To Austin, Keira, and Owen.
The following publications describe portions of this dissertation:


Abstract

Targeted delivery of pharmaceutical compounds enhances efficacy of the drug at the site of activity while reducing interactions with off-target tissues and organs. Using this technology, scientists have been able to take drugs discarded years ago due to harmful side effects and reformulate them for use. In this work, we have been motivated by enhanced efficacy of camptothecin in lung cancer treatment through the use of passive targeting within the capillary bed in the lungs. We have developed a nanocomposite, drug loaded hydrogel microparticle to deliver drugs to cancer tissue in the lungs through venous filtration. Critical to the success of this method is the control of the size and modulus of the hydrogel particles injected. In order to closely control the size, we have developed a glass capillary microfluidic device and a shear emulsification process for generation of drug loaded hydrogel microparticles from 12 μm to 100 μm in diameter.

We developed a low cost, modular glass capillary microfluidic device, using commercial components, to simply and reproducibly generate microparticles using microfluidic droplet generation. We then studied the effects of multiple parameters on the generation of small droplets using the device. After establishing the microfluidics, a bulk emulsification technique was developed to generate large quantities of microparticles for in vivo studies in mice. A thiol-acrylate Michael addition polymerization was used to solidify our droplets, containing fluorescent nanoparticles for imaging, into particles. We performed in vivo pilot studies in mice to demonstrate the targeting of the microparticles. A dosing strategy was developed using particles produced through bulk emulsification methods. We then compared our bulk emulsification methods with the dosing of monodisperse hydrogel particles to determine the benefits of microfluidic particle
production. We found that small particles, 36 μm in diameter, stay in the lungs for a week, with nonspecific targeting to the liver and spleen. Larger particles tended to lodge in the lungs for up to seven weeks undergoing degradation by macrophages, with small satellite particles targeting other organs. Monodisperse particles, 91 μm in diameter, generated by microfluidics had even better targeting of the lungs due to removal of satellite particles.
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Chapter 1: Introduction and Thesis Objectives

1.1 Motivation

Lung cancer is the leading cause of cancer-related death in the United States. Although it is not the most common type of cancer, only 15 percent of patients diagnosed with lung cancer survive beyond 5 years.\(^1\) This high mortality rate is attributed to the fact that lung cancer is often discovered at late stages, with 77 percent of new patients being diagnosed having tumors that have already metastasized. At that point, the 5-year survival rate drops to less than 10 percent.\(^2\) Non small cell lung cancer (NSCLC) accounts for 85 percent of all lung cancer cases diagnosed. Currently, the methods for treating this disease depend on the stage of the disease, the ability to perform surgery, and health of the patient.\(^1\) Early stage NSCLC is often treated with resection followed by chemotherapy. Late stage NSCLC is normally treated with a combination of radiation and chemotherapy, because surgery would result in loss of too much lung function.\(^3\) Late stage NSCLC treatment has increased morbidity due to systemic chemotherapies with large quantities of expensive drugs, causing side effects including pulmonary toxicity and death.\(^4\) These side effects normally occur because the concentration of the drug in the local tumor environment cannot sustain therapeutic levels due to rapid clearance, forcing the dosing to be very high.\(^5\) Chemotherapy has been shown to be effective in some cases, but there is a need to develop a smarter drug delivery system that can target and treat NSCLC with lower drug concentrations to reduce costs and side effects.

Camptothecin (CPT) is a naturally occurring small molecule topoisomerase inhibitor that has been proven to be effective against many types of cancer cells \textit{in vitro}.\(^6\) The high level of
Topoisomerase inhibition activity is seen when CPT is stable in a lactone form. However, at biological pH, the fifth ring in CPT opens to create a carboxylate form, which has much lower antitumor activity. Due to its high toxicity and low solubility in water, it is unable to be effective as a systemic treatment for NSCLC in its natural form.\(^7\)

In this thesis, we describe a method for development of a two stage drug delivery system involving biodegradable composite gel microparticles (cGMPs) generated using conventional emulsification and advanced microfluidic emulsification techniques. The cGMPs are designed to passively lodge in the blood capillaries within the lung, and then degrade and release drug. Drug release from the gel microparticle may be controlled by the release from the encapsulated nanoparticle, or may occur by release of the entire nanoparticle from the gel matrix as it degrades. We will describe a method to fabricate microfluidic devices for emulsification, how to use microfluidics to fabricate our cGMPs, how to characterize the mechanical properties of our cGMPs, and how to look at the performance of our delivery system \textit{in vivo}.

1.2 Previous Work

This thesis is based on a foundation of drug delivery research over the last decade involving many researchers in the Sinko and Prud'homme groups.

Initial work to understand the effects of microparticle embolization within the lungs was the basis of the thesis of Hilliard Kutscher.\(^8\) In his thesis, he developed a model for the ventilation perfusion of a mouse and how it was affected by the introduction of solid particles within the lungs. In order to understand the toxicological effects of dosing microparticles into mice, studies were performed using different quantities and sizes of microparticles to determine the maximum tolerated dose of the microparticles. Interestingly, it was shown that the maximum
tolerated dose of solid polystyrene microparticles was correlated well to the volume of the particles dosed. Both small numbers of larger particles and large numbers of small particles gave similar results in terms of dose toxicity if they had equal volumes. This work allowed us to decide on dosing quantities for in vivo studies based on their calculated maximum tolerated dose values with solid particles.

The thesis work by Nathalie Pinkerton developed the chemistry utilized within the drug delivery system both for the gel microparticle and the nanoparticle prodrugs containing camptothecin.\(^9\) It was shown that a Michael addition gelation of precursor emulsion droplets was a gentle and tunable chemistry for generating polyethylene glycol (PEG) hydrogel microparticles. Sensitive molecules and dyes would not degrade as they typically did when using a free radical polymerization in UV light. The gelation chemistry also allowed for a tunable modulus and particle morphology based on the precursor concentration and polymerization conditions. As well, initial in vivo tests showed that gel microparticles targeted the lungs of mice after injection. Building upon these results, we were able to reformulate the microparticles for higher drug loading and proceed to in vivo tests.

1.3 Background

1.3.1 Microfluidic droplet generation

Microfluidic droplet generation involves the manipulation of microscale low Reynolds number two phase flows in order to form monodisperse droplets.\(^9,10\) This method of producing complex monodisperse droplets is much more precise and controllable compared to other methods, such as shear emulsification.\(^11,12\) The droplets are formed due to the manipulation of surface instabilities within small scale two phase flows, and their size can be controlled by tuning
of the capillary number.\textsuperscript{9} By using PDMS soft lithography, microfluidic devices can be made rapidly and very inexpensively once a master has been created.\textsuperscript{13} The advantage of this process is devices can be generated inexpensively and reproducibility for quick emulsion generation.\textsuperscript{9} However, PDMS is very sensitive to certain solvents, which can cause swelling of the polymer leading to changes in device dimension, which affects droplet generation. As well, these devices cannot handle high pressure. In order to avoid these shortcomings, microcapillary devices have been developed for microfluidic droplet generation. Being made out of glass, they can be fabricated and functionalized to handle a variety of solvent and operating conditions. However traditional fabrication of microcapillary devices is serial and "art-dependent", which will be described in more detail later on in this chapter.

There are currently two main device geometries used to generate droplets in passive microfluidics, flow focusing and the T-junction.\textsuperscript{9} These techniques have many advantages compared to the more specialized techniques involving electric or magnetic fields, although integration of switches and other active controls is becoming more common.

In a flow focusing device, the dispersed phase is bounded on both sides by the continuous phase and sent through a pinch point. At this point, the dispersed phase breaks up due to the perturbation generated by the pinch point, leading to a surface tension driven instability and droplet break-up.\textsuperscript{14} A schematic of the basic aspects of a flow focusing device along with an actual device is shown in Figure 1.1. This process has advantages compared to the T-junction because it can produce very small droplets (less than 10 microns in size), and does not allow the droplets to interact with the channel walls. However, there is an issue with this process when
trying to make polymerizing gels. The orifice size of the pinch point is often very small in order to create small droplets, so impurities or prepolymerized gel fragments can jam and clog the device. For capillary flow focusing devices, it is necessary to have a reusable glass capillary device that can be cleaned when clogged.

T-junction droplet generation is when two streams are brought together perpendicularly to form drops. The stream that is the dispersed phase flows into the continuous phase, and as the two streams meet, the droplets are sheared off of the dispersed phase stream. This method of droplet generation cannot make droplets as small as flow focusing. There are, however, fewer issues with clogging and the process can be scaled up easily by running parallel dispersed phase streams into one large continuous phase stream.15

A variation of flow focusing called co-flowing is another method to produce droplets. Co-flowing is when the dispersed phase flows between two continuous phases without a pinch point. At long distances from the initial phase contact, the dispersed phase eventually becomes unsteady due to Rayleigh instabilities and breaks up into droplets. This process has less control on the size and monodispersity of the droplets formed.9
Other techniques that have been developed involve the use of external forces to force breakup such as thermal, magnetic, or electric fields.\textsuperscript{16-20} Changing the temperature of the interacting fluids can change the viscosity and interfacial tension, thus tuning the drop size in the microfluidic device.\textsuperscript{16} Adding a laser into a microfluidic channel with two phase flow can cause a local shift in temperature that acts as a switch for starting and stopping droplet generation.\textsuperscript{17} Magnetic forces can be used in a situation where there is a ferrofluid or phase with magnetic nanoparticles in solution. By using magnetic pulses, droplets of magnetic fluid can be pulled into the continuous phase. Their size can be tuned by the strength of the pulse.\textsuperscript{18} Electrical forces can also be used at interfaces of flows to produce droplets. Dielectrophoresis can pull droplets from a reservoir into a continuous phase at a certain rate.\textsuperscript{19} As well, electrowetting on a dielectric can create droplets by tuning the wetting of a fluid on a surface.\textsuperscript{20} All of these methods are interesting ways to make monodisperse droplets, but they require extra processing steps and active control, which complicates the droplet generation process. To avoid complicating operating steps, we adopted the flow focusing technique in our work.

1.3.2 Fabrication and application of glass capillary microfluidic devices

As mentioned briefly before, glass capillary microfluidics offers many advantages over traditional microfluidic droplet generation using PDMS devices.\textsuperscript{21} PDMS and other parallel-fabricated devices typically require the use of a clean room and sophisticated processing equipment. Whereas glass capillary devices are generated using only glass capillaries, syringe needles, epoxy, a microscope slide, and some tubing. The only equipment necessary is a flame to generate orifices and a microscope to look at the orifice size. It is very easy to gather the parts for building capillary devices, yet there is very little discussion about the difficulties of fabricating traditional capillary devices.\textsuperscript{22} The fragile nature of the glass capillaries make them very hard to
handle. As well, it is not a simple process to properly epoxy the device together in registration. Too little epoxy and the device will leak, while too much epoxy and there is a risk that the very small orifices will be filled with epoxy and clog the device. If you are successful with the epoxy step, you still have to find a way to remove the air bubbles from the device so that uniform droplet generation can occur. Due to these complexities, it often takes hours to build devices and more than half of the devices are unusable. Even someone trained in device building cannot consistently produce perfect capillary devices. When it became necessary for us to heavily rely on capillary microfluidics for emulsification, we developed a new microfluidic device that is described in Chapter 2.

1.3.3 Emulsion-templated fabrication of particles

Using microfluidics to generate monodisperse emulsions of polymer precursor solutions enables the formation of complex monodisperse microparticles upon polymerization. This technique has been shown to successfully and efficiently capture nanoparticles, cells, and therapeutic agents within drug delivery particles.

Typically particles formed using microfluidics use decoupled emulsification and polymerization steps. This means that particles can be generated without a time dependent reaction occurring. Particles are generated this way using a variety of decoupled solidification steps including evaporation, UV polymerization, and subsequent reaction induction. Although easy to implement into a microfluidic process, these methods often create conditions that can compromise the contents of the microfluidic droplets due to heating for solvent evaporation or radical generation for UV curing.
It has also been shown that polymerization techniques using softer gelation chemistries can be implemented into microfluidic droplet generation. These techniques typically involve the mixing and generation of droplets of precursor polymer solutions that are undergoing a very slow polymerization, such as a Michael addition reaction which will be discussed in the following sections. Often these devices cannot be run continuously, rather only for a certain "operating window" before the precursor solution polymerizes to a point where droplet generation is not possible. This is due to increasing precursor solution viscosity and finally gelling and clogging of the device.

1.3.4 Michael addition gelation of polyethylene glycol

In recent years, hydrogels, such as poly (ethylene glycol) (PEG), have become interesting materials for use in drug delivery because of their biocompatibility. Hydrogels are polymeric networks that can absorb and imbibe water within them. The network can be tuned by changing the polymer building blocks to change the strength, pH, charge, or degradability of the entire gel. For example, adding poly (lactic acid) (PLA) into a PEG hydrogel polymer matrix causes the gels to become biodegradable through hydrolysis of the PLA block. The chemical structure of this hydrogel is shown in Figure 1.2. As well, the mesh size of the hydrogel network can be tuned to control the diffusion of particles and drugs within the gel, thus allowing precise control of the release rate.

The Michael addition reaction to form PEG hydrogel networks typically involves the polymerization of a PEG containing nucleophilic end groups, such as thiols, reacting in a stepwise fashion with PEG precursors containing vinyl groups under basic conditions that activate the nucleophile. It has been shown that this reaction is versatile and gentle, allowing for
the creation of PEG hydrogel networks containing cells, biologics, or drug molecules. Because of its stepwise nature, the strength of the network is directly proportional to the amount of precursor molecules in solution. The higher the concentration, the denser and stiffer the network will become. As well, network strength can be controlled through the separation of the crosslinks by increasing the polymer molecular weight.\textsuperscript{35} Such flexibility in polymer tunability, along with ease of encapsulation make PEG hydrogels formed using a Michael addition reaction an attractive route to fabricate particles for advanced drug delivery.

1.3.5 Characterization of microparticle hydrogels

Often it is necessary to characterize mechanical properties of hydrogels in order to understand how they will perform at biological conditions within the body. Large solid gels can have their modulus and other properties measured by a rheometer.\textsuperscript{37} Recently it has been shown that hydrogel microparticles can also have their individual modulus measured by doing bulk rheological measurements on the particles slurried in water at close packing.\textsuperscript{38} However limitations still exist due to the need for large quantities of materials and particle slipping during measurements. Therefore interest has grown in developing methods for direct characterization of single microparticle mechanical properties using techniques such as osmotic compression and AFM indentation.
For decades, researchers have been using osmotic compression to impose an isotropic pressure on polymer particles.\textsuperscript{39} The theory is that if a particle is in solution, and small solutes are added to the solution, an osmotic pressure will be exerted on the particle if the solutes cannot enter the particle. The most important aspect of creating an osmotic pressure involves ensuring the molecules in the water do not “fit” into the pore of the hydrogel microparticle. Experiments have verified the role of the radius of gyration of the external stressing polymer and the mesh size of the gel.\textsuperscript{39}

In order to measure the compressive modulus of the particles, the same particles must be exposed to increasing concentrations of solute, typically 70K MW dextran solution. At each concentration, the volume of the particle is calculated using the diameter of the particle imaged under an optical microscope. By plotting the osmotic pressure in the solution against the volumetric strain on the particle a compressive modulus can be found from the slope.\textsuperscript{40} The equation is,

\[ \pi = K \left( \frac{\Delta V}{V} \right) \]  \hspace{1cm} (1.1)

where \( \pi \) is the externally imposed osmotic pressure (Pa). For the 70K dextran solution the osmotic pressure follows: \( \pi = c + 87c^2 + 5c^3 \), where \( c \) is in \% (w/w), as shown by Bonnetgonnet.\textsuperscript{39} \( \Delta V/V \) is the volumetric strain, and \( K \) is the compressive modulus. Using this method the compressive modulus can be found for hydrogels with different formulations, allowing us to characterize some of their mechanical properties to help understand their \textit{in vivo} performance.

The method of osmotic compression is relatively simple and easy to perform, but it only provides information on the compressive or bulk modulus. However for polymer gels in good solvents, the bulk modulus can be related to the shear modulus, \( G \), by\textsuperscript{41}
\[ K = \left( \frac{2}{3} \right) G \quad . \quad (1.2) \]

AFM indentation of hydrogel microparticles offers a way to measure the Young's modulus.\textsuperscript{40,42,43} AFM indentation involves axial compression of a soft sphere using an AFM cantilever with a small hard sphere attached.\textsuperscript{44,45} The cantilever is displaced into the soft sphere after contact until a maximum force is measured by the deflection of the cantilever. The force and displacement are measured throughout the experiment and Hertz theory is used to find the Young's modulus. Hertz theory is described in the following paragraph.

When two elastic spheres of radii \( R_1 \) and \( R_2 \) of Young’s modulus \( E_1 \) and \( E_2 \) are compressed under a given load \( F \), the radius of circular contact (\( a \)) between the spheres is related to the applied load by,\textsuperscript{46-48}

\[ F = \frac{4}{3R} E^* a^3 \quad \text{(1.3)} \]

where,

\[
\frac{1}{E^*} = \frac{1-\nu_1^2}{E_1} + \frac{1-\nu_2^2}{E_2}, \quad \text{and} \quad (1.4)
\]

\[
\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2} \quad . \quad (1.5)
\]

Here, \( E^* \) and \( R \) are the reduced modulus and radius of the system. \( \nu_1 \) and \( \nu_2 \) are the Poisson ratios of the two spheres. The radius of circular contact, \( a \), is related to the indentation \( \delta \) of the spheres by the relation \( \delta = a^2/R \). The contact radius is the most critical aspect to any
indentation measurement, but very difficult to measure in most experiments. Conveniently, eliminating the radius of contact, \( a \) from equation 1.3 results in,

\[
F = \frac{4}{3} E^* \sqrt{R} \delta^{3/2} .
\]  

(1.6)

We follow the notation that \( R_1 \) and \( R_2 \) are the radii of the hard particle and soft hydrogel microparticle, respectively, where \( E_1 \) and \( E_2 \) are their respective Young’s modulus. Since the hard particle has a high modulus \( (E_1 \sim O(10) \text{ GPa}) \) compared to soft hydrogel microparticles \( (E_2 \sim O(0.1) \text{ GPa}) \), the first term on the right hand side of equation 1.4 can be neglected resulting in,

\[
\frac{1}{E^*} = \frac{1 - \nu_2^2}{E_2} .
\]  

(1.7)

Essentially the Young's modulus of the soft particle can be found by accurately measuring the slope of the force-displacement curve that is given by the AFM data assuming a good fit to the slope and an accurate description of the contact area are possible.

The experiments by Briscoe et al. are some of the first experiments to measure the Young’s modulus of micron sized elastomeric spheres with very high resolution measurements of the deformation and loading force.\(^{47}\) They found that even in the presence of adhesion forces, Hertz theory was applicable at deformations up to a maximum strain of 10% to estimate Young’s modulus. Lekka et al. measured the Young’s modulus of hydrogel microspheres of diameter of about 850 µm of varying chemical composition by compressing them with a V-shaped sharp silicon nitride tip in AFM.\(^{45}\) The measured range of Young’s modulus was from 0.2 to 500 kPa depending on the chemical composition of the hydrogels.
Theoretical comparison of strains observed with sharp and blunt tips by Emilios et al. showed that the strains observed with sharp tips exceed more than 20%, the linear limit found by Briscoe, even at the lowest possible forces measured with AFM. They also showed that with blunt (spherical) tips, the induced strains are well within the linear regime even with large measured forces. Therefore, they used spherical colloidal probe tips to measure the Young’s modulus of thin films of poly (vinyl alcohol) gels. Mahaffy et al. measured the Young’s modulus of polyacrylamide gels using a spherical colloidal probe and found that it matches well with that measured from a commercial rheometer \( E = 2G' (1 + \nu) \). They found that the response of gels to indentation was linear up to a maximum strain of 20%. These studies have shown the applicability of a complex but direct method by AFM to measure the mechanical properties of gel microparticles directly.

1.3.6 Nanoparticle production and Flash NanoPrecipitation

Due to the increased need for delivery of hydrophobic or unstable drug compounds, such as camptothecin, to specific areas of the body, nanoparticle delivery systems have been engineered. Developing a nanoparticle drug delivery system involves creating a suitable stable nanoparticle, loading the nanoparticle with therapeutic or imaging agents, and functionalizing the outside of the particle for \textit{in vivo} retention and targeting. There are many methods to make these systems, normally involving multiple steps of synthesis and low amounts of drug loading. However, in our lab we have developed Flash NanoPrecipitation, a rapid mixing process that performs all three of these steps in a one-pot method with high drug loading.

