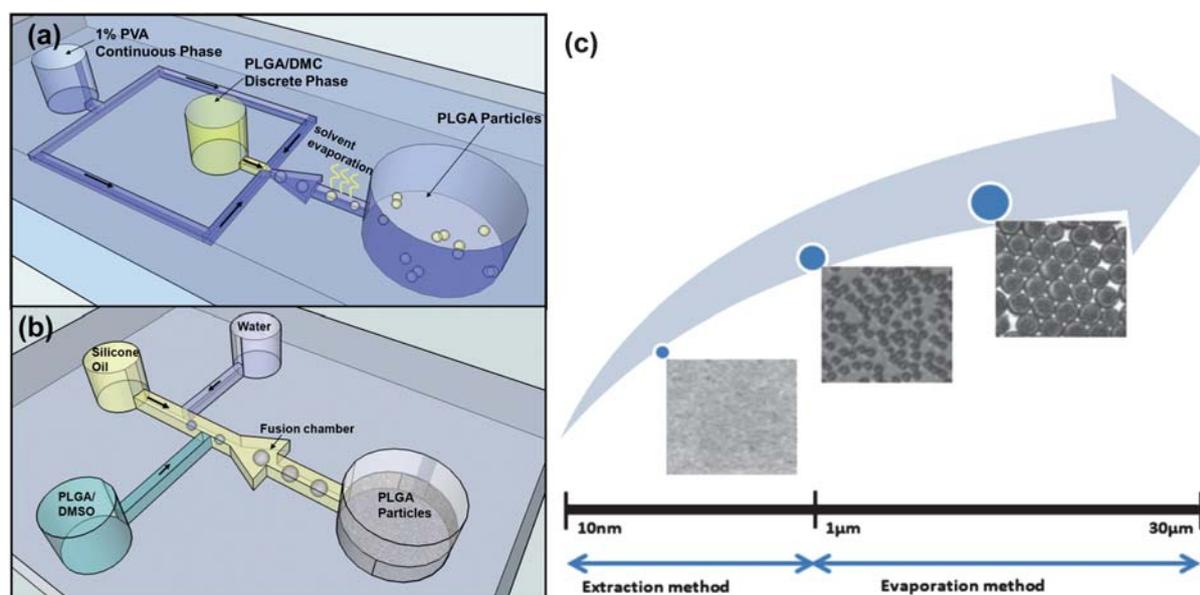


Fig. 1 (a) Schematic of device design for the solvent evaporation method. Channels are molded in PDMS and bonded to a glass slide. PVA solution shears PLGA–dimethyl carbonate to form PLGA droplets. The solvent rapidly evaporates away, allowing PLGA to polymerize into particles. (b) Schematic of solvent extraction channel design, which includes three inlets, a fusion chamber and a collection reservoir. Water and PLGA solution are sheared by silicone oil into monodispersed droplets, then fused in a widening chamber to initiate the solvent extraction. (c) Size graph showing that a wide range of particle sizes can be achieved with the extraction and evaporation methods.

microchannels were molded by pouring a mixture of PDMS elastomer base and curing agent at a 10 : 1 ratio over the SU-8 mold and baking for at least 4 hours in a 65 °C oven. A glass slide was bonded to the flat PDMS substrate by air plasma treatment. The bond was strengthened by placing the device in a 120 °C oven overnight. Since this is an oil-in-water droplet generation process and there is a favorable wettability between DMC and PDMS, poly(vinyl alcohol) (PVA) surface treatment is applied to the channels to make the device hydrophilic. PVA treatment has been shown to irreversibly adsorb to a number of materials including silicon capillary channels and some hydrophobic polymer surfaces.³¹ Wu *et al.* showed that repeated cycles of adsorption/drying and heat immobilization of PVA onto PDMS result in a hydrophilic treatment that lasts for several weeks.³² The treatment process begins by first flowing a 1% aqueous solution of PVA through the channel of interest and incubating for at least five minutes at room temperature. This provides time for PVA to self-assemble onto the surface. Excess PVA is vacuumed out of the channel, and the device is incubated at 100 °C for 5 minutes to heat-immobilize the PVA. This process of PVA adsorption, drying, and heating can be repeated twice to ensure sufficient coating and a longer lasting hydrophilic surface.