Conventional production methods for nanoparticles are classified as either top-down or bottom up. Top down methods are described as taking a larger piece of material, like a
microscale drug crystal, and breaking it down into much smaller pieces mechanically or chemically. These processes include etching, grinding, milling, and spray drying. These methods create particles with a very wide size range, have difficulty making O(100 nm) particles, and cannot produce nanoparticles with complex structure. Bottom-up methods make narrow-size ranged particles that are synthesized molecule-by-molecule through nucleation and growth regulated by thermodynamics. These methods include homogenization, co-precipitation, salting out, solvent emulsification-diffusion, supercritical fluid precipitation, dialysis precipitation, and self-assembly. There are some limitations to these processes as well. Due to the thermodynamic nature of the processes, there can be limitations on the amount of drug loading while the particles are being fabricated or after they have been formed. Upon drying, the particles may have strong physical interactions, leading to difficulties with reconstitution. Drug or dye loading can occur either during the formation of the nanoparticles, or post processing. Common techniques for loading drugs or dyes involve using carriers, such as Triton X-100 or an organic solvent, to partition the drug into the particle. After the thermodynamic limit of loading is reached, the carrier is removed from the system leaving loaded particles. Adding targeting receptors to nanoparticles is normally the last step before use. Often active targeting agents are covalently linked to the surface of the nanoparticles. These targeting agents include folate, monoclonal antibodies, aptamers, dendrimers, and integrins.
All three of the processes described above can be accomplished at once using Flash NanoPrecipitation (FNP). FNP is a process where an organic solvent stream containing a copolymer solute along with a drug molecule is rapidly mixed on the order of milliseconds with an anti-solvent stream, normally water, creating solute supersaturation ratios in the anti-solvent greater than 100.\textsuperscript{55,67} Because of the high supersaturation, the solutes along with the hydrophobic end of the copolymer rapidly precipitate with growth being controlled by diffusion-limited aggregation of both components. Due to the kinetically-trapped nature of the particles, loading can be much greater than the thermodynamic equilibrium loading amounts. A schematic of this process, adapted from Johnson et al, is shown in Figure 1.3.\textsuperscript{56} This process is also beneficial because the particles can be easily conjugated with targeting agents at the same time the particles are formed.\textsuperscript{68,69} In addition, because of the ability to put multiple solute compounds into the solvent phase, multifunctional nanoparticles can be formed containing imaging and therapeutic agents or drug cocktails.\textsuperscript{67,70} Due to the versatility of FNP to produce complex stable nanoparticles, this technology is used to formulate our model drug nanoparticles.
1.4 Thesis Objectives

In this thesis there are four core chapters involved with the creation of hydrogel microparticles for \textit{in vivo} delivery of drugs to mice with non small cell lung cancer.

In Chapter 2, the focus will be devoted to the development and fabrication of a simplified microcapillary microfluidic device for droplet generation. We will discuss device assembly as well as device operation with a focus on tuning droplet generation by changing the dynamic interfacial tension at the droplet break-up point. New studies are performed to show how dynamic interfacial tension can be tuned by interfacial area and age, as well as surfactant molecular weight.

In Chapter 3, applications of the simplified microcapillary device to produce hydrogel microparticles suitable for intravenous drug delivery will be discussed. Experiments will be discussed involving the fabrication of complex emulsions and particles using our device, reducing droplet size by precisely controlling the aqueous phase flowrate, and integrating a living Michael addition polymerization into the device to produce PEG hydrogel particles.

In Chapter 4, the focus of the thesis switches towards medical applications by discussing the production and characterization of hydrogel microparticles produced by bulk shear emulsification methods for use in pilot \textit{in vivo} studies. The focus of the chapter is on the optimization and characterization of a polymer and nanoparticle formulation to generate microparticles without compromising the two stage drug delivery approach. Experiments are performed to understand particle size, particle morphology, particle mechanical properties, and particle degradation, in an effort to optimize a particle formulation and production process suitable for pilot \textit{in vivo} studies.
In Chapter 5, the focus switches from particle production to particle drug delivery in living mice using model fluorescent hydrogel particles. The goal of these studies is to understand the biodistribution of cGMPs based on size and uniformity. The final study brings together all of the concepts of the thesis by generating uniform microfluidic hydrogel particles at a high enough quantity to perform an \textit{in vivo} test of their biodistribution.

Finally in Chapter 6, we conclude the thesis and discuss future directions of the current lung cancer project, including introduction of microparticles into mice with lung cancer models and eventually looking at camptothecin efficacy studies.

The appendices at the end of the thesis describe in detail some of the specifics with regards to building the simplified capillary device, the \textit{in vivo} performance of all gel microparticles tested, and a number of other projects that were undertaken during the primary thesis work.

1.5 References


Chapter 2: An “Off-the-shelf” Capillary Microfluidic Device that Enables Tuning of the Droplet Breakup Regime at Constant Flow Rates

Abstract

The fabrication of glass capillary microfluidic devices is technically challenging, often hampering use of the design. We describe a new technique, based on commercially available components, for assembling flow focusing capillary devices that can readily be taken apart and cleaned between uses. This design strategy allows for generation of both water-in-oil and oil-in-water emulsions in the same device after an ethanol rinse. The modularity of the device enables the adjustment of the tip separation between the two inner capillaries during droplet generation, which enables tuning of the age of the interface. Time-dependent surfactant diffusion to the interface changes the interfacial tension, thus providing an approach for adjusting the capillary number in addition to the usual method of changing flow rates. This design enables the tuning of the mode of breakup and the droplet size.
2.1 Introduction

Glass capillary microfluidics are an important tool in many different fields, including complex emulsion and particle generation\textsuperscript{1-6}, drug delivery\textsuperscript{7-9}, particle sorting\textsuperscript{10}, protein dynamics\textsuperscript{11}, and even tissue engineering\textsuperscript{12}. Due to their axisymmetric flow and ability to withstand organic solvents, when compared with their lithographically fabricated polydimethylsiloxane (PDMS) counterparts, glass capillary devices possess advantages for microfluidic applications. In particular, a circular tube is inserted into a square outer flow channel, which greatly simplifies alignment and centering of these devices.\textsuperscript{1,2,8} Recently designs have been introduced to simplify this fabrication process using commercially available components to set up co-flowing systems.\textsuperscript{13-15} These devices can produce large droplets, typically hundreds of microns in diameter, but lack the ability to generate the $O(10 \ \mu m)$ size droplets that can be produced using a flow focusing device.\textsuperscript{16,17} A summary of current devices used, including their construction and typical applications, is shown in Table 3.1.

However, limitations exist with the current design of capillary microfluidics. Conventional glass capillary devices for flow focusing microfluidic droplet generation are typically built in a manner similar to that shown in Figure 2.1(a).\textsuperscript{7,8} The devices are assembled on a glass slide and needles are epoxied over the capillaries in order to interface the device with tubing that injects the fluids. This fabrication method is technically challenging, and reproducibility in fabrication is more difficult than with lithographically produced devices.\textsuperscript{18} For both conventional capillary devices and PDMS devices, cleaning can only be accomplished by flushing and not by device disassembly. We have found this to be a significant problem for polymerizing fluids and in high-solids droplet generation.\textsuperscript{7}
In this thesis chapter, we present a flow focusing glass capillary device based on commercial chromatography components that can be assembled easily, can be disassembled for cleaning, and can be switched from oil-in-water to water-in-oil drop formation after rinsing with ethanol. A specific advantage of the design is that the separation between the capillary inlets can be adjusted during operation. This feature allows tuning of the “age” of the interface at the point of drop breakup. As a consequence of time-dependent surfactant diffusion to the interface, the interfacial tension can be varied to control the breakup regime. The device is shown in Figure 2.1(b), with a schematic of the components in Figure 2.1(c).

### 2.2 Materials and Methods

#### 2.2.1 Materials

MilliQ water is used for all aqueous phases in the microfluidic device running. Light mineral oil, Span 80, and ethanol were purchased from Sigma-Aldrich. ABIL EM 90 was graciously donated by Evonik Industries.
2.2.2 Fabrication of glass capillary device

A detailed description regarding the fabrication of the ‘off-the-shelf’ glass capillary device is described in Appendix A of this thesis. Briefly, the device includes two PEEK chromatography tees (IDEX Health & Science, P-713). The central square capillary (Vitrocom, 8290-050) bridges the two tees and ensures concentric alignment of the two round capillaries (Vitrocom, CV7087) that define the breakup zone (Figure 2.1(c)). The two orifices, one for injection of the inner fluid phase and one for the focusing of the flow, are created by flaming the ends of the glass capillaries, so that “art-dependent” capillary pulling and breaking using expensive equipment is not required.\textsuperscript{1,2} Alternatively, the focusing capillary can have a tapered

Figure 2.1. Design of a modular glass capillary flow focusing device. a) Image of common, non reusable glass capillary device. b) Image of modular, reusable microfluidic device built from commercial materials in minutes. c) Schematic of the construction of the modular glass capillary device. Scale bars are 1 cm.
tip to produce a different geometry for droplet generation. This will be discussed in greater detail in Chapter 3. The sealing of the square and round capillaries in the chromatography tees is accomplished by sliding a 10 mm section of soft PVC tubing (Tygon, R-3603 1/8” OD) over the glass tubing. The compression by the chromatography ferrules provides a pressure-tight seal. The continuous phase flows into the device from both directions in order to prevent the dispersed phase from interacting with the glass walls of the square capillary tube. Alternatively, three immiscible phases can be injected to create double emulsions, which are detailed in Chapter 3.

2.2.3 Running the microfluidic device

After completion of the device, connection to syringe pumps is accomplished by interfacing Intramedic polyethylene tubing to the top of the chromatography tees by simply sliding the smaller tubing within the larger PVC tubing and tightening the screw. The Intramedic tubing is then attached to disposable or glass syringes with 27 gauge needles. Typical aqueous solutions used in the microfluidic devices include solutions containing water, functionalized polyethylene glycol 600-1300 MW and block copolymer nanoparticles. Typical oil phases contain silicone oil (10-100 cSt) with 749 Resin surfactant or mineral oil containing chlorinated oil and ABIL EM 90 or Span 80® as a surfactant. Two syringe pumps were used (PHD 2000, Harvard Apparatus, Holliston, MA) to pump the oil and aqueous phases into the device. A 1 mL Norm-Ject plastic syringe was used to inject the aqueous phase, and two 10 mL Norm-Ject plastic syringes were used to inject the oil phase.

2.3 Results and Discussion

2.3.1 Producing W/O and O/W emulsions in same device
Due to the use of a flamed tip design for injection of the dispersed phase into the microfluidic device, a favorable contact angle is created for both water and oil that allows us to use a glass capillary without chemical functionalization. Most capillary microfluidic devices using pulled-tip glass capillaries have very small outside tip diameters. They require chemical functionalization in order avoid wetting by water along the injection tip, which affects droplet breakup. However, by using a flamed injection tip, the relatively flat interface that is perpendicular to the flow direction (Figure 2.1(c) insert) effectively pins the water/oil contact line. This result is shown by the red arrow in Figure 2.2(a). Using this feature, combined with the ability to disassemble and clean the device, both water-in-oil and oil-in-water emulsions can be produced in the same device after quickly rinsing the glass with ethanol.

To demonstrate this concept, we first used a pure water and mineral oil system with Span 80 surfactant at 2% (v/v) in the oil to produce droplets of water-in-oil as shown in Figure 2.2(a).
After operating the device, it was disassembled by removing the outer screw on the tees and removing the round capillary tubes. The individual components were washed with ethanol, dried by nitrogen, and reassembled. Injecting pure water as the continuous phase and mineral oil with 2% (v/v) Span 80 as the dispersed phase produced the oil-in-water emulsion shown in Figure 2.2(b).

2.3.2 Tuning the capillary number by adjusting interfacial tension

The importance of the ability to adjust the distance between orifices is shown in Figure 2.3. The two orifices are easily adjusted manually under a microscope during device operation, without any leakage occurring, by pushing or pulling the ends of the round capillaries. The orifices can be precisely positioned with an error of 20 microns or less. By changing the distance between the injection and collection orifices, the surface area of the dripping liquid drop can be increased. As shown in Figure 2.3(a-f), by holding the flow rates constant, we can change the droplet generation regime of our system. We used a pure water-in-mineral oil system containing 2% (v/v) ABIL EM 90 (Evonik Industries) as the surfactant and kept the continuous phase constant at 20 µL/min and the dispersed phase at 0.2 µL/min. We slowly adjusted the collection orifice distance and waited for the flow to become stable again after a few minutes. As we increased the collection orifice distance, the droplet generation regime of our system changed. This observation is due to the increased “age” of the interface at larger separations. The longer time enables surfactant diffusion to the interface to reduce the interfacial tension. This effect leads to transitions from geometry controlled dripping to tip streaming, then to dripping and finally jetting. Droplets around 20 microns in size were produced by dripping and are shown in Figure 2.3(e). It has been described elsewhere that changing of the droplet generation regime of
the microfluidic system is caused by a change in the capillary number (Ca) of the system.\textsuperscript{4,20} The capillary number, Ca, is defined,

\[
Ca = \frac{\mu_c V_c}{\gamma},
\]  

(2.1)

where \(\mu_c\) is the viscosity of the continuous phase, \(V_c\) is the characteristic velocity of the flow through the orifice, which is a function of the orifice size and volumetric flow rate, and \(\gamma\) is the interfacial tension. The capillary number determines the conditions for breakup in a particular breakup regime.\textsuperscript{16,20} Normally, the capillary number is changed by adjusting the continuous or dispersed phase flow rates. We see that adjusting the tip spacing, and therefore the age of the
interface, enables variation in the capillary number by varying the effective interfacial tension.

This new device enables more flexible control of the capillary number and drop breakup than can be achieved with flow rate control alone. We also ran a similar experiment using 2% (v/v) Span 80 as the surfactant, and it showed a similar trend towards an increase in Ca. The images of these experiments are shown in Figure 2.4 and were used to calculate the graph in Figure 2.5.
To ensure that the observed phenomenon was due to the surfactant in the system, a control experiment was performed using just water and mineral oil. The results of this experiment are shown in Figure 2.3(g-i). The continuous phase flow rate was considerably higher (ten-fold) in order to produce droplets by dripping. As the separation distance increased, the dripping does not change to jetting but instead transitions to geometry controlled dripping. This control of the interfacial tension, using diffusion controlled surfactant tuning by tip separation, demonstrates a unique capability of this new device.\textsuperscript{21,22}

2.3.3 Comparing flowrate ratio and tip separation

In an attempt to gain some knowledge about the dynamic interfacial tension of a water-in-mineral oil system containing 2\% (v/v) Span 80 as the surfactant, we varied the collection distance for three different flow rate ratios to learn how it affected the transitions between droplet generation regimes. Figure 2.5 is a graph of the droplet generation regimes for different orifice separations ($l_c$) that have been normalized to the collection orifice diameter ($d_c$) of 100 µm. Solid lines have been added to the graph to show major droplet generation regime transitions and the dotted line shows the more subtle transition from tipstreaming to dripping.

![Figure 2.5](image-url)

Figure 2.5. Mapping the change in droplet generation regime as the collection orifice distance ($l_c$) increases at a constant continuous phase flow rate ($q_c$) of 20 µL/min and a constant collection orifice diameter ($d_c$) of 100 µm. As the ratio of continuous phase flow rate to dispersed phase flow rate ($q_d$) is increased, more surfactant can reach the interface, thus reducing the interfacial tension faster and leading to a larger capillary number. Solid lines have been added to the graph to show major droplet generation regime transitions and the dotted line shows the more subtle transition from tipstreaming to dripping.

To ensure that the observed phenomenon was due to the surfactant in the system, a control experiment was performed using just water and mineral oil. The results of this experiment are shown in Figure 2.3(g-i). The continuous phase flow rate was considerably higher (ten-fold) in order to produce droplets by dripping. As the separation distance increased, the dripping does not change to jetting but instead transitions to geometry controlled dripping. This control of the interfacial tension, using diffusion controlled surfactant tuning by tip separation, demonstrates a unique capability of this new device.\textsuperscript{21,22}
microns. The continuous phase flow rate \((q_c)\) was maintained constant and the dispersed phase flow rate \((q_d)\) was reduced to maintain a constant continuous phase capillary number that only changed due to changes in the dynamic interfacial tension. The results show that as the flow rate ratio is increased, the transition between different droplet generation regimes occurs at a shorter separation distance. This result supports the argument that the age of the interface controls the effective interfacial tension; a lower interfacial tension produces a larger capillary number. Therefore, changing droplet generation regimes by tip separation is also dependent on the flow rate ratio of the system.

### 2.3.4 Comparing the dynamic interfacial tension of two different surfactants

After showing how the dynamic interfacial tension was a function of the flowrate ratio and tip separation, we thought it might be interesting to see if it was also affected by the type of surfactant. For this experiment we chose to use two nonionic surfactants, Span 80 and ABIL EM 90, with similar HLB values of 4.3 and 5 respectively. The main difference between the two molecules is the structure and the molecular weight. Since ABIL EM 90 has a molecular weight about 40 times larger than Span 80, along with a very bulky structure, we thought it would take longer for the molecule to diffuse to the interface and reduce the dynamic interfacial tension of the droplet generating system. If the reduction in interfacial tension is lower, it will lead to the need for larger tip separation in order to transition the break up into a new regime. As can be seen in Figure 2.6, the transition from geometry controlled dripping to tipstreaming is five times larger in tip separation for the ABIL EM 90 when compared to the Span 80. We believe this microfluidic device could be used in order to provide a dynamic ‘fingerprint’ on surfactant systems and how they affect droplet break-up. This type of surfactant characterization could have
implications for studying the droplet break-up dynamics in more complex surfactant systems similar to what are seen in commercial applications.

2.4 Conclusion

In this thesis chapter we have described an easy-to-fabricate, reusable microfluidic device for the production of monodisperse droplets down to 20 microns in size. The orifices produced by flaming relatively large glass capillaries avoid the more complex capillary pulling and scoring required for conventional flow focusing glass capillary devices. Moreover, the flat ends of the capillary tip effectively pin the oil/water contact line and enable oil-in-water or water-in-oil operation without functionalized glass surfaces. We have shown that the tip separation can be tuned in an operating microfluidic device, which leads to changes in the droplet generation regime. We believe that this device, with its simple design and assembly, will be useful to the community that builds and experiments with glass capillary microfluidics. The inherently serial nature of capillary devices compared with PDMS molded devices is still an issue. Scaling-up

Figure 2.6. Comparing the change in capillary number between two surfactants with very different molecular weights. At a flowrate ratio of 100, in the same microfluidic device that has been cleaned between uses, Span 80 and ABIL EM 90 are compared in terms of their effect on the capillary number as the dynamic interfacial tension is changed.
will require multiple devices to be fabricated individually. However, by shortening and simplifying the fabrication process with commercial materials, our device is an attractive alternative for these applications. A quantitative understanding of the role of surfactant diffusion to the interface, the axisymmetric flow field, and surfactant dynamics on the interface will require modeling and simulations. These further investigations would help more fully define the processes at play in this work. By using this device, we hope to further the understanding of the role of surfactants and dynamic interfacial tension in microfluidic droplet generation.

Through proof-of-concept experiments, we have shown that using this easily assembled and reconfigured glass capillary microfluidic device allows precise control of drop breakup during droplet generation. A user can adjust both flow rates as well as the dynamic interfacial tension of the system in order to find the optimum operating conditions for droplet production.

2.5 References


Chapter 3: Microfluidic Production of Complex Emulsions and Gel Microparticles Using an “Off-the-Shelf” Capillary Device

Abstract

The generation of complex emulsions and microparticles has become very important in many fields including drug delivery, tissue generation, and lab-on-a-chip applications. Of particular interest is the generation of uniform hydrogel particles near the size of a red blood cell. Using our simple "off-the-shelf" glass capillary microdevice to perform microfluidic droplet generation, we generated different emulsions and particles including double emulsions, nanocomposite single emulsions, and composite microgel particles. We also discuss the importance of device parameters and operating constraints on the minimum size that can be generated using both flow-driven pumps and a constant pressure compressed air driven flow. To generate hydrogel microparticles by a Michael addition reaction using microfluidics, our simple capillary device is modified for coflow mixing and post polymerization.
3.1 Introduction

There is interest in generating well controlled architecture in microparticles and emulsions for applications in drug delivery, tissue engineering and other lab-on-a-chip applications.\textsuperscript{1-3} In recent years, microfluidic droplet generation using glass capillary devices has made it feasible to generate many different types of complex emulsions and particles.\textsuperscript{4,5} The Weitz group at Harvard University has made many contributions to this area in particular.\textsuperscript{6,7} They have built devices that can produce droplets with different compartments by multiple emulsification, phase separation, or droplet dewetting.\textsuperscript{8-10} The ability to create emulsions with multiple phases allows for compartmentalization of different components into one droplet or particle. This can be useful when trying to deliver both hydrophobic and hydrophilic drugs to the same location in the body at the same time, or to regulate the interaction of two molecules to stop them from reacting until the emulsion is ruptured either by pH, temperature, or mechanical agitation.\textsuperscript{11} Not only can the emulsions and particles generated using microfluidics be complex, but they are very uniform in structure and monodisperse in size. The volumes of each compartment are identical in each droplet or particle, and the total particle size and morphology are also identical.\textsuperscript{5} This leads to control over the quality and reproducibility of the emulsions being generated. There are four distinct droplet breakup regimes in microfluidic droplet generation: geometry-controlled, tipstreaming, dripping, and jetting. Droplet breakup by dripping is preferred due to the high monodispersity of particles generated.\textsuperscript{12}

Microfluidic production of particles and emulsions has been performed both with small quantities of expensive reagents, and in large quantities to generate commodity-scale emulsions.\textsuperscript{13,14} Very expensive reagents and molecules, such as biomolecules, must be handled in very small quantities. Using microfluidic droplet generation to make microreactors for screening
biomolecular compounds allows for the use of very small quantities of molecules compared to large scale screening techniques.\textsuperscript{13} In terms of scaling up microfluidics to produce industrial-scale emulsions, typically the number of droplet break off points is increased either by making many devices or by incorporating many break up points into a highly parallelized device.\textsuperscript{15-17}

One application of microfluidic particle generation that has gained interest in recent years is the production of polyethylene glycol (PEG) hydrogel microparticles.\textsuperscript{18,19} PEG hydrogels are biocompatible materials that avoid an immune response in the body and allow for the growth of cells within their degrading matrix.\textsuperscript{20} By combining the emulsification control of microfluidics with the biocompatibility of PEG particles, very uniform particles for drug delivery can be produced.

The best method to produce hydrogel microparticles in a microfluidic device decouples the generation of the emulsion with the polymerization of the PEG hydrogels.\textsuperscript{5} This has been done by using a UV radical initiation polymerization of precursor PEG acrylates within the droplet after it is formed.\textsuperscript{21} Briefly, an initiator, such as Irgacure, is used to generate free radicals in the aqueous droplet upon exposure to UV light. These radicals attack the acrylate ends of the PEG molecules and activate them setting off and radical reaction to form a gel network. The simplicity of this reaction and the ability to "switch it on" after droplet generation has made it a very popular route to produce hydrogel microparticles. There is a large downside to this technique, however. The free radicals produced by the UV initiation can lead to degradation of drug or imaging molecules within the hydrogel matrix.\textsuperscript{21} If there is alteration of the structure of a portion of drug within the hydrogel, it will not pass FDA approval.\textsuperscript{22} As well, cells and other biological molecules are affected by exposure to UV light in high doses, such as what is needed to generate PEG hydrogels. There are other methods to produce PEG hydrogels that are different
from UV polymerization. These include chelation, copolymer precipitation, and Michael addition polymerization.\textsuperscript{19,23} We have focused on the Michael addition polymerization of PEG to form microparticles. The Michael addition reaction is typically a reaction between a nucleophile, such as a thiol and an electrophile, such as an acrylate.\textsuperscript{20,24} It is a stepwise reaction where the monomers of the hydrogel are built onto the existing network leading to a covalently bound polymer network.

Although very good for encapsulation of drugs and biological material within a hydrogel, the Michael addition reaction is not very compatible with microfluidics. As soon as the two reactants are mixed together in an aqueous phase, they begin to polymerize and change fluid properties such as viscosity and surface tension. Since viscosity affects the size of the droplets formed, this can lead to changes in droplet sizes over the course of particle production and negate the benefits of microfluidic droplet generation over traditional emulsification techniques.\textsuperscript{25} There have been a few attempts in the literature to produce microparticles using Michael addition microfluidics.\textsuperscript{18,19,26} None of the previous examples attempted to make large volumes of microparticles, and therefore long run times were not performed.

In this thesis chapter, I am going to discuss how our simple 'off-the-shelf' microcapillary device can produce complex nanocomposite double emulsions similar to what has been done in the literature. The advantage is that our simple device is easy to use and can be rapidly assembled, disassembled, and cleaned for re-use.\textsuperscript{27} As well, I will discuss how our device can be modified to operate in a constant pressure mode in order to precisely control the flowrate of the inlet streams in order for the production of red blood cell-sized droplets that contain PEG acrylate precursor, which can be further polymerized into particles. Finally, I would like to
discuss how we modified the microfluidic device in order to continuously produce hydrogel microparticles that can be used for drug delivery applications.

3.2 Materials and Methods

3.2.1 Materials

MilliQ water is used for all aqueous phases in the microfluidic device running. Light mineral oil, Span 80®, and ethanol were purchased from Sigma-Aldrich. ABIL EM 90® was graciously donated by Evonik Industries (Parsippany, NJ). Par Oil 10NR® chlorinated oil was donated by Dover Chemical Corporation (Dover, OH).

3.2.2 Fabrication and operation of microfluidic device

A detailed description with figures regarding the fabrication of the ‘off-the-shelf’ glass capillary device is described in Appendix A of this thesis. An example device and schematic are shown in Figure 3.1. Briefly, the device includes two PEEK chromatography tees (IDEX Health & Science, P-713). The central square capillary (Vitrocom, 8290-050) bridges the two tees and ensures concentric alignment of the two round capillaries (Vitrocom, CV7087) that define the breakup zone (Figure 3.1(b)). The two orifices, one for injection of the inner fluid phase and one for the focusing of the flow, are created by flaming the ends of the glass capillaries, so that capillary pulling and breaking using expensive equipment (i.e. capillary puller, microforge) is not required. Alternatively, the focusing capillary can have a tapered tip to produce a different geometry for droplet generation. The sealing of the square and round capillaries in the chromatography tees is accomplished by sliding a 10 mm section of soft PVC tubing (Tygon, R-3603 1/8” OD) over the glass tubing. The compression by the chromatography ferrules provides
a pressure-tight seal. The continuous phase flows into the device from both directions in order to prevent the dispersed phase from interacting with the glass walls of the square capillary tube.

3.2.3 Generating double emulsions using capillary device

In order to produce double emulsions using the simplified microfluidics device, the inlet fluids could be altered in order to create oil/water/oil double emulsions. This was done by changing the outer phase to a blend of chlorinated oil and mineral oil in order to match the density of our aqueous phase. Removing effects from gravity on droplet breakup enhanced stable production of double emulsions. Microfluidic double emulsions were produced in the glass capillary microfluidic device using the following conditions. The middle phase contained pure water with 1.35% (w/w) 250 nm latex nanoparticles (Surfactant-free poly methyl methacrylate, Arkema Inc.) which were fluorescently labeled using a solvent evaporation method described elsewhere28 and 1.35% (w/w) Pluronic F68 (BASF). The inner and outer phases consisted of a mixture of 64% (v/v) Chlorinated Oil, (ParOil 10NR, Dover Chemical Corp.) 34% (v/v) mineral oil, and 2% (v/v) ABIL EM 90 surfactant (Evonik Industries). The solutions were flowed together though an orifice of 100 microns.

3.2.4 Building of pressure pump

In order to generate microfluidic droplets with a much smaller size, a pressure driven flow based off of house compressed air was used. The setup of the pressure pump was designed after a setup used by the Doyle group for generating particles using stop flow lithography, however our pump was modified for use with large quantities of fluid.29 The pump is connected to the house air using a high speed regulator (SpeedAire, Dayton, Niles, IL) followed in series by a low flow precision regulator (Type 90 AA, Controlair, Inc., Amherst, NH) in order to finely
control device pressure. 1/4" ID PVC tubing (VWR) and L and T joints (VWR) are used to join the regulators to the barbed PVC ball valves (Grainger), needle valves (Swagelok), and 25mL pipettes (Sigma) containing the solutions. Different independent pressures can be achieved by using the same regulator, but running multiple lines off of it and placing a pipette filter (1 mL Filter Tip, Fisher) within the tubing to create a pressure drop. Each line leads to the solution pipettes and a needle valve is used for regulating the pressure near the injection pipette. Pressure gauges are place at the injection pipettes and near the precision regulator to measure the pressures during droplet generation. The 25 mL pipettes are fitted with some 1/4" PVC tubing and a cut 5 mL syringe barrel for attaching the 27 gauge needles to interface with the microfluidic device tubing. A schematic of the pump and actual picture are shown later in the chapter in Figure 3.8. A vacuum line was also fitted onto the pump in order to fill the pipettes before device running and to decrease local pressure if a vent is not releasing enough pressure and slower flows are required.

3.2.5 Fabrication of modified device for Michael addition reaction

There were two different important fabrication aspects for using microfluidics to generate PEG hydrogel microparticles by a Michael addition reaction. We used both an aqueous coflow setup and a droplet inline polymerization. A schematic of the aqueous coflow device is shown in Figure 3.11 and a schematic of the inline polymerization is shown in Figure 3.14. The coflow device was built by modifying the injection capillary to contain two separate fluids up until just before droplet breakup. This was done by adding a third chromatography tee to the front of the device. The front end of the injection capillary was connected to the tee using Tygon tubing similar to the simple device. A long thin square capillary with an outer diameter of 400 microns is connected to polyethylene medical tubing and slide into the other end of the tee and fed down
the injection capillary until it is appropriately close to the droplet breakup area. The small glass capillary is tightened in the tee using more 1/8 ID Tygon tubing. One aqueous phase is fed into the device from the top of the chromatography tee and one is fed in from the side opposite the injection capillary. This is shown later in the chapter in Figure 3.11.

In order to increase the stability of the droplets after droplet generation, amine base can be added to the oil phase post droplet generation to initiate the polymerization of the particles while still in the microfluidic device. This will allow solidification of the particles before collection and settling. A chromatography tee is attached to the collection tube of the microfluidic device using Tygon tubing. The collection tube is fed completely through the tee and out the other side. A square capillary similar to the one used in the droplet break-up regime is attached to the tee fitting over the end of the collection tube coming out of the tee, allowing outer fluid to coflow with the collection stream.

3.2.6 Fluorescent confocal microscopy of particles

Fluorescent confocal microscopy was done using a Leica SP5 confocal microscope. Particles were collected onto a glass slide and placed into the microscope and imaged quickly. If a cover slip was used, it would cause coalescence of the double emulsions, so it was avoided during imaging.

3.2.7 Optical microscopy and particle sizing

Optical microscopy was performed using either a Nikon G100 light microscope or a Balplan light microscope with an attached CMOS camera (Edmunds Optics (Barrington, NJ)). Samples were collected on glass slides and imaged immediately. Droplets were sized manually using ImageJ (NIH) or by using their particle sizing software.
3.3 Results and Discussion

3.3.1 Configuring “off-the-shelf” capillary device to generate complex emulsions

The modular design of the “off-the-shelf” capillary device is ideal for allowing the manipulation of geometries and orifices to create very different emulsions and break up conditions. There are three inlet streams and one outlet stream on the capillary device, allowing
both single (Figure 3.1(b)) and double emulsions (Figure 3.1(c)) to be generated simply by changing inlet stream components. As well, capillaries of different sizes and shapes can be swapped out to change droplet break-up.

3.3.1.1 Nanocomposite single emulsion droplets

Figure 3.1(b) shows a schematic of how single emulsions can be generated using our microfluidic device. Oil is flowed in from the two outer streams while an aqueous phase is flowed in through the injection capillary. Break-off occurs at the entrance into the collection capillary by a combinations of end-pinchching and Rayleigh instability effects.

The unique utility of the device is shown by the replacement of the flamed collection tube with a pulled collection tube as shown in Figure 3.2. This type of collection tube transition can be performed without disrupting device operation. The flamed collection tip acts as a pinch point in the microfluidic two phase flow. At this point fluid rapidly speeds up and the interface of the two fluids is increased producing a filament of the inner phase that experiences Rayleigh instabilities. This is followed by an equally fast decrease in fluid speed beyond the pinch point. This decrease leads to formation of droplets bunched together in the collection tube which can result in drop coalescence. Some benefits of a flamed tip collection tube include a decrease in the pressure drop along the device and a larger operating regime for dripping due to the propensity for the filament to undergo end-pinching. Pressure drops in microfluidic devices can be very high, and in situations where liquids are viscous and the orifice is small and long, the syringe pumps will have difficulty maintaining flow rates as they are increased. Changes in pressure may also affect the droplet generation over long periods of time, which in turn will affect the monodispersity of the emulsion produced.
Figure 3.2. Glass capillary devices with different collection tube geometries. a) A flamed tip collection tube is fabricated by tip flaming. This geometry creates a pinch point which facilitates good droplet break up by end pinching. b) A pulled capillary collection tube is generated by capillary pulling and scoring. This geometry does not pinch the two-phase flow and therefore breaks up the droplets by more of a Rayleigh instability. Scale bar is 100 µm.
Figure 3.3. Generating nanocomposite single emulsion droplet using off the shelf glass capillary devices. a) Break-up tip of an aqueous phase filled with fluorescent block copolymer nanoparticles. b) Confocal image of 25 micron droplets formed using a glass capillary microfluidics device showing the fluorescent nanoparticles incorporated. c) Optical image of water in oil droplets showing the uniformity of the droplets formed using microfluidics.
Typically the droplet generation regime that produces the most monodisperse droplets is the dripping regime. In order to produce the smallest, most uniform droplets or particles for drug delivery applications, it is necessary to have a large, stable dripping operating window for the microfluidic device. Inducing end-pinching by using a flamed device will destabilize the filament formed through the pinch point and lead to uniform break up. If the pinch point is made longer to confine the filament in a longer uniform capillary, as occurs with a pulled tip, break-up occurs due to Rayleigh instability. This would mean that the droplet generation tends towards a jetting regime for a pulled tip.

There is a major drawback with the flamed tip capillary collection tube. After droplet generation, emulsion drops are crammed together by the slowing of the flow. This means that emulsions that do not have immediate stability are subject to coalescence after generation. There is also the possibility that droplets could interact with each other to flocculate or polymerize together depending on the physical and chemical properties of the entire emulsion.

As previously mentioned, there are positives and negatives to generating droplets with a tapered pulled capillary as well. The high pressure drop and stabilization of the jetting regime create only a small operating window for a dripping regime, leading to difficulty generating stable, monodisperse emulsion droplets over long periods of time. Jetting typically generates droplets with 5% polydispersity, whereas dripping is close to 1-2% polydispersity. Due to the modular nature of the 'off-the-shelf' device, both types of collection tubes can be interchanged on the fly based on the needs of the operator.

Incorporation of nanoparticles into the emulsion droplets is easily done through formulation of the precursor solution that is injected into the microfluidic device. One of the
benefits of microfluidics is the ability to work with very small quantities of solutions and manipulate them accordingly. Block copolymer nanoparticles were fluorescently labeled with EtTP5, a stable pentacene derived near-IR dye synthesized by the John Anthony group in Kentucky.\textsuperscript{30,31} Fluorescent microscopy in Figure 3.3 shows both the fluorescence of the nanoparticles during droplet generation and the fluorescent nanocomposite and monodisperse emulsion droplets. Also shown is a bright field microscopy image of another emulsion generated using microfluidics to demonstrate how uniform droplet generation can be.

3.3.1.2 Interfacial effects with nanocomposite emulsions

Nanoparticles are known to interact with interfaces in water and oil emulsions.\textsuperscript{32} Therefore it is important to consider their interaction with surfactants during microfluidic droplet generation. Nanocomposite microfluidic droplet generation may involve multiple components that can affect the properties of the emulsion including viscosity, interfacial tension, and density. The aqueous phase used for nanocomposite emulsions typically includes water, block copolymer nanoparticles, PEG precursor if polymerization is desired, and sometimes a surfactant. There are two common oil phases used for microfluidics to make water in oil emulsions. They are silicone oil with a silicone-based surfactant or resin, and mineral oil with a small molecule surfactant like Span 80\textsuperscript{®} or a larger polymeric surfactant. In order to achieve the greatest stability in our emulsions to ensure monodispersity after collection, we typically use silicone oil with RSN-749\textsuperscript{®} fluid as a polymeric stabilizer. Mineral oil can be used with a polymeric surfactant such as ABIL EM 90\textsuperscript{®} to provide good stability initially, but we observed coalescence over long periods of time in settled emulsions. During overnight curing of the droplets for drug delivery applications, it is necessary to have very good stability. RSN-749\textsuperscript{®} fluid is a very effective
stabilizer of our emulsion interface as it creates a solid-like interface between the aqueous phase and the silicone oil. There is an interaction that occurs between the PEG in the block copolymer and the polysiloxane in the surfactant the locks the polymer in place. This solid-like interface is...
noticeable during microfluidics, as the interface between the two fluids will buckle and crumple under high shear conditions typical in droplet generation. This is shown in Figure 3.4. When droplets are collected from the device and dried in air, they crumple into non-spherical shapes on the glass slide. Although the solid-like interface is useful for stability of microfluidic emulsions, it can cause problems with the generation of uniform droplets. RSN-749® fluid can build up on the droplet break up point to create non-spherical particles and fibers. This typically occurs under high shear conditions (high capillary number) when the continuous phase is at a high flow rate and the aqueous phase is moving relatively slowly. The non-spherical dripping regime that arises decreases the operating window for producing uniform droplets with this type of system. The dripping regime is very limited because as you attempt to decrease droplet size by increasing shear, you induce non-spherical droplet generation. As well, the jetting regime dominates break-up for most flow conditions due to the formation of a stable jet with a solid interface. Therefore it is necessary to balance stability of your emulsion system with the practicality of your operating conditions to optimize your droplet production.

3.3.1.3 Production of double emulsions

As shown schematically in Figure 3.1(c), simple rearrangement of the input streams allows for the generation of double emulsions using our simple "off-the-shelf" glass capillary device. Again we can formulate block copolymer nanoparticles within our aqueous phase and send it in as the middle stream in our microfluidic device. As the middle phase flows to the orifice for break-up, it will carry the inner phase with it, leading to simultaneous break up of both
Figure 3.5. Microfluidic production of double emulsions with glass capillary devices. a) Production of a microfluidic double emulsion by jetting using a glass capillary device with a 100 µm orifice. Inner and middle phase flow rates were 0.5 µL/min, while the outer phase flow rate was 10 µL/min. Insert shows fluorescent nanoparticles in the middle phase. (i.e. aqueous outer phase of the double emulsion droplet) b) Screen shot of Supplemental Video 1 showing generation of a double emulsion by a device with a 250 µm orifice. Inner and middle phase flow rates are 5 µL/min and the outer phase flow rate is 25 µL/min. c) Monodisperse droplets generated by device in b. Scale bars are 100 µm.
phases by the outer phase and generating a double emulsion. In our case, we have made a double emulsion with fluorescent latex nanoparticles (NPs) in the shell of our core-shell structured double emulsion.