Fig. 1a shows a schematic of the micro channel design, which includes an inlet for PLGA dissolved in DMC and inlet for the continuous water phase, a shear junction, and an outlet reservoir. PLGA was dissolved in DMC at concentration of 0.5–5 wt% and housed in a Becton–Dickinson solvent compatible syringe, and connected to the microfluidic device with Teflon tubing. The continuous phase consisted of a 1% PVA solution in water. PVA was added to the continuous aqueous phase as a stabilizer to prevent droplet fusion and as a wetting agent to prevent the solvent from wetting the PDMS walls. The inlet flow rates were controlled by two separate syringe pumps. Due to the high volatility of DMC, the solvent evaporates quickly and results in micron-sized PLGA particles within a minute. At the outlet reservoir, PLGA microspheres were collected by a pipette and transferred to a storage vial. PLGA microspheres were sputter-coated with Au–Pd alloy in preparation for scanning electron microscope (SEM) (Philips USA) imaging. Sizes of droplets and microspheres were measured by ImageJ software.

Solvent extraction device

The microchannels for the solvent extraction approach were prepared using PDMS soft lithography as described in the previous section. After molding and bonding to the glass substrate, the PDMS channels underwent surface thermal treatment for faster hydrophobic reconstitution,⁸ which favors water-in-oil droplet generation. Fig. 1b shows a schematic of the solvent extraction channel design, which includes three inlets, one each for silicone oil with 1% span 80, PLGA in DMSO, and water; T-junctions, a fusion chamber, and outlet reservoir. The PLGA–DMSO solution, silicone oil continuous phases, and aqueous solution were injected into the channels by three syringe pumps. Tygon tubing was used to connect the reagents to the microfluidic device.

PLGA was dissolved in water-soluble dimethyl sulfoxide (DMSO) at a concentration of 1 wt% and sheared by silicone oil at the T-junction into monodispersed droplets. The surfactant

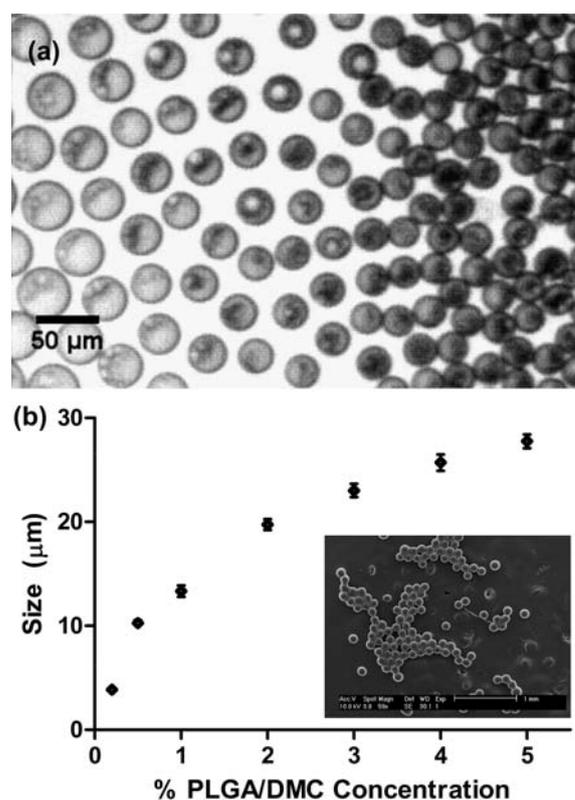


Fig. 2 (a) At the outlet reservoir, PLGA–DMC droplets shrink to form hard PLGA microspheres. (b) PLGA microsphere sizes increased proportionally with the PLGA concentration in DMC solution. Inset shows SEM image of monodispersed PLGA particles prepared by the evaporation method from a 3% PLGA solution.

span 80 was added to the oil phase at 1 wt% to prevent the PLGA–DMSO droplets from sticking onto the PDMS channel walls or from fusing to each other, yet still allowed fusion between the water and PLGA–DMSO droplets. As shown in Fig. 2, droplets of water are generated and introduced to the PLGA–DMSO droplet at the extraction junction. Upon fusion with the aqueous droplet, PLGA nanospheres are immediately precipitated out in the aqueous droplet. PLGA nanospheres were collected by pipette, centrifuged to remove excess solvent, and resuspended in pure water. The particles then underwent a vacuum-drying process and Au–Pd alloy sputter-coating for SEM imaging and laser light scattering.