When generating double emulsions, the particular properties of the injection fluids can significantly affect double emulsion generation. Some considerations include density matching the inner and middle phases, using a middle phase with high wet-ability on glass capillaries, and picking a system with low interfacial tension between the inner and middle phases. It is important to density match the inner and middle phase to ensure that the fluids flow coaxially through the droplet generation orifice and break up. If the inner oil phase has a different density, it will tend to phase separate with the water and rise to the top of the capillary creating a coflow stream and mixing with the outer phase oil. We accomplish density matching in our microfluidic devices by mixing our mineral oil with chlorinated oil to increase density. The solution is typically 34% (v/v) mineral oil, 64% (v/v) chlorinated oil, and 2% (v/v) ABIL EM 90®. This matches the density with water/PEG mixtures in the aqueous phase. It is very important for the middle phase to wet the glass capillaries well for good double emulsion formation. You can think of double emulsion droplet generation similarly to blowing bubbles. The middle phases needs to wet the glass capillary and provide a film around the inner phase prior to droplet generation. If the inner phase wets the capillary more than the middle phase, it can wet around the middle phase and create simple single phase drops that do not have a second internal liquid phase. Low surface tension between the inner and middle phases is very important for uniform double emulsion droplet break up. If there is a high surface tension between the two phases the inner phase will not convect through the orifice and break up. Rather, the inner phase will break up either before or after the middle phase and generate double emulsions with multiple droplets.
inside or single emulsions with no inner phase. You can attempt to tune this fluid property to generate larger double emulsion droplets with many small uniform droplets within. These types of droplets can have interesting drug delivery features.

3.3.2 Generating red blood cell sized UV polymerizable hydrogel particles.

An important aspect of drug delivery using hydrogel microparticles is to be able to fabricate uniform particles at a similar size scale to particles typically found in biology, such as red blood cells (RBC). In some cases, microparticles smaller than RBCs are of interest. This ensures that the drug delivery vehicle can move through the circulatory system like a RBC, and maintain its drug releasing properties. In other applications, particles larger than a red blood cell are desired to specifically target organs of interest by filtration for drug delivery applications as discussed throughout this thesis.

3.3.2.1 Incorporation of functionalized polyethylene glycol into aqueous phase

Polymers, such as PLGA, chitosan, alginate, albumin have been used to fabricate RBC sized hydrogel microparticles. We chose polyethylene glycol (PEG) to generate our hydrogel microparticles for drug delivery applications since it is biocompatible and can be readily functionalized to create gels. PEG is biologically inert, and considered safe for use in biological applications. To produce hydrogel microparticles by microfluidics, we need to incorporate PEG oligomers into the aqueous phase of our emulsion generating system.

3.3.2.2 Considerations with regard to light sensitivity and solution viscosity

Microfluidic droplet generation of pure water systems is straightforward, as the viscosity of the aqueous phase is low, the interfacial tension is high to promote Rayleigh breakup, and there is no change in fluid physical properties over time. PEG precursors for hydrogel formation
include PEG diacrylate (PEGDA), PEG triacrylate (PEGTA), and PEG trithiol (ETTMP). By adding PEG acrylate precursor to the water phase, along with an initiator, PEG hydrogels can be generated by UV polymerization. This simple polymerization technique works well as it essentially decouples the droplet generation from the droplet polymerization, as polymerization will not happen significantly until a UV light source is exposed to the particles. However, if exposure of light to the polymer occurs during droplet generation, it can begin polymerization of the solution and change the viscosity of the solution significantly.

Droplet generation is affected by the viscosity of the PEG solutions, creating conditions that are difficult for generating small monodisperse droplets as hydrogel precursors. Typically high concentrations of PEG are necessary for the polymerization of droplets into hydrogels. For 1000 MW PEG triacrylates you need about 30% (w/w) in the aqueous phase to form a fully crosslinked gel after polymerization. Adding 30% (w/w) PEG to an aqueous phase will increase the viscosity of the phase by about 10 fold to close to 10 centistokes. As well, any pre-polymerization of the PEG could create increases in viscosity in the solution prior to droplet break-up. The changes in viscosity will affect the viscosity ratio of the microfluidic system, which will have direct implications on the minimum size that can be effectively generated in the device under stable conditions. Due to these circumstances, it is very important to avoid exposure of precursor solutions to light before and during droplet generation.

3.3.2.3 Parameters affecting small viscous droplet generation over long periods of time

Producing very small, uniform droplets using microfluidics without a second processing step, such as drying, is very challenging. There are multiple factors that affect how small a droplet can be generated while still maintaining the stable dripping regime. As droplets produced during dripping are more uniform in size, it is desirable to generate droplets in this regime. In
order to create the smallest possible droplets, the microfluidic device should operate at the highest possible capillary number and flow rate ratio without transitioning into jetting. The capillary number where the droplet generation will transition is controlled by the geometries of your capillary orifices and by the flow conditions of your outer phase. When limited by flow conditions, it is possible to change the device geometry by changing the tip separation distance (Figure 3.6) to reduce droplet size. Closer tip separation allows for the production of smaller droplets, which is likely due to the higher shear flow of the continuous phase and the higher dynamic interfacial tension as described in Chapter 2.

The viscosity ratio plays a big role in how high of a flowrate ratio can be used while still producing uniform drops. The more viscous the continuous phase is compared to the aqueous phase, the easier it will be to create smaller droplets as the continuous phase generates more shear stress for droplet generation. The trade-off is that the production must be slow as viscous oils will reach the critical capillary number for dripping to jetting transition at a lower flowrate. Figure 3.6 shows an example of droplet generation using a very viscous oil phase to generate small droplets over 2 hours. The aqueous phase flowrate is 0.05 µL/min and the oil phase flowrate is 8 µL/min, leading to very slow generation rates.

Creating small droplets based on flow conditions, geometries, and viscosity ratios enables the smallest possible size, however, if droplets must be produced over a long period of time, fluctuations in flowrate must be avoided.
At any instant, the droplets being generated are monodisperse; however over time the fluctuations, mainly due to the resolution of the syringe pump, will cause polydispersity in the collected material. An example of a fluctuation of flow is shown in figure 3.6(b). The fluctuation was large enough to transition the droplet generation briefly to jetting which greatly changes the particle size.

The difficulty in generating small droplets has to do with the step size of a pump motor. For drops of 10 microns in diameter, using a standard 1mL syringe, every step of the motor becomes larger than the droplet of fluid coming out. Fluctuations arise as the fluid cannot be pumped in small enough volumes to continuously produce droplets. This leads to pressure build-up.
ups and oscillating changes in flowrate. A typical graph showing instantaneous droplet size over time is shown in Figure 3.7, comparing a larger 3 mL glass syringe with a smaller 0.05 mL glass syringe. At the larger syringe size, the motor must advance only a few microns every step before it expels a volume equal to the droplet size. Since the motor cannot advance small enough, the fluctuations are large. These fluctuations have been described recently by the Shum group. Equal fluctuations are observed when using plastic or glass syringes. It was thought initially that the plastic syringes, which are somewhat compliant, might flex under pressure and cause larger fluctuations, but they had a similar fluctuation pattern to glass syringes of a similar diameter. However, as shown in Figure 3.7, the much smaller diameter syringes, which have to move much further O(100 µm) per droplet, have much lower fluctuations during droplet generation. This leads to much more stable flows and the ability to produce red blood cell sized droplets using microfluidics.
3.3.2.4 Developing pressure pump to generate RBC-sized droplets

As shown previously, mechanical pumps are easy to use and can produce relatively small particles with minimal fluctuations using very small syringes. However, with the constraints on the syringe diameter, only 50 µL of emulsions can be produced at a time. Therefore, to potentially address scale-up to large volumes we investigated a compressed air driven flow to provide a constant pressure flow for a large volume of fluid and avoid fluctuations due to a pump.34

A compressed air microfluidic pump was built based off of design specifications from the Doyle group at MIT.29 Our setup was modified to include a vacuum line to fill the fluid reservoirs and we used reservoirs that contained milliliters of fluid as opposed to microliters. A schematic of the device and an actual photo are shown in Figure 3.8. After turning on the compressed air line and adjusting the bleed valves, fluid could be pumped at constant pressure into our simple microfluidic device. After a number of runs, we found that it was necessary to

![Figure 3.8. Schematic and image of compressed air pressure pump for connection to microfluidic devices. The schematic is adapted from the Doyle group at MIT for which the design is based around. Of particular importance for uniform droplet generation is the resistance on the aqueous phase inlet line using a 50 micron bore in order to reduce fluctuations in the line. The image shows the actual device used in experiments.](image-url)
add a large resistance to the inlet of the aqueous phase to generate a large pressure drop in order to eliminate the effect of changing hydrostatic pressure caused by the liquid column in the inlet reservoir. We added a 50 µm diameter, 50mm long piece of glass tubing (bore) to the aqueous inlet stream before the device. It was calculated that the pressure drop in the tube was ~1 psi, larger than the positive pressure from the liquid column. Without the glass restrictor, the aqueous flow rate could not be slowed down enough to produce RBC sized droplets. Using the restrictor, stable microfluidic dripping was able to produce 2-3 micron droplets over the course of an hour with a very narrow size distribution as shown in Figure 3.9. The oil pressure was regulated at 5 psi and the aqueous pressure was varied between 1.5-3 psi to generate droplets. Due to the complex geometries of the device, exact flow rates were not known, but they appeared low during generation. Production time was restricted due to clogging of the 50 µm bore as it was difficult to avoid dust contamination of the device without the use of a clean room. These particles could potentially be polymerized using UV light and used as RBC sized drug delivery carriers.

![Image](https://via.placeholder.com/150)

*Figure 3.9. Production of very small droplets using a pressure pump configuration. a) Very small and stable droplet generation was possible with the use of a significant pressure drop in the AQ injection line. Droplets broke up in a manner similar to stable tipstreaming. b) Collected droplets containing PEG precursor solution were very uniform and around 3-5 microns in size, similar to a red blood cell. Scale bars are a) 100 µm b) 50 µm with inset 10 µm.*
As will be shown later, droplets of these very small sizes were not required for the drug delivery application. Therefore, syringe-driven flows were used to generate 30-100 µm drops for cGMP studies.

3.3.3 Generating Michael addition polymerizing particles

Although UV polymerization schemes for generating hydrogel microparticles offer the versatility of decoupling the droplet generation from the polymerization, there are downsides to the method. UV light can have adverse effects on the dyes, drug molecules, and even cells that could be encapsulated in the PEG hydrogel network. Previously, we have shown that UV polymerization of our block copolymer (BCP) nanoparticles containing EtTP5 dyes underwent serious fluorescent degradation due to the free radicals produced during UV polymerization.21 As well, networks formed by UV polymerization were typically filled with defects, creating soft particles that would not fully encapsulate our BCP NPs. Therefore it was necessary to look into additional hydrogel polymerization schemes to produce particles. Michael addition hydrogel polymerization has been investigated by several groups, particularly Hubbel et al; as a radical free polymerization method for PEG hydrogels.20,24,35,36 This polymerization approach involves using a PEG acrylate molecule and reacting with a di- or trifunctional thiol molecule under basic or acidic conditions to create a hydrogel network. We have shown that using PEG triacrylate 1100 MW, we could generate PEG hydrogels using dithiothreitol (DTT) and control the polymerization time by pH.21 By controlling the gelation time, we could create a two hour window for running microfluidics using our device. So simply mixing the two precursor acrylate and thiol molecules into a buffered solution at pH 4.3, we could generate hydrogel particles during microfluidics. Although particles were produced, we were unable to maintain stable microfluidic flow due to the slow change in viscosity of the aqueous phase over time. So
although particles could be collected, they were not of consistent size to be useful for drug delivery applications.

Premixed reactants gelled because the reaction proceeded during the 120 minutes that were required to express 60 µL of aqueous phase at a flowrate of 0.5 µL/min, which produced small droplets. Therefore, a more complicated device that could mix the acrylate and thiol precursor solutions prior to droplet generation was fabricated and is shown in Figure 3.10. Initially it was thought that mixing the two solutions in a simple tee before droplet generation could suffice for continuous production of particles as shown in Figure 3.10(left). However,
residence time along the walls of the tee junction led to polymerization which then caused polymerization throughout the entire tee and clogging of the device. As shown previously, in order to produce small particles for drug delivery, relatively slow flowrates (0.5 µL/min) are needed in the aqueous phase. This means that mixed solutions near the wall can have very long residence times during long continuous device running. Because of this, the tee junction will eventually clog in a few hours.

Another method is to adjust the inlet streams to inject the two different aqueous phases to mix right before droplet break-up as shown in Figure 3.10(right). This method offers less chance for residence times to clog the entire inlet stream but there are still limitations. By using both inner and outer inlet streams on one side of device to inject the aqueous phase, there is no ability to tune the dynamic interfacial tension by adjusting tip separation, as discussed in detail in Chapter 2. Then the operator of the device is left with fewer controls to generate droplets effectively. Although polymerization will take longer, it will still occur on the glass walls of the outer square capillary before break-up and on the collection orifice. Eventually, after longer periods of time (~4 hours), the device will clog similar to what is shown in Figure 3.10(bottom).

In order to reduce the residence time, it would be ideal to mix the precursor solutions immediately before break-up and have a “mixing section” at the end of the injection capillary to ensure uniform composition of the microfluidic droplets. For the DTT and PEG triacrylate system, we set up a device by adding another commercial Tee to create a complex injection tube containing a smaller inner tube for the second reaction mixture. This is shown in the top of Figure 3.11. The device was fabricated by simply connecting the end of the injection capillary to a third PEEK tee and then threading a 0.2 mm ID square capillary within it. By fitting the small Intramedic tubing over the end of the small square capillary, we ensured a leak-free fit within
the larger 1/8" PVC tubing and the ferrule. We connected one aqueous stream to the 0.2 mm ID square capillary and the other aqueous stream to the top of the third tee, allowing us to flow both streams into the round injection capillary without any mixing until close to the droplet break up point. In essence the two aqueous reactant streams are incorporated into each drop and mixing occurs inside the drop. This flow is shown in the bottom of Figure 3.11. By using the injection

Figure 3.11. A dual injection coflow design for Michael addition microfluidics. By mixing the two aqueous streams just prior to leaving the injection tube, a much cleaner and continuous droplet production could be achieved. The device is shown with a schematic of the third PEEK injection tee above and 30 µm droplet breakup detailed below.
capillary, we can control the wetting of the aqueous phase and contain buildup of polymerizing polymer to within it, while keeping the rest of the device free of buildup. Therefore if the device does clog, we can simply swap out the complex injection capillary with another one without

Figure 3.12. Stable production of milligram quantities of microfluidic hydrogel particles. Our simplified design for coflow of precursor solution led to the ability to run particle production uniformly over 24 hours to produce measurable quantities of microfluidic hydrogel particles. The particles were around 30 microns when dry and 50 microns when hydrated. Inset shows dry particles.
ruining the entire device. As well, the injection capillary creates a “mixing section” induced by the flow field of the continuous phase, leading to an area of very good mixing between the phases as the droplets are breaking up. We can also adjust the orifice separation distance to add another level of control for microfluidic operation.

Using this new design, we generated particles that are about 30 microns in size and swell to 50 microns in water as shown in Figure 3.12. The aqueous phase flowrate for both streams was 0.25 µL/min and the oil phase flowrate was 11 µL/min. The stream compositions were 50% (w/w) PEGTA and 50% (w/w) water in AQ1; water with 108.5 mg/mL DTT and 0.1% (w/w) EtTP5 NPs in AQ2; and 75% Par Oil 10NR, 23% (w/w) Min Oil, and 2% (w/w) ABIL EM 90 in the oil phase. Production of these particles can be close to 7.5 mg per hour of dry powder at those flowrates, which is relatively low for bulk production, but would be acceptable for a few pilot in vivo studies. However, the monodispersity and removal of satellite droplets is far superior to bulk emulsification techniques. Numbering up would increase the production rate of droplets at this size range, but the complexity of running multiple devices would need to be better understood.

As discussed previously with UV polymerized particles, small size and high monodispersity are important factors for producing particles by microfluidics. If the polydispersity in the particles is too high, the increased amount of time necessary to generate particles makes microfluidics no longer advantageous, compared with bulk emulsification methods such as shear emulsification, as a route for particle formation. We attempted to produce small particles around 10 microns in diameter using our microfluidic device. We initially tried to address this issue by using the pressure pump discussed in the previous section and modifying it to inject two aqueous streams. We found that the two aqueous phases were
competing with each other to go through the orifice and not flowing through at the same time. One phase would build pressure, and then force the other out of the orifice, and then the other phase would build pressure and do the same. We were unsuccessful with reducing these oscillations using the pressure pump. Therefore we moved back to flow driven pumps with very low flow rates, and very small syringes. We found the very low flow rates with two mixing aqueous streams caused greater fluctuations in the droplet size when compared to a single aqueous stream. This is shown in Figure 3.13. The size fluctuated between 5 and 30 microns and the particles produced were similar in monodispersity to what could be made with bulk emulsification. The droplets swell by 50% in diameter after hydration in water after polymerization, so making particles below 20 microns in size once hydrated was a great challenge using our device. Therefore, we have found that microfluidics is optimum for making hydrated hydrogel particles down to about 40-50 microns in size. However, below this limit,
bulk emulsification techniques, such as shear emulsification with post polymerization steps to remove satellite droplets become a better, more practical method.

When developing microfluidic systems to generate monodisperse emulsions, the next step is to turn those monodisperse emulsions into monodisperse particles. This can be affected greatly by lack of droplet stability after droplet generation, causing larger particles to be generated due to coalescence of droplets as they flow downstream and collect in the receiving reservoir. In order to improve particle size distribution after generation, some simple steps can be taken to stabilize the emulsion. Typically larger polymeric surfactants will stabilize the particles downstream, so they should be used in a high concentration in the oil stream. For mineral oil, we used ABIL EM 90® at 3% (v/v), and for silicone oil we use RSN-749® fluid at 5% (v/v). As discussed earlier, the RSN-749® interface is so stable that it creates a solid-like structure that can retard droplet break up. Another issue with surfactants to consider is their effect on the dynamic interfacial tension of the system. As shown in Chapter 2, Span 80® will decrease interfacial tension more than ABIL EM 90® at similar concentrations. So sometimes adding a little bit of small molecule surfactant, such as sodium dodecyl sulfate or Tween 80®, to the aqueous phase can help to reduce interfacial tension more rapidly and allow for easier droplet break-up if that is desired.

Another approach to stabilize the particles is to induce gelation relatively quickly after the particles are formed and before they are collected downstream. For the Michael addition reaction, this can be done by adding an amine base, such as triethylamine (TEA) or diisopropylethylamine (DIPEA), to the oil phase of the emulsion at 14.4 mg/mL after the droplets have been formed. Using our simple microfluidic system, we can do this by two
Figure 3.14. Microfluidic generation of composite gel microparticle for drug delivery. a) Photograph of "off-the-shelf" microfluidic device used for generation of PEG microparticles for drug delivery. Schematic depicts the post droplet generation coflow polymerization section to generate hydrogel particles from droplets before collection from the device. Particles were solid before exiting the device. b) Optical microscope image of particles being generated in the microfluidic device over the course of an hour. c) Optical image of uniformly polymerized particles generated by Michael addition polymerization in the microfluidic device. Scale bars are 100 µm.
different methods, either adding amine to the outer oil phase inlet stream or by adding a downstream tee to mix in amine base to the preformed emulsion.

The more elegant and somewhat riskier first approach would be to use the second oil inlet stream. In this setup, the oil stream not in contact with the aqueous "mixing section" contains the amine base. Due to the laminar flow associated with microfluidics, the two oil phases do not mix before entering droplet breakup, but rather they coflow through the orifice. This means that the amine can only travel into the inner oil stream by diffusion, and due to the flowrate of the oil phase and the diffusion length to reach the aqueous phase, it would be hundreds of microns downstream from droplet break up before the amine can influence the polymerization. What makes this method somewhat risky is the difficulty of not introducing the oil with amine until the device is running smoothly, as a fluctuation or brief stop in flow will cause immediate gelation of the aqueous phase and clog the device. As well, if the aqueous phase does not stay axisymmetric in the collection tube, due to density issues or an obstruction in the orifice, it could contact the oil with amine much closer to break off and lead to device clogging. When working with very precious or expensive material, where device clogging could cause the loss of the entire batch, it is better to design the device in a way that limits exposure to amine base until the droplets are hundreds of millimeters away from the break up orifice.

Using two different precursor molecules PEG diacrylate 600 and PEG trithiol 1300 MW, we generated particles using the device shown in Figure 3.14. Due to the pH control of the PEG trithiol, these two precursor molecules can be mixed together and will not begin to polymerize causing viscosity changes and phase separation for 2 hours in a vial without the addition of a catalyst, which is typically the addition of an amine to the oil post droplet generation. In order to introduce the oil containing the amine, which in this case is mineral oil with DIPEA, we added a
third commercial tee to the device downstream of the collection tube. This tee allowed the collection tub to pass completely through it and adds a second larger square capillary to the device downstream of the third tee. Oil with amine was able to coflow inside the square capillary but outside of the round collection tube. At the end of the collection tube, approximately 300mm downstream and seconds after droplet breakup, the oil with amine mixes with the emulsion and instantaneously polymerizes the droplets into hydrogel particles before they exit the device. The particles can be rinsed with hexane and sterile water containing Tween 80 and be injected as drug delivery vehicles. This will be discussed in greater detail in Chapter 4. Both co-mixing and post polymerization methods can produce hydrogel particles for collection at the end of the device. In each case, it is important to consider whether or not device clogging will be an issue and if particles are stable enough to reach the end of the collection tube without coalescence.

3.4 Conclusions

Throughout this thesis chapter, ideas and applications were presented for the use of simplified capillary devices to be used as complex emulsion generating tools.

The beginning of the chapter focused on generating complex nanocomposite emulsions. Both single and double emulsions were demonstrated using the same microfluidic device. The versatility of the device was also displayed through the ability to change collection tube geometry during device operation. This change from a flamed collection tip to a pulled collection tip has dramatic effects on the type of droplet break up occurring in the device. Flamed tips favor the dripping regime, while pulled tips favor the jetting regime. We also discussed limitations in surfactant use and how interfacial affects could limit droplet operating windows.
We then went into detail on the production of red blood cell sized hydrogel microparticles (~5 µm) by UV polymerization routes. Using our device, we discussed the critical parameters, such as capillary number and viscosity ratio, which affected the ability to generate very small droplets using microfluidics. We also introduced the concept of fluctuations in aqueous flow due to motor limitations in the syringe pumps. To increase monodispersity of small droplets generated over long times, we built a pressure driven flow device based on a design in the literature. We showed that this pump could greatly increase stability when generating small droplets using microfluidics, however production rates were estimated to be slow and the device could only run up to 2 hours maximum before clogging of the 50µm bore stopped the device.

To wrap up the chapter we introduced the topic of developing a method for incorporation of a Michael addition polymerization into the microfluidic device. We discussed some of the earlier designs and how the devices evolved to be more effective at producing stable droplets. Although able to produce monodisperse particles undergoing a Michael addition polymerization, the minimum size of stable particles generated were only around 30-40 microns in diameter. As discussed in the next chapter, these particles will find use in pilot studies for drug delivery.

3.5 References


Chapter 4: Preparation and Characterization of Composite Gel Microparticles for In Vivo Drug Delivery and Imaging

Abstract

Microfluidics can generate very uniform, complex particles that have the potential for in vivo drug delivery, however the quantity of material generated is very small due to milligram per hour production rates. Pilot in vivo studies and larger trials to understand toxicology and dosing schedules in mice need grams of hydrogel microparticles. Therefore it is necessary to additionally develop a method to generate gram-scale quantities of relatively uniform particles using shear emulsification. A simplified method for generating these particles is discussed in this chapter. We also describe how particles were generated for in vivo studies using microfluidics to generate doses with a much narrower size distribution. As well, optimization of the microparticle formulation was undertaken in order to increase drug loading by studying the phase separation of nanoparticles from the gel phase in emulsion droplets before polymerization. Finally, characterization of the mechanical properties of the gel microparticles is performed to understand the variability based on size for different production methods.
4.1 Introduction

In order to study the proper cGMP dosing particle size and quantity in mice, a gram-scale quantity of hydrogel material must be generated. Unfortunately, microfluidics can only produce relatively small quantities of materials over long periods of time. Therefore it is necessary to generate particles using a controlled shearing emulsification technique, so that gram scale quantities of materials can be generated relatively quickly. With size control and minimal polydispersity, shear emulsification can be used as a method to produce large quantities of particles. Bibette and coworkers developed a method and guidelines for generating particles using homogeneous shear fields.¹²

This emulsification method has been used previously by our group to produce cGMPs for the first in vivo test in a mouse.³ In that method, a coarse emulsion was made and put into a cup and bob geometry in a rheometer. The rheometer was run on a controlled shear setting to produce uniform final droplets. The method was effective, but did have some limitations. When using viscous oil, large quantities of material were left inside the cup after emulsion collection from the device, greatly reducing yield. Due to the need for shearing in a rheometer, the emulsion is taken out of its container for post-processing. A one pot method would be better, particularly when handling sensitive or toxic cancer drugs, such as camptothecin, in the pre-polymer phase.⁴

Nanoparticle loading within the cGMPs is a critical aspect of the formulation and design. Mixing a PEG precursor with highly concentrated nanoparticles leads to phase separation in the aqueous phase, forming two separate aqueous phases: a nanoparticle-rich phase, and a PEG macromer-rich phase. This leads to non-uniform droplet break up both in terms of particle size
and composition. When observing droplet breakup, clear droplets are generated containing no fluorescence, followed by large droplet formation with large aggregates of nanoparticles within. In this chapter, we will present a reformulated cGMP can be fabricated and have nanoparticle loadings as high as 33% (w/w) dry mass. We will also discuss how these new formulations can be polymerized by the diffusion of amine base through the oil phase in order to generate Janus-type gel microparticles.

In drug delivery, it is very important to characterize the material being introduced into the body. Both the size and the stiffness, or modulus, of the gel microparticles may play a role in the \textit{in vivo} performance.\textsuperscript{5-7} Both shear emulsification and microfluidics have good control over size which can be characterized by optical microscopy. Characterizing the modulus of microparticles is a bit harder to do and an extensive discussion was given in the background section of this thesis. We developed a compressive modulus measurement technique based on osmotic compression using dextran solutions to characterize our particles.\textsuperscript{8} We also fabricated particles for AFM indentation experiments in order to measure their Young's modulus. Those results and their limitations are also described in this chapter.

4.2 Materials and Methods

4.2.1 Materials

Polyethylene glycol diacrylate 600 MW (PEGDA) and PEG triacrylate 1176 MW were generously donated by Sartomer (Exton, PA). Thiocure® ethoxylated trimethylolpropane tri(3-mercaptopropionate) 1300 MW (ETTMP) was generously donated by Bruno Bock (Marschacht, Germany). EtTP-5 was synthesized as described previously.\textsuperscript{9} The block copolymer, 1.6k MW polystyrene-b-5k poly(ethylene glycol) and the 1.6k MW polystyrene homopolymer was
purchased from Polymer Source (Dorval, Quebec). ABIL EM 90 was a sample from Evonik Industries (Parsippany, NJ). Dow Corning 749® Fluid was a sample donated by the Dow Corning Company (Midland, MI). Diisopropylethylamine (DIPEA), silicone oil, mineral oil, Tween 80®, dithiothreitol (DTT), and solvents were purchased from Sigma-Aldrich. Dextran 70K MW was obtained from Fluka.

4.2.2 Production of cGMPs by shear emulsification

The fabrication of the cGMPs required the combination of three components, block copolymer nanoparticles, ETTMP 1300, and PEGDA 600, into an aqueous phase, followed by emulsification and polymerization in a continuous oil phase. Alternatively PEGTA could be combined with DTT in order to polymerize the polymer matrix.

The BCP nanoparticles are formulated using the process of Flash NanoPrecipitation (FNP). Briefly the block copolymer and hydrophobic components of polystyrene and a hydrophobic dye are dissolved into an organic solvent such as THF. The organic stream is then rapidly mixed in a confined impinging jet mixer with the antisolvent water and then diluted ten-fold in a beaker. The rapid supersaturation of the hydrophobic materials drives nucleation and aggregation of the hydrophobic components. The hydrophobic end of the block copolymers caps the nanoparticle and terminates growth, generating kinetically stable PEG coated nanoparticles with the size range of 50-300nm depending on parameters. After dialysis to remove the organic solvent, the nanoparticle solution is concentrated to 1.5-15% (w/w) nanoparticles depending on the formulation or required loading.

Typically cGMPs are made in 3 mL batches to enable homogeneous emulsification inside the 15 mL BD Falcon® centrifuge tubes. First 210 µL of nanoparticle solution is added to the
bottom of the centrifuge tube. Then 39 µL of PEGDA 600 is added to the NP solution and lightly vortexed. Finally 48 µL of ETTMP is added to the aqueous phase and lightly vortexed. Then 3 mL of silicone oil 5cSt-50cSt (depending on the size of particles desired) containing 5% (v/v) 749® Fluid is added to the centrifuge tube. The centrifuge tube is then lightly rocked back and forth to create a coarse emulsion around 2-3 mm in diameter with no large droplets in the solution. The centrifuge tube is then pressed onto a SP Vortex Mixer (American Scientific Products) on level 10 for 60 seconds to create the primary emulsion. 1.5 mL of silicone oil with 749® Fluid doped with 200 µL per 10 mL DIPEA (16.6 mg/mL) is added to the fine emulsion to shift the pH and initiate polymerization, and the emulsion is lightly vortexed for another 60 seconds. Hexane is then added to the emulsion to settle the particles and wash out the silicone oil. The particles are settled and the silicone oil/hexane mixture is decanted. The particles are washed three more times with hexane, then 1 time with 1% (v/v) Tween 80® in water. The particles are then settled and washed with 0.1% (v/v) Tween 80® solution 3 times. The settling process removes most of the smaller satellite drops. The particles are then put through a nylon screening filter with 200 µm pore size (Small Parts, Logansport, IN) to remove larger aggregates. Typical particle yield after settling and filtration is 80% by dry polymer mass. TGA (Perkin-Elmer) is performed on the solution to determine the mass concentration of the cGMPs minus the weight of the Tween 80®. TGA is run by ramping at 10°C/min up to 110°C, holding for 45 minutes to evaporate most of the water, then ramping at 10°C/min up to 800°C to decompose all materials. The concentration is found by comparing the water evaporated to the polymer degradation in the pan.
4.2.3 Production of cGMPs by microfluidic emulsification

To produce cGMPs using microfluidic droplet generation, an "off-the-shelf" microcapillary device using commercially available chromatography components was fabricated, as we have described previously.\textsuperscript{12} The concept behind the device is that drop formation is achieved on the reactive solution under pH conditions (pH≈4.3), where the reaction is retarded; the solution viscosity does not change under the long times required to process adequate drop volumes.\textsuperscript{13} Constant viscosity is required during the process. An increase would change the capillary number and subsequent breakup mechanism, changing droplet size.\textsuperscript{14} In the same microfluidic device, downstream from the drop formation constriction, a second solution stream introduces an amine, DIPEA, to shift the pH and initiate rapid polymerization. This is shown in the schematic in Figure 3.14 in Chapter 3. This rigidifies the gel particles and enhances their stability for collection from the device.

The aqueous phase was injected into the device through the injection capillary at a flow rate of 1.25 µL/min for two hours to produce a batch. Mineral oil with 5% (v/v) ABIL EM 90 was used as the continuous phase and was injected through the top of the first Tee assembly at 40 µL/min. Silicone oil was not used because the 749\textsuperscript{®} Fluid created such a stable interface, that nonspherical drops were formed. Mineral oil with 5% (v/v) ABIL EM 90 containing 16.6 mg/mL DIPEA was introduced into the second Tee assembly at 20 µL/min in order to coflow with the produced droplets and polymerize them through diffusion of the DIPEA into the droplets. Running the device required the start-up of each component individually. The mineral oil phase was introduced into the device first, followed by the mineral oil with DIPEA phase. It was important to have the mineral oil phase running in order to convect the DIPEA out of the device and not towards the droplet generation constriction of the device. Shifting the pH in the droplet
break-off zone could cause polymerization of the aqueous stream and clog the device. The aqueous stream is slowly introduced last and the device is run until the droplet sizes generated begin to change due to polymerization of the aqueous injection stream occurring over time. This occurred at about two hours. At this point all fluid flow rates were increased and the device is flushed. Then the device is taken apart, rinsed with ethanol, and ready for another batch of material. We were able to produce about 40 mg of particles in a two hour long run.

4.2.4 Osmotic compression of cGMPs

To characterize the compressibility of the cGMPs, osmotic compression of the cGMPs was performed as described previously. An external polymer solution of known osmotic pressure (i.e. concentration) was placed in contact with the gel. The polymer is large enough to be excluded from entering the gel mesh, and so the gel is compressed to equilibrate with the external osmotic pressure of the polymer solution. From the change in gel volume the gel modulus was calculated according to the equation:

\[
\pi = K \left( \frac{\Delta V}{V} \right),
\]

(4.1)

where \( \pi \) is the externally imposed osmotic pressure (Pa). For the 70K dextran solution the osmotic pressure follows: \( \Pi = c + 87c^2 + 5c^3 \), where \( c \) is in % (w/w), as shown by Bonnetgchonnet. The volume change from the initial volume is \( \Delta V \) and is measured in the regime where the volume change is less than 25% so that linearity is maintained. \( K \) is the bulk modulus, which for polymer gels in good solvents is related to the shear modulus, \( G \), by

\[
K = \left( \frac{5}{3} \right) G.
\]

(4.2)
cGMPs were settled to the bottom of a Petri dish and the Tween solution was washed with pure water. Particles stuck to the bottom of the dish were noted. Aqueous solutions containing different concentrations of 70k MW dextran were added sequentially to the Petri dish. The particles attached to the plate were imaged at each concentration and their size was recorded. The osmotic pressure at each dextran concentration was calculated as has been done previously. The relative change in volume of the particles was plotted against the calculated osmotic pressure. The volume changes were linearly regressed against the osmotic pressure with the slope yielding the bulk modulus of the cGMPs.

4.2.5 AFM indentation of cGMPs

AFM indentation was performed by our collaborators in the Dagastine lab at the University of Melbourne to complement the characterization work in our lab.

Dried cGMPs were rehydrated by adding them to a glass vial containing 10 ml of DI water leading to aggregates or clumps of cGMPs. These aggregates of cGMPs were redispersed by vortexing (Lab Dancer, from IKA) the vial for 10 minutes followed by sonicating (Unisonics, Australia) in an ice bath for 4 to 5 hrs. The temperature of sonicator was maintained at room temperature by continuously adding more ice to sonication bath. The cGMPs were immobilized on glass substrates in order to carry out AFM indentation experiments with silica colloidal probes. The circular glass bottom of AFM fluid cell was ozone cleaned (UV/Ozone ProCleaner Plus, Bioforce Nanosciences) for 10 minutes to remove any organic debris as well as to make the surface hydrophilic. A 500 μL solution of dispersed cGMPs was then spread on the glass surface and left in a laminar flow hood for a day to dry. The redispersed cGMPs solutions as well as the glass bottom with dried cGMPs were stored in a lab refrigerator for further use.
Before carrying out the indentation experiments with the silica colloidal probe, the glass bottom with dried cGMPs were attached to the fluid cell and 1 mL of DI water was added to rehydrate the cGMPs. Some of the dried cGMPs that were loosely anchored to the glass bottom were dislodged with the addition of water; whereas cGMPs that were firmly anchored remained on the surface and swelled. The cGMPs were left in the fluid cell for at least 16 hrs to reach equilibrium swelling prior to AFM measurements. The anchoring of cGMPs to glass bottom of the AFM fluid cell was further tested by adding 500 μL of DI water to the fluid cell where the swollen CGMPs were observed (via 40X inverted microscope objective, Nikon Eclipse Ti-U).

The silica colloidal probe used for indentation experiments was prepared by the method described in Ducker et al. A spherical colloidal silica particle was attached to V-shaped silicon nitride MLCT cantilevers (Bruker) by a small amount of two part epoxy adhesive (Super Glue Corp) with 30 minute delayed setting time. In this method, a MLCT cantilever was lowered into a small amount of adhesive and immediately used to pick up a colloidal silica particle deposited on a glass slide. The cantilever with the particle glued at the end was left at room temperature to allow the adhesive to dry and set for 24hr. The diameter of the colloidal probe (silica particle) ranged from 18 to 35μm. The spring constants of the probes were measured by method of Hutter-Bechhoefer and ranged from 0.03 to 0.15 N/m. The AFM head is slowly lowered into the fluid cell containing cGMPs swollen in DI water in order to ensure that no air bubbles were trapped on the colloidal probe. Before carrying out indentation experiments on swollen cGMPs, the sensitivity of colloidal probe was measured in water by indenting the bare glass bottom of fluid cell until a constant compliance was achieved. The constant compliant region was used to calibrate the optical lever sensitivity. The individual swollen cGMPs of appropriate size (≥5 μm) were located and the diameter was measured by recording an optical
image with an inverted microscope. The colloidal probe is then engaged and slowly lowered onto to the cGMP until a finite deflection of probe is recorded. The vertical alignment of the colloidal probe with that of cGMP is achieved further by adjusting the position of fluid cell with a micro-manipulation stage.

The cGMP was then indented with the colloidal probe, generating force-distance curves with a specified maximum applied force onto the cGMP. An example curve is shown later in Figure 4.6c. As all the AFM indentation experiments were carried out in a fluid cell with aqueous solution, it is important to eliminate hydrodynamic effects resulting from the permeability of the gel and drainage of water, so all force-distance curves were obtained at velocities below 100 nm/s. The maximum force of indentation was varied from 0.5nN to 80nN. The indentation experiment was repeated 10 times on each cGMP particle to check the reproducibility of the measurement. The alignment of the colloidal probe with that of the cGMPs did not change after 10 experiments. The diameter of the cGMPs also did not change after the experiments. For each sample type, 10 different individual cGMPs were located on the glass surfaces and the indentation experiment was repeated on each of them. Typical force distance curves of the indentation experiments were generated. These force versus Z-sensor curves were converted into force versus indentation data that was analyzed by linear elastic theory (i.e. Hertz theory). Some adhesion was observed between the silica particle and cGMPs in the retraction portion of the force distance curve. Thus for the analysis of the elastic properties of the cGMPs, only the approach portion of the force-distance curve was used to estimate the Young’s modulus.
4.2.6 Optical microscopy

Optical images of particles were taken using a Nikon Eclipse E200 equipped with an EO-4010C (Edmunds Optics) camera to capture high resolution images. Confocal microscopy on the particles was performed by using a Leica TCS SP5 confocal microscope.

Analysis of particle size was done by using ImageJ "analyze particles" subroutine. The data was processed and graphed using Origin.

4.3 Results and Discussion

4.3.1 Generation of cGMPs for in vivo biodistribution using shear emulsification

Michael addition fabrication of gel microparticles offers a route for simple and effective encapsulation of sensitive molecules for drug delivery by avoiding exposure to UV radiation. In order to create our cGMPs using a Michael addition polymerization we add two different PEG precursor molecules with different ends groups, namely a PEG-trithiol (ETTMP 1300) and PEG 600 diacrylate (PEGDA) (Figure 4.1a) These two molecules undergo a thio-ene Michael addition polymerization when exposed to basic conditions. However at acidic conditions (pH of 4.3) in the water/PEG mixture, the reaction takes longer than 4 hours to occur in a bulk solution. Therefore, the window for emulsification is large enough to decouple the emulsion generating process from the polymerization process. Another important reason for using this reaction scheme is the degradability of the thio-ene linkage, which will hydrolyze over time and break down the gel network, releasing the drug nanoparticles contained within.
Figure 4.1. Fabrication of composite gel microparticles by shear emulsification. a) Schematic of fabrication process. The aqueous phase contains two PEG precursors and block copolymer nanoparticles in water. The solution is added to silicone oil of varying viscosity containing a polymeric silicone surfactant. After coarse emulsification the vial is vortexted at 3000 rpm for 60 seconds. Excess silicone oil containing DIPEA is added to the fine emulsion to polymerize the particles. b) Optical image of 36 micron cGMP-S generated for drug delivery. c) Optical image of 97 micron cGMP-L generated for drug delivery. Scale bars are 100 µm.
The *in vitro* degradation of these particles at 37°C is on the order of 1 week, which will be discussed later. We will use this to compare with *in vivo* results presented in the next chapter.