Results and discussion

Monodisperse PLGA microsphere synthesis by solvent evaporation approach

At the shear junction, the PLGA solution was sheared by the continuous water phase to generate monodisperse droplets. DMC is volatile and evaporates rapidly upon exposure to air. As the solvent evaporates away, it leaves behind a concentrated solution of PLGA which crosslinks spontaneously to form a polymer matrix. The PLGA–solvent droplets experience up to a tenfold reduction in size, depending on the initial PLGA concentration, resulting in the formation of particles as small as

3 μm in diameter. Fig. 2a shows the transition from liquid droplets to solid particles at the outlet of the device, a process that takes less than one minute. The size distributions of PLGA–DMC droplets and PLGA microspheres are less than 3%. The SEM images of PLGA microspheres prepared from 3% PLGA–DMC concentration are shown in the inset of Fig. 2b.

The final particle size depends on the initial PLGA concentration as shown in Fig. 2b. With the same initial droplet sizes, larger particles are formed with higher concentrations of PLGA. The diameter of the particles ranged from approximately 3 μm to 30 μm demonstrating a proportional increase in particle volume with respect to the PLGA concentration. The volumes of PLGA microspheres are increased proportionally to the PLGA concentration in DMC solution. For example, when the concentration increased from 0.5% to 5%, the volume increased twenty fold from 0.52 to 9.2 pl. This indicates the matrix structure of PLGA microspheres decreases in density with higher PLGA concentration, with the solvent evaporation method, resulting in a larger matrix and greater cargo capacity.

Solvent extraction approach for PLGA nanosphere synthesis

After fusion with a water droplet, DMSO in the PLGA–DMSO droplet is rapidly extracted into the water. PLGA nanospheres are precipitated out immediately due to supersaturation of PLGA. Because of the small volume and rapid mixing that occurs inside of the droplets, the solvent extraction process occurs quickly and immediately after the fusion process. The uniformity in droplet size allows large numbers of PLGA particles to be generated with fidelity across different droplets as the reaction conditions in each droplet are virtually the same. Therefore homogeneous nano-sized particles can be generated. Fig. 3 shows consecutive processes of droplet merging; in Fig. 3c, small PLGA spheres can be observed. Fig. 4 shows the SEM image of PLGA nanospheres prepared by the solvent extraction approach. The sizes of the PLGA nanospheres are in the 100 nm range and appear to have a very narrow size distribution. Nanolitre-droplets have defined volume when solvent was

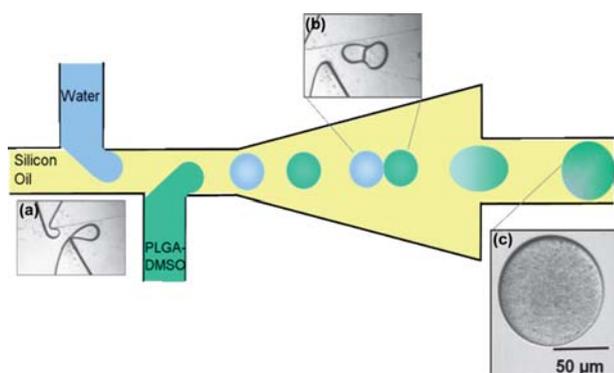


Fig. 3 (a) PLGA–DMSO and water droplets are formed in T-junctions. (b) At fusion chamber, PLGA–DMSO droplets merge with the water droplets and DMSO is extracted into the aqueous phase, resulting in immediate PLGA particle formation. (c) Each droplet acts as a micro-reactor to produce hundreds of PLGA particles.

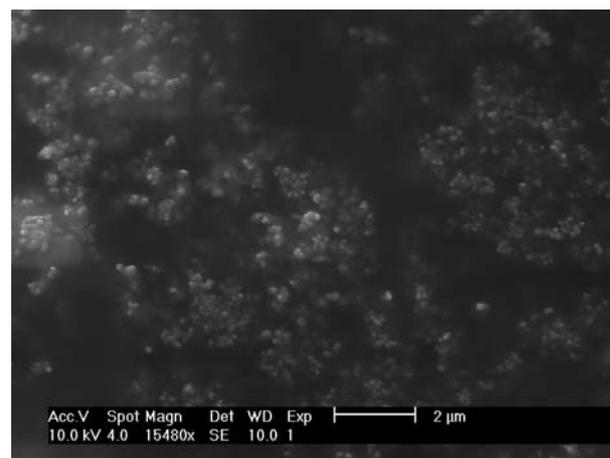


Fig. 4 SEM image of PLGA nanoparticles prepared by the solvent extraction approach. The blur is due to the presence of silicone oil.

extracted into the aqueous phase, resulting in rapid precipitation and homogeneous nanoparticles. Each droplet acts as a micro-reactor in which numerous nanospheres are produced. The rapid nanosphere precipitation is governed by the Marangoni effect,³³ which explains that the precipitation is due to interfacial turbulence between the interfaces of the disperse and continuous media, formed from complex phenomena such as flow, diffusion, and surface tension variations.³⁴ Another advantage of the solvent extraction approach is that it avoids the use of toxic organic solvents which are normally required for polymer particle synthesis.