4.3.1.1 Vortex mixing emulsification

We mix the two precursor PEG molecules with block copolymer (BCP) nanoparticles in order to create our aqueous phase for emulsification. The BCP nanoparticles are fabricated using our Flash NanoPrecipitation process, which allows for the encapsulation of hydrophobic molecules and dyes within the core of our nanoparticles. We use model polystyrene-polyethylene glycol 1.6k-5k molecular weight copolymers containing imaging dyes for biodistribution. These nanoparticles do not degrade *in vivo*, however a true drug delivery vehicle would use nanoparticles with a poly(lactic acid)-PEG BCP nanoparticle embedded in it, which will hydrolyze over time.\(^\text{19}\)

The oil phase in the shear emulsification process is silicone oil containing a large silicone-based polymeric stabilizer (749® Fluid) that will generate emulsion droplets with very stable interfaces, reducing the occurrence of coalescence. Emulsification of the aqueous phase can be achieved more controllably by using a rheometer shearing process, yielding very uniform particles.\(^\text{13}\) However, careful pre-emulsification of droplets to approximately 2 mm in diameter followed by 60 seconds of vortex mixing at 3000 rpm can yield similarly uniform droplet sizes compared to rheometer shearing. The simplicity of vortex mixing, along with the ability to keep the solution inside the same vial, avoiding lost materials in the rheometer, leads to a desirable emulsification method for the scale up of cGMPs required in our *in vivo* studies. The size of the final emulsion can be controlled based on the viscosity of the silicone oil used. Sizes can range from 15 microns using 100 cSt silicone oil, up to 80 microns using 5 cSt silicone oil, using the same pre-emulsifying formulation and under the same vortexing conditions. The different size
emulsions are shown in Figure 4.1. To increase the size further, the vortex mixer can be slowed down to produce sizes of about 120 microns, but more satellite particles begin to appear as mixing becomes less uniform.

After the emulsions are formed, the particles are rapidly polymerized by the addition of base-containing silicone oil to the continuous phase of the emulsion. This is done immediately after emulsification to reduce the chance of droplet coalescence. DIPEA doped silicone oil (16.6 mg/mL) is added to the continuous phase of the emulsion and instantaneously polymerizes the PEG precursor droplets into particles under gentle mixing conditions in the vortex mixer. An example of the versatility and uniformity of particles fabricated by this method is shown in Figures 4.1b and 4.1c, showing 36 µm and 97 µm particles formed by shear emulsification. The 36 µm cGMP-S particles were generated using 50 cSt silicone oil and 3000 rpm on the vortex mixer, while the 97 µm cGMP-L particles were generated using 5 cSt silicone oil and 1500 rpm on the vortex mixer. What is noticed is that as the size of the particles increases, the polydispersity increases, making shear emulsification less useful for larger particle fabrication.

4.3.2 Generation of cGMPs for in vivo biodistribution using microfluidics

In this chapter, we will only briefly discuss the use of microfluidics to generate particles for in vivo experiments, mostly focusing on generating material for dosing. A more detailed discussion of microfluidic production is given in Chapter 3.

4.3.2.1 Microfluidic post break-up polymerization

Microfluidic droplet generation provides a useful tool for making larger, more uniform, particles for drug delivery applications. An example of a microfluidic device has been described in Chapter 3, Figure 3.14. For these experiments, mineral oil is used as the continuous phase,
because the 749® Fluid in the silicone oil creates such stable interfaces, that it creates non spherical particles during droplet generation as discussed earlier in Chapter 3. This device is used to generate around 40 mg of particles that we were able to use for \textit{in vivo} biodistribution studies.

Due to the Michael addition reaction occurring in the precursor solution, a single phase device can only be run for approximately two hours before polymerization begins to affect the viscosity and surface tension during droplet generation, leading to changes in droplet size. Therefore, running small batches of aqueous phase over an hour at a time generated the most uniform particles for drug delivery, even when mixing more than one batch of particles together. Particle sizes generated ranged between 80 and 140 microns depending on the flow rates of the dispersed and continuous phases. For \textit{in vivo} biodistribution, particles were generated at 91 microns.

\textit{4.3.3 Generating cGMPs with very high loadings of nanoparticles}

It is desirable to be able to deliver the most amount of drug in each cGMP that is injected into the body. The more drug in each particle, the less particles need to be dosed, leading to less trauma on the lung tissue being targeted. As well, high loadings of nanoparticles containing imaging agents will allow visualization of the cGMPs deep within the tissue of the mouse after injection. It is very important to optimize the formulation of the cGMPs to maximize nanoparticle loading, while also avoiding phase separation and other sources of non uniform emulsification.
4.3.3.1 Phase separation during emulsification

Using the ETTMP 1300 (1300 g/mol MW) and PEGDA 600 (600 g/mol MW) Michael addition reaction to form hydrogels allows for 5-fold higher loading of BCP NPs within the polymer matrix without phase separation when compared to formulations using PEGTA (1176 g/mol MW) and DTT. Through numerous studies looking at the phase separation of NPs in PEG solutions, we found that acrylated end groups and high concentrations of PEG were leading to the phase separation of nanoparticles and PEG and poor emulsification of materials. This separation is due to depletion flocculation and/or hydrophobic effects. In our original formulation, we used 30% (w/w) PEGTA with a stoichiometric amount of DTT to form gel networks. Such high concentrations of triacrylated PEG lead to aqueous phase separation of emulsion droplets around 1.5% (w/w) nanoparticles in the solution. Our new formulation reduced to amount of acrylated PEG necessary to form gel networks and allowed us to increase nanoparticle loading by an order of magnitude to 15% (w/w) in solution (33% (w/w) dry basis).
before phase separation occurred. Figure 4.2 shows different formulations of the PEG/nanoparticle aqueous phase. Figure 4.2a is an aqueous phase with no phase separation, and Figure 4.2b is one in which phase separation has occurred. It is clear that the two different phases will have very different emulsification characteristics. The NP-rich phase is very viscous and will form large droplets, while the PEG-rich phase is less, so there will be small droplets with low loadings of nanoparticles. Such inconsistent particle composition and size will not work in in vivo studies. Therefore it is critical to maintain homogeneity until polymerization of the emulsion similar to what is shown in Figure 4.2c.

4.3.3.2 Phase separation during polymerization

Even with a homogenous aqueous solution of highly loaded nanoparticles, interesting morphologies are seen post-polymerization. In all particles, there is some visible phase
Figure 4.4. Polymerization of cGMPs on a glass slide to form Janus-type particles. a) A uniform pre-polymerized emulsion is spread onto a glass slide under the microscope. The aqueous phase contains 11.5% (w/w) PEGDA, 20% (w/w) ETMP 1300 and 7% (w/w) NPs and is not separated. b) Upon addition of amine base DIPEA to the side of the microscope slide, the particles are polymerized by the diffusion of the base. All particles first polymerize from the side of droplet first interacting with the diffusing base front. Polymerization across the droplet pushes all of the nanoparticles to one side creating Janus-type particles. The nanoparticle-rich side is still polymerized as the nanoparticles do not release from the microparticle upon resuspension in water. The particles are spun as the base mixes with the oil, so they reorient after the base front move past. Scale bar is 50 µm.

separation that gives the particles core shell or Janus-like morphology, but the nanoparticle loading remains very high. Figure 4.3 shows an example of a coarse but uniform emulsion that was polymerized using a base post-emulsification. From the images it is clear that as the base diffuses in the aqueous phase, polymerization is triggered at the point of first interaction with the base. In a well-mixed emulsion, the base enters the particles from all sides most of the time, creating a PEG-rich polymerized shell and a nanoparticle-rich core.

However, there are some particles that have a Janus-type (two-sided) structure where it seems that the aqueous phase was predominately contacted with base from only one side, leading to a cascade of polymerization from that side to the other, creating a PEG-rich face and a nanoparticle-rich face that is a still an intact particle. Clearly these particles were not in a very well mixed part of the vial when the base was added.
The Janus-particle polymerization due to one-sided diffusion of base in the silicone oil was demonstrated in the following experiment. An emulsion, shown in Figure 4.4a, was spread onto a microscope slide. Then the amine base, DIPEA, was gently added to one side of the slide and the particles all polymerized first from the side of the diffusing base front. The particles all polymerized into Janus-type structures as shown in Figure 4.4b.

Although Janus-structured and core shell structured particles are interesting in an applications standpoint, for drug delivery, they present challenges. Typically in drug delivery, you want a drug to come out of your matrix in a constant fashion over a long period of time. We know that the release of drug from the nanoparticles is controlling the release rate. If we have uniform nanoparticles throughout the matrix, we will see a uniform release of drug around the cGMP. Nanoparticle clearance rates may also be affected by the structure. Having core-shell particles may create a bursting nanoparticle release profile and Janus particles would likewise have a faster release profile than if the nanoparticle were spread uniformly throughout the matrix. So it is better to generate particles without these phase separated structures. We have been able to do this by lowering the amount of base added to the emulsion in order to slow down the polymerization and allow the diffusion of base to reach the middle of particles. This allows polymerization to start from all over in the particles and trap nanoparticles uniformly throughout the network. These types of particles were shown in Figure 4.1c.

4.3.4 Characterization of cGMPs used for in vivo studies

To understand the effect of particle properties on in vivo performance, it is necessary to develop reliable and accurate characterization methods. Size and modulus are two key characteristics that we believe may affect the way a particle interacts in vivo. In this chapter we
will discuss how we measured particle properties, while in Chapter 5, we will discuss their effects on \textit{in vivo} performance.

4.3.4.1 Characterizing size by microscopy

Optical microscopy was used in order to image the size of the particles generated from both shear and microfluidic emulsification. Figure 4.5a shows the size distribution for a few cGMP batches used for \textit{in vivo} studies in Chapter 5. Optical images are processed and analyzed using ImageJ and Origin software. Typically cGMPs are imaged under a 100x magnification and usually at least 100 particles are sized per batch. It is clear that the large particles generated by shear emulsification have a very broad size distribution and that microfluidic emulsification is a very effective tool for generate much more uniform large particles. However, at smaller particle sizes, shear emulsification can generate particles just as uniform as microfluidics.

4.3.4.2 Characterizing mechanical properties by osmotic compression

Osmotic compression was used as the method for determining basic mechanical properties of our cGMPs for comparison. The method is based on the concept of adding dextran to water with a high enough molecular weight that it cannot penetrate the PEG network. This leads to an osmotic pressure on the PEG particles that is described by the equation,

\[ \Pi = 286c + 87c^2 + 5c^3 \]  

where \( \Pi \) is osmotic compression and \( c \) is the concentration of dextran in \% (w/w).\textsuperscript{15} As the concentration of dextran is increased the compressive pressure on the particles is increased leading to a reduction of volume of the particles which can be measured optically by a change in diameter. This is shown in Figure 4.5b. As described previously, if the changes in osmotic pressure are plotted against the volumetric strain of the particle, then the slope of the line fitted to
Figure 4.5. Characterization of cGMPs for in vivo drug delivery. a) Size distributions of different formulations of cGMPs used for injection into mice for ex vivo biodistribution studies. Microfluidic particles are a considerable advantage for uniformity of particles larger than 40 microns in size. b) Optical microscope images of osmotic compression of cGMPs in order to determine their bulk modulus. c) Graph of change in volume versus calculated osmotic pressure of the dextran solution. The slope of the linear fit to the data gives a measurement of the bulk modulus. Scale bar is 100 µm.
the data is the bulk modulus. Multiple particles can be measured in a given batch to generate the average bulk modulus. Typically measurements are taken from a starting dextran concentration to avoid nonlinear affects from the pure water case. It is also important to avoid refractive index matching, which can make optical measurements of PEG particles very difficult. An example of this data, generated to characterize cGMPs generated by microfluidics, is shown in Figure 4.5c.

4.3.4.3 Characterizing mechanical properties by AFM indentation

AFM indentation was another mechanical property measurement technique used to generate Young's modulus data of cGMPs in order to understand the difference in stiffness versus different amounts of PEG and nanoparticles in the formulation. The particles were generated using the controlled shear emulsification technique in silicone oil, using PEGTA and DTT as the reactants to polymerize the gels. We generated particles and our collaborators in the Dagastine group in Australia generated the force curves and analyzed the data. A schematic and actual image of the experiment is shown in Figure 4.6. Discussion of the force curve analysis is given in the background section of Chapter 1 and an example is shown in Figure 4.6c.
Although an interesting technique that can directly measure the change in shape of particles due to force and displacement, the assumptions in the analysis and the forcing of data to fit analytical equations make it hard to measure accurate modulus data. As shown in Figure 4.7, there is no obvious trend in modulus as the PEG concentration is increased from 30 to 50%. The average modulus value is in the expected range if it is assumed that the Young's modulus is approximately 3 times the shear modulus (~50 kPa on bulk gels). However, the measurement error is very large for particles of the same composition. Therefore we did not use this technique to measure mechanical properties of particles that were injected into mice.
4.3.5 Determining effects of pre-injection conditions on cGMPs

When generating cGMPs for in vivo experiments, it is important to understand what effect the fabrication and storage conditions have on the size, modulus, and nanoparticle loading of the cGMPs being used for drug delivery. For the ultimate storage of the camptothecin-conjugated prodrugs, it is necessary to store the samples in a stable form. The camptothecin hydrolysis can be prevented by either freezing or lyophilizing the sample. We need to understand the stability of the gel particles in these conditions and the rate of gel degradation.
4.3.5.1 *In vitro* degradation of NP-loaded cGMPs over two weeks

It is important to know how long particles remain stable and how quickly they will break down in an aqueous environment. Therefore *in vitro* tests were run. Three different batches of microparticles were generated by shear emulsification in silicone oil using ETTMP 1300, PEGDA 600 and fluorescent nanoparticles with EtTP-5 as described in the methods section. The particles were polymerized by pH switching through the addition of DIPEA to the oil phase. They were then washed and resuspended into water. Finally, the particles were incubated for 14 days in vials containing 1X PBS at 37°C. At each time point, the vials were centrifuged and the liquid was decanted from the vial and imaged for fluorescence. As the hydrogels would break down, more fluorescent nanoparticles containing EtTP-5 could diffuse out of the networks and increase the fluorescence detection in the decanted liquid. This is shown in Figure 4.8. A stock solution of nanoparticles at the theoretical maximum encapsulation efficiency was also run to show 100% NP release for each time point. The results indicated that all of the nanoparticles were out of the hydrogels by day 7. We also observed at day 7 that there were no more cGMPs visible in the suspension. After 3 days, it was hard to even run compressive modulus tests on the particles because of their swelling and softening. In Figure 4.8, no batch of cGMPs reached the theoretical maximum, but they all stopped increasing in decanted nanoparticle fluorescence by day seven. The spread in the encapsulation efficiency between each batch was most likely due to slight differences in experimental procedure in formulation and polymerization. Further confirmation came from the fact that microparticles were no longer visible by optical microscopy at day 7. So our assumption is that we should see changes in the biodistribution profile of our particles within the first 7 days of injection. This is studied in Chapter 5.
Figure 4.9. Graph of change in bulk modulus versus the amount of days particles were frozen at -80°C. The freezing and subsequent thawing of particles did not generate a significant change in bulk modulus, indicating that the hydrogel network remained unchanged.

Another interesting point from the data, which affects material handling, is the fact that very few nanoparticles are released within the first 24 hours. This is important because we typically will allow the particles 24 hours to thaw after freezing or to re-equilibrate after washing. We also know that heat will accelerate hydrolysis, so room temperature degradation will be even slower in comparison. However, when generating material for \textit{in vivo} experiments, we keep the particle exposure to room temperature water to less than 48 hours from polymerization to injecting. This is done by storing the material frozen until it is needed.

4.3.5.2 Effect of freezing and thawing on the bulk modulus of cGMPs

In order to avoid degradation of cGMPs before dosing into mice, the particles were frozen in a freezer at -80°C. It was important to show that the particles did not change their structure due to freezing, so bulk modulus measurements using osmotic compression were performed before and after freezing and thawing for different amounts of time on the same batch of cGMPs. The bulk modulus of the particles was found to be 34.5 kPa. Figure 4.9 shows the
results of the test, which indicate that freezing and thawing cGMPs did not have a measurable effect on the bulk modulus. Particles that were frozen maintained a similar size to the original particles, whereas particles at room temperature that were degrading would swell over time. Therefore doses are typically frozen until needed for \textit{in vivo} studies.

4.4 Conclusions

In this chapter we presented the methods for producing uniform particles for \textit{in vivo} experiments at both the large scale using shear emulsification and at the small scale using microfluidics.

As well as presenting the process for production of cGMPs, we also discussed the importance of phase separation within our cGMP pre-polymerized aqueous droplets due to high concentrations of nanoparticles in the solution. We also discussed how to formulate around this issue and make particles with very high loadings of nanoparticles, which will have very nice advantages in \textit{in vivo} applications. We also showed that the direction of diffusion of amine base in emulsions can lead to the production of core-shell and Janus type microparticles which may have interesting properties, but are not as well-suited for controlled \textit{in vivo} studies.

Finally we discussed how we characterized the size and mechanical properties of our gel microparticles. Osmotic compression proved to be a reliable way to measure the bulk modulus of our cGMPs. With our collaborators, we also looked at AFM indentation as an elegant direct measurement method, but found that the error was too high to draw any real conclusions about the material properties at this time.
With the ability to produce and characterize cGMPs for drug delivery applications, we are able to test multiple controllable microparticle parameters and look at the performance in mice.

4.5 References


Chapter 5: *In Vivo* Performance of Composite Gel Microparticles Generated by Microfluidic Droplet Generation

Abstract

The intravenous delivery of composite gel microparticles offers a platform for localized treatment of lung cancer. We describe a method for fabrication of composite gel microparticles with average diameters of 35 to 100 μm using shear emulsification and microfluidic droplet generation. We characterized the particles and describe the performance of these particles *in vivo*. Long term biodistribution of the particles shows clearance of the particles in 7 weeks. Focusing on the one-week biodistribution, we found that larger, uniform particles produced by microfluidics provided optimal targeting of lung tissue. We also demonstrated that highly loaded gel microparticles containing a long wavelength fluorophore allow *in vivo* analysis of the biodistribution of gel microparticles without the need for *ex vivo* organ analysis.
5.1 Introduction

Targeting therapeutics for localized treatment of lung cancer would increase effectiveness and decrease off-site exposure of healthy tissue to cytotoxic drugs.\textsuperscript{1-4} There are a number of approaches for delivering drugs to the lungs, including inhalation, direct injection, surgical insertion and intravenous delivery.\textsuperscript{5-8} Delivery by inhalation has challenges in terms of the ability to deliver adequate doses of therapeutics to the disease site.\textsuperscript{9-11} Direct injection and surgical treatments, although effective, are invasive, leading to high morbidity in patients already weakened by the disease.\textsuperscript{1,4}

Recently, there has been interest in targeting the lungs by IV injection of microparticles, larger than erythrocytes, and having these microparticles deposit in the lungs by venous filtration.\textsuperscript{12-15} The delivery of particles to the lungs by venous filtration has FDA approval for albumin particles used as imaging agents, demonstrating the strategy is viable for therapeutic delivery.\textsuperscript{16-18} Localizing the delivery of anti-cancer drugs attached to intravenously-dosed microparticles potentially decreases the dose required to achieve efficacy and decreases side effects.\textsuperscript{12} However, those studies were performed using non-degradable polystyrene spheres, which will not clear the lungs over time to allow for multiple dosing. Therefore, we developed composite gel microparticles (cGMPs). In previous, preliminary experiments, delivery to the lungs was demonstrated, and \textit{ex vivo} degradation of the PEG gel was demonstrated over a seven day period by measuring the modulus of the gel.\textsuperscript{15} In that study, neither long term \textit{in vivo} clearance, nor the biodistribution of the cGMPs were addressed.

In this study, we present \textit{in vivo} results on the biodistribution and fate of cGMPs. In the last chapter we described modification of the PEG hydrogel scaffold to make it similar to
implanted PEG hydrogels and allow the nanoparticle loading to be increased to 33% (w/w) dry basis.\textsuperscript{19} The nanoparticles in the cGMPs were imaged using two highly hydrophobic dyes with emission maxima at 670 nm and 830 nm, which are encapsulated in the nanoparticles embedded in the PEG gel. The longer wavelength dye enables whole animal imaging of biodistribution. In this chapter, the biodistribution and clearance are presented. The \textit{in vivo} biodistribution of 50 µm cGMPs and the encapsulated nanoparticles were studied over the course of 12 weeks post injection. The 150 nm nanoparticles, which are encapsulated in the cGMP showed minimal capture in the lungs and clearance through the spleen and liver if not incorporated into a gel particle. The 50 µm cGMP showed capture in the lungs and a slow clearance with a half-life around 42 days.

cGMPs were formed using shear emulsification and microfluidics. Shear emulsification is a scalable process and is used to produce cGMPs with sizes of 36 µm and 97 µm.\textsuperscript{15,20,21} Droplet fragmentation during shear causes some smaller satellite drops. To understand the importance of particle size distribution on short time fate and clearance, we produced cGMP particles with a size of 91 µm using microfluidics to compare against the particles produced by shear emulsification. This produces particles with essentially ideal monodispersity, but it is not readily scaled. Efficient capture in the lungs is demonstrated, with the larger 91 µm monodisperse particles showing essentially complete retention in the lungs, while the polydisperse 97 µm particles had slightly less lung targeting due to satellites. The 37 µm cGMPs showed capture in the lungs with some bypassing to the spleen in relatively short times. Pathology was also performed to understand the physiological effects of the particle dosing on the lung tissue. Finally, qualitative \textit{in vivo} biodistribution of cGMPs in living mice was performed using high loadings of fluorescent nanoparticles in the cGMPs.
5.2 Materials and Methods

5.2.1 Materials

Polyethylene glycol diacrylate 600 MW (PEGDA) was generously donated by Sartomer (Exton, PA). Thiocure® ethoxylated trimethylolpropane tri(3-mercaptopropionate) 1300 MW (ETTMP) was generously donated by Bruno Bock (Marschacht, Germany). EtTP-5 and LW2 were synthesized as described previously.22 The block copolymer, 1.6k MW polystyrene-b-5k poly(ethylene glycol) and the 1.6k MW polystyrene homopolymer were purchased from Polymer Source (Dorval, Quebec). ABIL EM 90 was a sample from Evonik Industries (Parsippany, NJ). Dow Corning 749® Fluid was a sample donated by the Dow Corning Company (Midland, MI). Diisopropylethylamine (DIPEA), silicone oil, mineral oil, Tween 80 and solvents were purchased from Sigma-Aldrich.

Six week old male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were acclimated for at least 1 week prior to experiments and kept on 12hr light dark cycles in a temperature controlled vivarium. All animal studies were performed in accordance with the Rutgers University animal advisory committee guidelines.

5.2.2 Fabrication of cGMPs

The fabrication of the cGMPs required the combination of three components, fluorescent nanoparticles, ETTMP 1300, and PEGDA 600, into an aqueous phase, followed by emulsification and polymerization in a continuous oil phase.

The fluorescent nanoparticles are formulated using the process of Flash NanoPrecipitation (FNP).23,24 The procedure is discussed in Chapter 4.
The fabrication of cGMPs by shear emulsification is discussed in detail in Chapter 4. After the particles are generated, the particles are then washed 3 more times with sterile Tween 80 solution and resuspended to the approximate dosing concentration in sterile 0.1% (v/v) Tween 80 solution. TGA is performed on the solution to determine the mass concentration of the cGMPs minus the weight of the Tween 80 to get the exact concentration. The particles are settled in order to remove excess Tween solution to get to the final dosing solution of about 0.9 mg cGMPs per 100-125 µL solution. The particles are allowed to hydrate overnight and then are stored in a -80°C freezer until dosing. Bulk modulus measurements demonstrated that freezing and thawing did not measurably alter the gel structure as shown in Chapter 4. Before dosing, the particles are thawed over 24 hours and resuspended by 30s of probe tip sonication. Settled suspensions are resuspended by 30s vortexing before pulling doses into syringes.

The production of cGMPs using microfluidic droplet generation is also discussed in detail in Chapter 4. We were able to produce about 40 mg of 91 µm particles for dosing in a two hour long run. After the polymerized droplets are collected, they are rinsed and sterilized for dosing similar to the method described for particles generated by shear emulsification.

5.2.3 Biodistribution of cGMPs in mice

For long-term biodistribution, mice were dosed with either cGMPs or just BCP nanoparticles containing the dye EtTP-5 and monitored over the course of 8 or 12 weeks to look at the location of the fluorescent nanoparticles in different organs. 28 male CD-1 mice around 30 grams in weight were dosed with 0.9 mg of cGMPs via a tail vein injection. At each time point four mice were euthanized along with a control mouse to normalize the fluorescent signal. Lungs, liver, spleen, kidneys, and heart were imaged in an FX-PRO (Bruker) at 460 nm
excitation and 700 nm emission filter sets for a 60 second exposure. Organ fluorescence was analyzed by outlining the organ and calculating the average fluorescence. The average control organ fluorescence was subtracted from the dosed organ fluorescence to give the final organ fluorescence. For all experiments control organ fluorescence remained very consistent allowing comparison of organs from different time points.

For one week biodistribution studies, 16 male CD-1 mice, 20 g in weight, per formulation were dosed. Four mice were euthanized along with a control mouse at each time point. In order to reduce autofluorescence, the long wavelength dye, LW2, was used and analysis of the biodistribution was done using an FX-PRO (Bruker, Billerica, MA) at 730 nm excitation and 790 nm and 830 nm emission with 60 second exposure. The brighter 790 nm emission was used to generate semi-quantitative graphs of biodistribution because it was possible to outline the control mouse organs for normalization; while the 830 nm emission was used to generate the qualitative images since there was no autofluorescence in the lungs.

5.2.4 Pathology of mice post-treatment

Studies were conducted in young, sexually mature male CD-1 mice. Animals were assigned to one of 4 dose groups (vehicle, 36, 91, 97 μm) and evaluated at 4 time points (30 min, 1, 3, or 7 days). Mice were administered vehicle or 0.9 mg particles intravenously and then underwent fluorescent scan evaluation under anesthesia prior to necropsy. Lung and heart were collected at necropsy; fixed in 10% buffered formalin, embedded, sectioned, and stained with H&E. Heart tissues were evaluated only at the 3 day time point. Lung tissues were evaluated at 30 min, 1, 3, and 7 days for the incidence and severity of histopathological changes and the extent of particle deposition.
5.2.5 Characterization of cGMPs

Optical images of particles were taken using a Nikon Eclipse E200 equipped with an EO-4010C (Edmunds Optics) camera to capture high resolution images. Confocal microscopy on the particles was performed by using a Leica TCS SP5 confocal microscope.

Analysis of particle size was done by using ImageJ "analyze particles" subroutine. The data was processed and graphed using Origin.

In order to characterize the compressibility of the GMPs, osmotic compression of the cGMPs was performed. The details of this technique were discussed in Chapter 4.

5.3 Results and Discussion

5.3.1 Long term biodistribution of cGMPs compared to BCP nanoparticles

The main objective for using a microparticle based delivery vehicle for IV injection is to ensure that the drug particles are deposited only into the lung tissue, reducing the side effects in other organs such as the liver and spleen. However it is also important to determine how long it will take for the cGMPs to clear from the lungs in order to understand how often the materials can be dosed in a clinical setting. Healthy mice were dosed in groups with either cGMPs or just nanoparticles and euthanized at different time points out to 12 weeks in order to determine the clearance rate for the particles from the lungs as well as the accumulation in any of the non-target organs. The dye encapsulated in the nanoparticles for these experiments was a very robust dye, EtTP-5, used typically for organic solar cells. It has an excitation around 460 nm and emission around 635-670 nm and very little degradation from exposure to light. However, due to the excitation wavelength, some auto fluorescence of the tissues is observed. To remove
Figure 5.1. *Ex vivo* images of biodistribution of cGMP-50µm over the course of 12 weeks post-dose. These images were used to generate the graphs in Figure 5.2. Clearly the fluorescence decreases in all organs over the course of 12 weeks.
autofluorescence from the data, control mouse organs with no dose are imaged alongside of the dosed mouse organs and used to subtract autofluorescence from the organ. Some non target organs in dosed mice, such as the kidney actually have less fluorescence than the control giving a slight negative value. For these experiments we just assume a negative value to mean there is no dye in the organ. For the data analysis, we used the average fluorescence of the outlined organ and subtracted it from the average fluorescence of the control organ to get a final value for the biodistribution graph. Figure 5.1 shows an example image of one of the three mice analyzed for each time point in the long term study. Using the dye with the shorter wavelength of 670nm emission led to some autofluorescence in the control mouse where no particles had been dosed. The average fluorescence from each organ in the control was subtracted from the fluorescence of each dosed organ to obtain the values plotted in Figure 5.2. Qualitatively it is clear that the fluorescence in the lungs gradually clears out between weeks 6 and 8.

Figure 5.2. Long term fluorescent biodistribution of cGMP-50µm and BCP nanoparticles. a) Graph of biodistribution of cGMPs around 50 µm in size (Figure inset, scale bar is 50 µm) over 12 weeks post injection at 45 mg/kg. The particles significantly degraded and fluorescence in the lungs decreases around 6-7 weeks. This was much longer than our in vitro results. At 1 week post dose there is accumulation in liver and spleen of mice due to smaller satellite particles. b) Control dose of same concentration of nanoparticles as in cGMP dose. Fluorescence intensity has been normalized between week 1 liver signal with cGMPs to magnify the graph about 3.5x. Most of the fluorescence past 8 weeks is seen in the liver and the spleen, with only some initial fluorescence in the lungs possibly due to some aggregated nanoparticles or macrophage engulfment.
In the graph in Figure 5.2a, it is clear that the microparticles initially target the lungs. By performing an exponential fit on the data from weeks 2 to 12, a time constant of 4.3 weeks can be extracted. Interestingly, the complete clearance of fluorescence from the lungs takes approximately 7 weeks to occur. This is in contrast to our in vitro degradation time of around 1 week in PBS buffer at 37°C discussed in Chapter 4. One reason for this may be due to confinement of the particles by the blood vessels and macrophages, creating a barrier around the particle that does not let material leave the site of the embolism. Another interesting point is the residual amount of fluorescence that is still significantly present in the lungs at 12 weeks post dose. Histological findings discussed later reveal that this result may be due to macrophage uptake and storage of PEG hydrogel fragments, which has been observed before with in vitro models.25

For the nanoparticle control group, the organ fluorescence is very low despite similar doses of fluorescent nanoparticles given to the mice. This might indicate other clearance sites for the nanoparticles, such as the blood pool, skin, and the gastrointestinal tract. The fluorescence biodistribution for the nanoparticles was normalized to the cGMP graph using the one week liver signals to increase the intensity approximately 3.5 times. The nanoparticles tend to accumulate more in the liver and spleen as opposed to the lungs. The initial nanoparticle fluorescence in the lungs might be from some aggregated nanoparticles that lodged, however by week 4 there was no more signal found. The NP control clearly shows how the passive targeting system of the cGMPs can potentially create a localized increase in the drug concentration within the lungs, and still be mostly cleared from the lungs within about 7 weeks. This means the cGMP could be a good candidate for long term treatment of lung cancer as a depot delivery system.
Off target organs such as the spleen and the liver, which typically collect foreign material in the body, do show significant fluorescence particularly at 1 week post dose in the long term study (Figure 5.2a). This is most likely due to the presence of small satellite particles formed during our shear emulsification process. Some satellites can be seen in the inset in Figure 5.2a. Satellite particles can create off-target delivery of toxic anti-cancer therapies since they are not filtered. As well, if particles can get through the lungs, they can pass into the coronary arteries and lodge, leading to cardiac infarction. Therefore it is desirable to reduce the number of satellite droplets in our emulsification process to avoid these affects. There are two ways of reducing satellite particles. One is by making larger particles using larger emulsions and employing multiple separation steps, i.e. settling, to remove satellite particles post polymerization. The other, more elegant process is by making the particles using microfluidic droplet generation. Several groups have already begun to experiment with the development of drug delivery particles using microfluidics and a Michael addition reaction to generate hydrogel drug and cell delivery particles.26-28

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Diameter (µm)</th>
<th>Bulk Modulus (kPa)</th>
<th>IV Dose(mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGMP-36µm</td>
<td>36 ± 5</td>
<td>42 ± 3</td>
<td>45</td>
</tr>
<tr>
<td>cGMP-97µm</td>
<td>97 ± 31</td>
<td>73 ± 4</td>
<td>45</td>
</tr>
<tr>
<td>cGMP-µF-91µm</td>
<td>91 ± 6</td>
<td>88 ± 13</td>
<td>45</td>
</tr>
</tbody>
</table>
5.3.2 Ex vivo cGMP short term biodistribution study

To understand the factors that lead to off-target organ biodistribution over the first week of injection, we designed an experiment where three different groups of mice would be dosed particles of the same formulation, but containing different sizes and uniformities. This will allow us to answer the critical questions about how size and polydispersity affect biodistribution of particles in mice organs over the first week. With our initial coarse emulsion used for the long term study, there were a lot of satellite particles, which may have led to the large fluorescence in the spleen and liver at the one week time point. The microfluidic drops, with narrower size distribution, will address the question of the role of average size versus size polydispersity.

Figure 5.3. Images of one week in vivo biodistribution of cGMPs of different size and uniformity. Left circle contains heart (top), spleen (middle) and kidneys (bottom); center circle contains lung inflated with formalin; right circle contains liver with gallbladder removed. The cGMP-36µm particles targeted the spleen and the liver within 24 hours of dosing. The larger cGMP-97µm and cGMP-µF-91µm formulations both targeted the lungs much better over 1 week. The cGMP-97µm formulation did show some fluorescence in the spleen and liver at 24 hours, which may have been due to satellite particles.

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Table 5.1 shows the characteristics of the three different cGMP particles that were injected into mice for our one week biodistribution study. The detailed experimental characterization is discussed in Chapter 4. After particles were generated as described in the experimental section, they were isolated into sterile 0.1% (v/v) Tween 80® solutions where their size and modulus were measured after 24 hours. As shown in Table 5.1, the cGMP-36µm was smaller in size and produced using quite a uniform emulsion from vortex mixing. cGMP-97µm and cGMP-µF-91µm are both larger particle formulations that are generated in different ways. cGMP-97µm particles were made by vortex mixing and show a very large size distribution due to the presence of satellite particles from the shear emulsification method, which were not completely removed even after multiple settling attempts. cGMP-µF-91µm particles are generated using the microfluidic droplet generation scheme shown in Chapter 4. Because of the uniform method of emulsion generation, there are no satellite droplets in the emulsion, therefore the size distribution of the particles is much narrower. The modulus of cGMP-36µm was significantly lower than that of the larger particles, which may have to do with the rate of diffusion of DIPEA into the droplets to polymerize the particles. Smaller particles will polymerize more rapidly by diffusion and create a less uniform network leading to a slightly lower bulk modulus.\textsuperscript{15}
Figure 5.4. Semi-quantitative graphs of the *in vivo* biodistribution of cGMP formulations over the course of one week. a) cGMP-36µm formulation shows that the particles accumulated largely in the spleen and liver after the 30 minute time point. b) cGMP-97µm formulation shows accumulation mostly in the lung with a small amount of nanoparticles in the spleen and liver at the 24 hour time point. c) cGMP-µF-91µm showing significantly less accumulation in the spleen and liver compared with the other two formulations at 24 hours. The graph was broken in order to maintain the same scale as the other studies.
The *in vivo* biodistribution in the mice is shown in Figure 5.3 to compare the three different batches of particles. Previously, we determined that a 45 mg/kg dose was safe for all particle sizes to ensure that no deaths were seen in the mouse groups. For each formulation, 16 mice were dosed, 4 mice each for time points of 30 minutes, 24 hours, 72 hours, and 168 hours. Initially we attempted to use EtTP-5 as the dye for visualizing the organ biodistribution of the nanoparticles within the cGMPs, however we found that bleeding in all groups at 24 and 72 hours greatly reduced the fluorescence of the organs as blood would mask the fluorescent signal from EtTP-5. At longer times, when the bleeding had subsided, the fluorescence would increase in the lungs, giving us inaccurate results. Therefore, in order to gain an understanding about the short term biodistribution, we switched to a custom made long wavelength dye with an excitation peak at 700 nm and emission peaks at 790 nm and 830 nm. We imaged the organs with both filters because the 830 nm filter greatly reduced autofluorescence, while the 790 filter was easier for analyzing the autofluorescence of the control groups for normalization of data.

In Figure 5.3, it is clear that there is a difference between the three groups with regard to biodistribution over the course of 1 week. Figure 5.4 shows quantitative graphs of the average fluorescence of each organ imaged after subtracting the average autofluorescence of the control organs. The small uniform particles tended to make their way out of the lungs over the course of a week and accumulated in the liver and spleen. Both sets of larger particles remained in the lungs. However the cGMP-97µm particles, containing some percentage of small satellite particles, did have an increase in fluorescence in the liver and spleen at 24 hours. The microfluidic particles had a considerably smaller proportion of fluorescence in the liver and spleen at 24 hours, due to the lack of satellite particles. There is a small amount of fluorescence seen in the liver at 72 hours for the cGMP-µF-91µm group. We imagine that this fluorescence
could be from nanoparticles that were released out of the GMPs before and during cGMP injection. It is also interesting to note that the fluorescence of the lungs at the initial 30 minute time point for both larger particle batches is high and then is constant in the 72hr and 168hr time points. We believe this could be caused by bleeding within the lungs after dosing, due to either optical attenuation from this localized hemolysis or due to oxidation of the fluorescent dye by reactive oxygen species (ROS) associated with the early damage and inflammatory response.

From these in vivo results, we can see that if we want the particles to clear the lungs rapidly, we would use smaller cGMPs, whereas if we want to solely target the lungs for long depot deliveries, we would use much larger cGMPs and try to remove satellite particles formed. Microfluidics can generate the right size particles without satellites, but fabrication of microfluidic particles is a challenging production problem. Scalability of microfluidic droplet generation using polymerizing precursors has not been demonstrated and will need further development before it can be a viable technique. Careful shear emulsification can yield particles almost as good as microfluidics, and may offer a better route towards commercial production of cGMPs for drug delivery purposes, with better particle separation techniques utilized to remove satellites.

5.3.3 Pathology of cGMPs used in ex vivo studies

Pathology was performed on all mice dosed in the 12 week long term study and in the 1 week short term study to understand better the physiological response to injection of hydrogel microparticles. In preliminary dosing experiments it was found that doses greater than 2 mg in the 30 g mice resulted in some morbidity during the first 48hrs, with greater morbidity occurring for the smaller cGMP particles. Morbidity was associated with internal hemorrhaging in the
lungs. Based on these preliminary studies, doses of 0.9 mg were delivered for which no morbidity was observed for any of the particle sizes. Figure 5.5 shows an example of pathology performed on mice dosed with cGMPs in a pilot run. As shown in Figure 5.5a, the particles are uniformly distributed in the lungs, and did not “logjam” to form domains with high cGMP concentrations. (The fluorescent dye has a blue color, which is visible in the histologically stained slides, and is circled in red in the Fig. 5.5a.) Particles of larger sizes tended to lodge in larger arterioles in the lung capillary bed. Figure 5.5b, shows one possible side effect from satellite particles that could pass through the lung capillary bed. In this slide of cardiac tissue an embolism in a coronary artery is observed. This event was observed in only a single mouse with shear emulsified large, polydisperse particles. It was not observed in the cGMPs of equivalent size produced by microfluidics, which did not have satellite drops and which appeared completely retained in the lungs. While any thrombi going to the heart can cause occlusion of the vasculature, myocardial hypoxia and death, thrombi to the lung are not generally considered a life threatening condition. This is because the lung has dual blood supplies carrying variable levels of oxygen and nutrients; the pulmonary and bronchial artery systems. If pulmonary arteries become occluded, oxygen and nutrients are still supplied via the bronchial arteries.

Due to the use of the stable EtTP-5 dye, microparticle matter could be visualized throughout all 12 weeks. Interestingly over the course of the treatment, the hydrogel material was seen accumulating in macrophages close to embolism sites created by the particles. This visualization confirms our hypothesis of why fluorescence is maintained in the lung for a long time. Macrophages engulf particulate material containing dye filled NPs and retain material in the lungs indefinitely. We did not assay lymph nodes to see if a significant amount of internalized PEG gel was translocated to other sites. Long term, there were no pathologies.
associated with the hydrogel particles in the lungs and all lungs began to look normal again after the first week post dose.

5.3.3.1 Pathology of 1 week term study mice

For the 1-week short-term study, pathology scoring was used to more quantitatively understand the effect of different size particles on the acute response. The full score sheet from the pathology findings are recorded in Appendix C at the end of this thesis.