Evaluation of PLGA nanoparticles

The diameter of the PLGA nanoparticles was determined by dynamic light scattering (DLS, Malvern Instruments, UK). The particles were centrifuged and resuspended in DI water prior to being loaded into the DLS. Size measurements show that the particles are monodisperse and sizes can be controlled by the ratio of PLGA–DMSO and water droplets fused together, as shown in Fig. 5. The diameter of the particles ranged from approximately 70 nm to 0.5 μm and there was a proportional increase in particle volume with respect to the PLGA concentration. The higher the concentration of PLGA, the larger the particle size. This can be attributed to the higher quantity of PLGA monomers that leads to slower solvent diffusion, and larger particle formation.³⁵

We also demonstrated the ability of the particles to encapsulate and release a sample compound and penetrate tissue. For this experiment, fluorescein dye (Standard Fluka, Sigma-Aldrich) was added into the PLGA–DMSO solution at a final concentration of 0.05 wt%. During the solvent extraction process, PLGA precipitates out of DMSO and entraps fluorescein dye as it polymerizes and forms the polymer matrix.

Fluorescein is released from the particles by diffusing out of the small cavities in the polymer matrix which widens as PLGA undergoes hydrolysis. The degradation rate of PLGA is determined by the ratio of lactic acid and glycolic acid that comprises the copolymer. The greater the number of glycolide units, the faster the polymer degrades, and thus the more rapidly

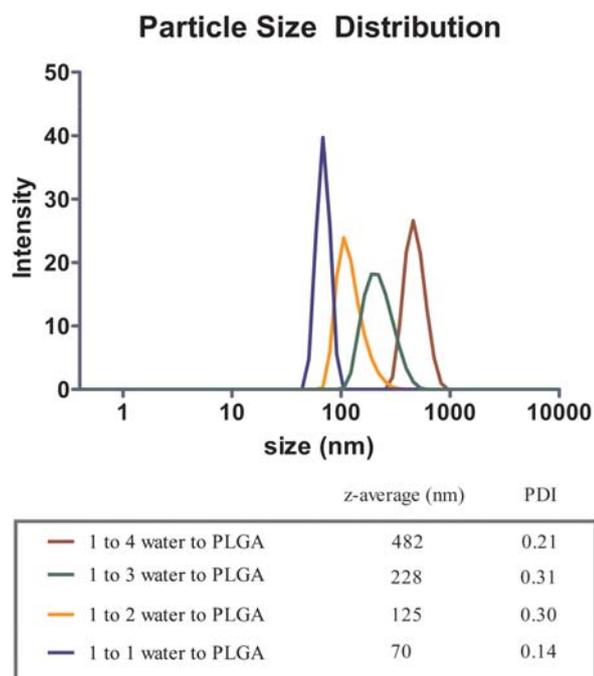


Fig. 5 DLS measurements of nanoparticles formed *via* the solvent extraction method. A range of particle sizes can be formed based on the ratio of the water and PLGA–DMSO flow rates.

the contents can be released. This enables better control over the encapsulation and timed release of drugs, proteins, or peptides for drug delivery.

To evaluate the drug delivery capabilities of the synthesized nanospheres into tissue, a preliminary penetration test was performed on the cornea of a rabbit. We observed particles as deep as 25 μm below the corneal surface. First, corneal surface ablation was performed to form a well on the anterior corneal surface. Then the PLGA nanosphere solution was applied to the cornea for 10 minutes. Fig. 6a shows a multiphoton microscopy image of the particles in corneal tissue. The particles show up as green spheres due to the entrapped fluorescein, and collagen is represented by the magenta color. The particles are located predominantly on the stromal surface, with a few particles that could be detected deeper in the stroma. The multiphoton microscopy images shown are sample XY and XZ slices through a large 3-D dataset showing the anterior stroma extending some 50 μm into the cornea. Some particles were found as deep as 25 μm into the stromal surface, showing that the particle size is small enough to penetrate through tissue. Additional experiments would be required to fully characterize particle penetration capabilities but this demonstrates the potential of the particles to pass through the surface of the tissue.

Characterization of the release profile was conducted by measuring the amount of fluorescein released from the particles over time. The particles were synthesized as previously described and suspended in phosphate buffered saline (PBS). The particles were then loaded into a chamber in which the external solution was separated from the particles with a dialysis membrane with molecular weight cut off of 10 000. As the particles undergo hydrolysis, fluorescein is released into the surrounding medium.

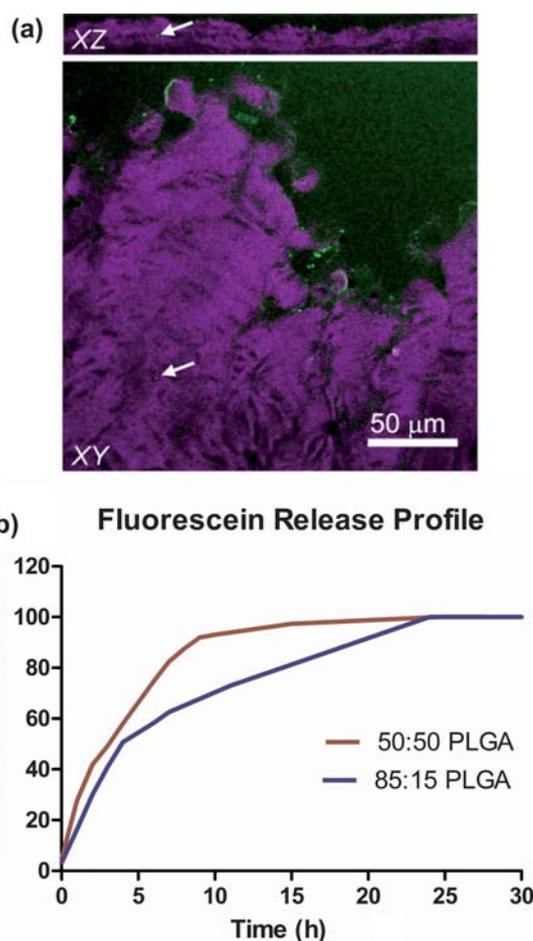


Fig. 6 (a) A series of Z-stack images were collected using multiphoton microscopy. Representative images of 3 μm PLGA particles prepared by solvent evaporation approach, on a corneal stroma surface. The collagen fibers are shown in magenta and the PLGA particles are shown in green (arrows). (b) Release profile of fluorescein from 70 nm particles over 24 hours.

The dialysis membrane prevents the particles from entering the sampled solution and affecting the measurements; while at the same time allows the small molecular weight fluorescein to freely diffuse through. At one hour intervals, the fluorescence of the external solution was measured and plotted by normalising to the final fluorescence intensity. The release profiles of 70 nm PLGA nanoparticles of 85 : 15 and 50 : 50 lactide to glycolide monomer ratios are shown in Fig. 6b. The release profile shows a biphasic release pattern, where there is an initial burst release of fluorescein that had been coating the surface of the PLGA particles, then a steady release as the particles disintegrate. The release of fluorescein from PLGA 50 : 50 (glycolic-to-lactic) is faster than that of PLGA 85 : 15, which is expected since the degradation rate of PLGA 50 : 50 is also greater. Nearly all of the fluorescein is released at the 10th hour for PLGA 50 : 50, whereas it takes nearly 24 hours for PLGA 85 : 15 to fully release its cargo. This system can be fine tuned by altering the composition and potentially the size of the PLGA particles to vary its degradation rate. The attachment of targeting moieties to the particle surface can also be implemented to improve its specificity.

Conclusion

We have presented microfluidic droplet devices that are capable of synthesizing monodisperse PLGA particles of varying sizes. Using two microfluidic droplet-based approaches and tuning of the concentration of PLGA, we are able to make particles that are up to three orders of magnitude different in size. Not only do the devices enable fine control over the synthesis of drug carriers, they also offer the potential of being integrated into more complex microfluidic systems. The release profile and penetration tests demonstrate the potential of microfluidically formed PLGA particles for applications in therapeutic delivery. Future work includes further characterization of the degradation kinetics of the PLGA nanoparticles and the release profile for larger molecules.

Lastly, to be competitive against conventional synthesis methods, scaling up of the microfluidic processes is necessary. There has been recent work in developing methods to scale up droplet based processes including droplet splitting³⁶ and parallel generation schemes.^{37,38} Due to the simple nature of the methods developed here, we believe it is possible to implement our process with such scale up methods to dramatically improve the throughput of our microfluidic methods.

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