At the 30-minute time point, most particles in the 91 μm and 97 μm groups were thromboembolic, a substantial number of 35 μm particle emboli were in patent (nonclotted) vessels and still ~36 μm. Inflammation, as assessed by neutrophil and macrophage accumulation, was generally mild and similar between the three particle groups. No necrosis was observed in any treatment groups. Pulmonary hemorrhage in the 35 μm and 90 μm groups had low incidence (one of four mice) and severity while the 97 μm group had high incidence (four of four mice) and variable severity (scores of 1-3). In our preliminary experiments using 12-15 μm cGMPs we observed even greater levels of hemorrhaging damage. The damage was associated with particles

Figure 5.5. Pathology images of lung and heart tissue of mice dosed with cGMPs for a pilot study. a) Image of lung tissue showing distribution of hydrogel particles within the capillary bed. Particles are well dispersed and typically lodged in larger pulmonary arterioles. b) The cardiac tissue of one mouse shows the signs of an infarction potentially caused by the lodging of a cGMP within the coronary artery. This type of result stresses the need to reduce the number of satellite particles with a given dose.
that were small enough to enter the \( \sim 8 \mu m \) alveolar capillaries. These capillaries, where oxygen transfer occurs, have the most delicate epithelia. It appears that larger particles, which are captured in larger capillary vasculature are more benign than smaller particles. This was contrary to our original hypothesis that making particles only slightly larger than RBCs would distribute the particles more uniformly, and prevent blockages and occlusions that might produce necrotic regions. The branching and secondary perfusion in the capillary bed in the lungs appears to make this not a significant problem as is shown below. One potential reason for the lung severity observed by dosing the shear emulsified cGMPs is that a relatively small mass of satellite particles in the \( \sim 12-15 \mu m \) size range could be the source of pulmonary hemorrhaging. However, as was noted, after 1 week these tissues rehealed and presented normal physiology.

**By day 1,** the most notable findings were the appearance of necrosis and enhanced neutrophil levels. There were perivascular neutrophilic cuffs 2 to 10 cell layers thick and increased numbers of enlarged, vacuolated alveolar and interstitial macrophages. The extent of the necrosis was slightly greater in the 91 \( \mu m \) and 97 \( \mu m \) groups compared to the 35 \( \mu m \) group. Minimal-to-mild multifocal necrosis (hypereosinophilia, karyorrhexis, karyolysis, cell debris) was centered on thromboemboli. Though no groups had detectable free microparticles in vessels or extracellular spaces, most macrophages in particle-treated groups contained increased amounts of cytoplasm and/or cytoplasmic vacuoles that resembled microparticle material. The observation of slightly greater sizes around the thromboemboli for the larger particles has been weighed against the greater number of sites that occurs when the smaller particles are injected. There are approximately 20 times more particles injected when 36 \( \mu m \) particles are administered, relative to 97 \( \mu m \) particles at the same mass. This is the probable origin of our observation in the original screening studies that larger particles resulted in lesser morbidity.
By the 3rd day, necrosis has largely resolved in particle-treated groups. Hemorrhaging in all dose groups was less than at Day 1 but did continue to persist. Neutrophil numbers in the all dose groups were comparable to Day 1. Particulate matter was no longer discernible. Possibly due to the lack of color as the LW2 dye used in these experiments was less stable compared to the EtTP-5 dye used in the long term study. Alveolar macrophages contained variable numbers of cytoplasmic vacuoles. There were no signs of particle treatment-related infarction, inflammation or necrosis in the hearts of the mice at day three.

At the 7th day time point, particle-treated mice no longer exhibited hemorrhage or necrosis. All dosed groups had similar, mildly increased macrophage scores. The neutrophil scores for the 35 μm group were similar to control levels while the scores for the 91 μm and 97 μm group were minimal. There was no discernible particulate matter in lung tissue. This indicated that the lungs were returning to their normal state.

To summarize, at 30 minutes, the larger particles (91 μm and 97 μm) caused more lung pathology (venous thromboemboli, hemorrhage, inflammation) than the smaller particles (35 μm) because they had greater potential to occlude microvasculature. The lower damage scores for the smallest particle emboli (35 μm) correlated with unclotted, patent vessels still containing free microparticles. Treatment-related pulmonary scores were greatest at Day 1. While macrophage, neutrophil, necrosis, and particle scores were comparable between the three treatment groups at this time, the 97 μm group has slightly higher hemorrhage scores. Day 3 was the last time point that microparticles could be detected with light microscopy. By Day 7, most treatment-related damage (necrosis and hemorrhage) had resolved. No groups had signs of cardiac thromboemboli, infarcts, vasculitis or necrosis.
While any thrombi going to the heart can cause occlusion of the vasculature, myocardial hypoxia and death, thrombi to the lung are not generally considered a life threatening condition. This is because the lung has dual blood supplies carrying variable levels of oxygen and nutrients; the pulmonary and bronchial artery systems. If pulmonary arteries become occluded, oxygen and nutrients are still supplied via the bronchial arteries. This is why the Day 7 lung findings appeared to be reversible and the rodents were well on their way to recovery.

The use of particles larger than lung capillaries precluded any particles from passing through the lung to the heart where they could cause life threatening and irreversible cardiac damage. The larger the particle, the less likely the particle will pass to the heart.

Small sample sizes, intragroup variability and the acute nature of the study precluded robust statistical analysis or toxicity assessment. It appears as though treatment-related changes in all groups were reversible with 36 micron particle causing slightly less pulmonary damage.

5.3.4 In vivo imaging of cGMPs to look at biodistribution

Although ex vivo biodistribution is the standard for biodistribution studies in mice, in vivo imaging for biodistribution studies would offer many benefits for scientists attempting to develop drug delivery systems. Fewer mice would be needed for each iterative experiment with changes in the formulation. Additionally, the same mouse can be studied over the course of treatment to determine how particles are clearing the body and the mouse to mouse biodistribution variation can be studied with the same dose.
The ETTMP and PEGDA formulation used for our cGMP experiments was optimized to allow for very high loadings of nanoparticles in the aqueous phase before phase separation will occur. We have been able to formulate cGMPs with up to 33% (w/w) nanoparticles by dry weight of the cGMPs using our new formulation. This is a five-fold improvement over the previous formulations used with smaller dithiol linkers. We loaded the similar nanoparticles, containing the long wavelength dye used in the one week ex vivo studies, into cGMPs at 5% (w/w) NP by dry cGMP mass. This was a little more than 3 times more loading than what was

Figure 5.6. Proof of concept experiment showing the ability for in vivo imaging of the biodistribution of cGMPs of different sizes without euthanizing animals 30 minutes post dose. All animals had autofluorescence in the fat below the waistline. a) Small 12 μm cGMP-IV particles tended to accumulate in the liver as well as the lung. b) 37 μm cGMP-IV particles had stronger fluorescence in the lungs and less fluorescence in the liver. c) 66 μm cGMP-IV particles had a lot of fluorescence in the lungs and almost no fluorescence in the liver. This trend seems very consistent with our one week in vivo results. Scale bar is 100 μm.
used for the one week *ex vivo* studies. At this concentration we could dose particles at 67 mg/kg and image the particles *in vivo* in the mice at 30 minutes post dose, which is shown in Figure 5.6. By imaging the mice through their shaved chest, we could distinguish the difference in biodistribution between very small cGMPs and much larger cGMPs. This feasibility study was
only performed on two mice per group out to 30 minutes, but it shows the potential of being able to track the biodistribution of cGMPs within living mice. It is clear from the figure that the smaller particles target the liver and spleen of the mouse at much higher concentrations than the larger particles do, confirming our ex vivo results. Interestingly, it is only possibly to see half of the lungs using this technique through the shaved chest. This is due to the heart blocking the other side of the lungs. The long wavelength light is unable to penetrate into the lungs through the heart.

At much higher nanoparticle concentrations in the cGMPs, around 15% (w/w), it is possible to see the fluorescence in the lungs from all sides of the mouse, even looking through the mouse hair. This is shown in Figure 5.7. We believe that this pilot study shows that deep tissue in vivo biodistribution is possible, using cGMPs with high loadings of imaging nanoparticles, without the need for euthanizing mice at multiple time points.

5.4 Conclusions

In this chapter we have described two methods for making composite gel microparticles (cGMPs) for controlled delivery into the lungs of mice by venous injection and filtration: shear emulsification, and microfluidic droplet generation. Drops between 12 and 100 microns could be produced. The standard deviation of the smaller drops (36 μm) was 14% of the mean, and increased to 32% for the larger 97 μm drop. Microfluidics produced uniform drops with 91 μm mean sizes with standard deviations of only 7%. Nanoparticles could be encapsulated in the drops at a mass loading of 33% (w/w) (dry basis). In this study we encapsulated a long wavelength fluorescent dye in the nanoparticles to image fate and clearance. In our continuing studies camptothecin prodrugs will be encapsulated to treat non-small cell lung cancer. The
aqueous phase of the drop contained a PEG macromer pair that gelled by Michael addition reaction between a vinyl and thiol groups.

Three fate studies were conducted: a twelve week study, a more detailed one week study, and a 30 minute study to test *in vivo* whole animal imaging with a longer wavelength fluorophore. *Ex vivo* fluorescence measurements on organs was used to evaluate cGMP concentrations. The major conclusions were:

**12 week study:** cGMPs of 50 μm were captured selectively in the lungs and cleared to the liver and spleen. In all studies the cGMPs were uniformly distributed throughout the entire pulmonary tissue. In contrast, directly injected nanoparticles of 150 nm circulated with only a minor accumulation in the lungs and accumulation in the liver and spleen. The 50 μm particles cleared from the lungs in 7 weeks. Residual fluorescence after 7 weeks resulted from alveolar macrophage uptake of the degraded gel particles and encapsulated fluorescent nanoparticles, within the macrophages, remaining in the lungs.

**1 week study:** The 36 μm cGMPs accumulated in the lungs in the first 30 minutes, but were cleared from the lungs to the spleen with a half time of 42 hours. This is similar to clearance profiles that have been observed with 30 μm PEG microparticles previously.\(^\text{14}\) Larger 91 and 97 μm cGMPs had a longer retention in the lungs with 50% of the fluorescence remaining after 168 hrs. The 91 μm cGMPs were prepared to eliminate a population of smaller satellite droplets, since preliminary studies with 12 μm cGMPs showed that smaller particles, which can avoid filtration in the lungs may be captured in coronary arteries (Figure 5.5b), which is to be avoided. There was no evidence of the satellite particles affecting biodistribution, clearance or pathology for these larger particles.
All cGMPs produced localized minimal-to-mild necrosis, perivascular neutrophilic cuffs 2-10 cell layers thick, and increased neutrophil numbers around individual cGMP particles. The level of severity around larger particles was slightly greater than around the smaller particles, but at equal dosing, there were 20 times more small particles injected than large particles. The initial response to the captured particles resolved by day 7 and at that time point none of the mice demonstrated hemorrhage or necrosis. All groups had similar, mildly elevated macrophage scores, and similar low neutrophil scores.

Whole animal in vivo imaging study: Using higher loadings of the long wavelength fluorophore EtTP-5 in a 67 mg/kg dose of cGMP with 33% (w/w) nanoparticle loading, it was possible to directly observe accumulation of cGMPs in the animal (Figure 5.6). In this test, 12 μm cGMPs accumulated significantly in the liver; the 37 μm cGMPs localized primarily in the lungs with some fluorescence observed in the liver, and the 66 μm particles had strong lung fluorescence and fluorescence in the liver that was comparable to the control animal. This indicates that live animal imaging may be an effective way to follow clearance using fewer animals with greater precision than is achieved with ex vivo assays.

In summary, the approach to encapsulate nanoparticles in larger gel microparticles appears a promising route for targeting therapeutics to the lung. The nanoparticle provides the drug release profile desired, and the clearance of the PEG gel particle occurs at longer time scales. The observation that the mice lungs have returned to normal states by day seven indicates that a weekly injection regimen could be followed. Faster clearance of the PEG gel network can be achieved using sacrificial lactide linkages as we have discussed previously.\textsuperscript{15} The next stage of our work involves the delivery of camptothecin from nanoparticles encapsulated in these cGMPs. Camptothecin is known to be an effective anti-cancer agent, but suffers from severe off-
site dose-limiting toxicity. With the successful dosing of 0.9 mg of cGMPs and our ability to load 40% nanoparticles in cGMPs, we believe this will provide a promising platform for camptothecin delivery.

5.5 References


Chapter 6: Summary and Recommendations for Future Work

6.1 Thesis Summary

The focus of this thesis was on the development of a microfluidic device for production of drug delivery vehicles targeting the capillary bed of the lungs.

In Chapter 2, we introduced a novel 'off-the-shelf' microcapillary device that could be fabricated in minutes and generate uniform emulsions. We also showed the versatility of this device for analyzing the behavior of surfactants at the interface during microfluidic droplet generation. This allowed us to prove that dynamic interfacial tension could be tuned by adjustment of the device geometry through tip separation.

In Chapter 3, we discussed how this simple microfluidic device could be used to generate uniform, complex emulsions and particles. We discussed qualitative aspects of device operation and different design criteria including collection tip geometry, surfactant choice, and fluid properties. We also discussed the issues with generating red blood cell sized droplets using a microfluidic device. Syringe pump motor fluctuations necessitated the use of a pressure pump to generate truly stable small microfluidic droplets. Finally, we talked about how to incorporate a Michael addition polymerization into a microfluidic device to continuously run the device and generate hydrogel microparticles.

In Chapter 4 we discussed the techniques to fabricate and characterize hydrogel microparticles for drug delivery applications. First we talked about generating large quantities of material by bulk emulsification and then how more uniform particles could be made by
microfluidics. We also discussed methods to characterize the particles including measuring the size and mechanical properties. We developed methods for modulus measurements of particles and had the most success using osmotic compression. These characterization techniques were used to look at the storage and handling of cGMPs before use in *in vivo* studies.

In Chapter 5, we discussed the *in vivo* performance of our hydrogel microparticles in mice. We showed that small particles clear the lungs in less than one week, while large particles will slowly clear with a half-life around 7 weeks. We showed that microfluidics can create the best combination of large particles without satellites to avoid off-target drug side effects. We followed up these experiments with pathology of the lung tissue to understand the long term impact of our hydrogel particles in mice. We found that the mouse lungs returned to normal in a period of one week and that macrophages in the lungs were engulfing hydrogel material. Finally in a pilot study we showed that formulating our cGMPs with high loadings of nanoparticles allowed us to visualize the biodistribution *in vivo* through the mouse hair due to the strong fluorescence of the formulated cGMPs.

### 6.2 Recommendations for future work

#### 6.2.1 Development of a surfactant analysis technique using microfluidic device

The concept of adjusting the tip separation on a microfluidic device in order to study the dynamic interfacial tension was introduced using our 'off-the-shelf' micro capillary device. To this point, most interfacial tension studies of microfluidic droplet generation involve running different devices with different surfactant concentrations to study change in interfacial tension. But this method runs into issues including device to device variability and interfacial surfactant saturation due to geometry effects.
Typically devices produced using PDMS stamping techniques can be made with reproducible dimensions, but devices with axisymmetric droplet generation, such as glass capillary devices, are hard to fabricate identically. Therefore, slight differences in tip separation and orifice size could have large impacts on the reproducibility of exact droplet generation conditions in each device. Our device can be used in all experiments by taking it apart and cleaning it thoroughly in between. More importantly, the device can study the interfacial tension of tip separation as opposed to different total surfactant concentrations.

The other issue with tuning of total surfactant concentration is the saturation of the microfluidic interface. During microfluidic droplet generation, surfactant can absorb and be concentrated at the tip of the break up zone due to shear by the continuous phase. This phenomenon is typically seen in tipstreaming regimes of microfluidic droplet generation. In high surfactant concentrations, there may not be an observable difference in the dynamic interfacial tension between two different tests due to saturation of surfactant at the interface. Only so much surfactant can absorb at the interface, and it may not be clear where that point is. As well, it is unlikely that the dynamic interfacial tension scales linearly with bulk surfactant concentration, so it would be difficult to indirectly study the dynamic interfacial tension by this method.

There are a number of interesting studies that can be done in order to study the dynamic interfacial tension in microfluidic droplet generation. Fundamentally, it would be interesting to compare how different surfactants change the interfacial tension as a function of tip separation by looking at droplet break-up transitions. Using a simple water in mineral oil system, similar surfactants, such as the Span® series of surfactants can be loaded into the continuous phase at differing concentrations and droplets can be produced at different tip separation distances. The
distance at which the dripping to jetting transition can be used as an indication to how quickly or slowly a surfactant changes the dynamic interfacial tension. This value would have direct correlation with the surfactants ability to diffuse through the oil and absorb to the interface. This analytical method would have implications in the understanding of surfactant dynamics and could have a broad impact in emulsion generation for many different industrial applications.\textsuperscript{4}

6.2.2 Continued in vivo studies of hydrogel microparticles in mice

After performing the initial long term studies of hydrogel particles being injected into mouse lungs and finding conditions to ensure survival and sufficient drug loading for drug delivery applications, it is important to move further in development and really build a good pharmacokinetic model. It is also fundamentally important to move the studies into mice that have compromised lung function due to the presence of real lung cancer.

We have a good idea of how long the microparticles are sticking around in the lungs of mice and what kind of damage they are doing, but it is still unclear what will happen when anti-cancer drug nanoparticles are introduced into the particles. It will be important to develop good methods for tracking both the microparticle and nanoparticle biodistribution in the mice and tracking drug concentrations in different organs over the short and long term time frames. It is unclear whether cancer drugs, such as camptothecin will burst release from the hydrogel upon lodging, or slowly release over the course of weeks. Understanding this behavior will allow us to develop a proper dosing strategy for injecting particles into cancer mice to achieve appropriate drug levels to ensure efficacy.\textsuperscript{5} Controlled experiments using stable dyes or metal tracer molecules will be necessary for dual loading with the drug to understand better the fate of all components in the drug delivery system. It will be important to develop careful analytical
techniques to analyze small quantities of drug and accurately track the biodistribution in the mice. These studies will be fundamentally important for the continued development of the drug delivery vehicle.

There are considerable questions regarding how well mice with compromised lung function will handle dosing of hydrogel particles into the lung capillary bed. Due to their large volume when swollen in solution, relatively low masses of cGMPs generate large volumes of material when compared to hard spheres such as polystyrene. Experiments done using polystyrene beads showed that the deterioration of lung function is correlated very well to the volume of material injected into the lungs, regardless of particle size. This explained why our low density cGMPs could cause such adverse effects in very healthy mice at relatively low doses, creating the need for very highly drug-loaded cGMPs. We hypothesize that mice with lung cancer causing compromised immune systems and lung function may have a very hard time dealing with the injection of our cGMPs. The path forward with this set of experiments is unknown, but a starting dose of cGMPs into the mice should be at least at a tenfold lower dose than what causes severe lung issues in healthy mice. Somewhere around 4 mg/kg would probably be a good starting point for mouse studies. Data regarding doses that caused death in mice can be found in the summary of in vivo experiment results in Appendix C.

6.3 References


Appendix A: Detailed Description of Glass Capillary Device Building

A.1 Introduction

Fabrication of glass capillary devices requires attention to small details that can sometimes be left out of publications. In this section, I felt it was important to go over every detail of building glass capillary devices to help future users of the device.

A.2 Glass Capillary Device Building

A.2.1 Selection of glass capillaries

One of the key aspects of glass capillary device building is the axisymmetric alignment of glass capillaries within a larger glass capillary. This is accomplished by using a square glass capillary and insertion of round capillaries within it. It is important that there is a difference in size between the inside diameter (ID) of the square capillary and the outside diameter (OD) of the round capillary. Typically, the ID of the square capillary should be at least 30-50 microns larger than the OD of the round capillary to ensure proper fitting of the capillary despite diameter errors during production of the capillaries. Traditionally, glass capillaries used for fabrication had to be special ordered 1.05 mm ID square capillaries and stock 1.00 mm OD round capillaries. However, by searching the suppliers, we were able to find stock sized square capillaries with a 0.9 mm ID (8290-050) and stock shaped round capillaries with a 0.87 mm OD (CV7087) sold by Vitrocom (Mountain Lakes, NJ). This became a critical source for purchasing inexpensive capillaries for device production.
A.2.2 Preparing glass capillaries for microfluidics

In order to create orifices for microfluidic droplet generation in the glass capillary microfluidic devices, the round capillaries must be treated by either pipette pulling or end-flaming.

Pulling pipettes can be done either by using a puller or by hand. Pipette pullers offer excellent control over the size and axisymmetry of the capillaries. The only drawback is that the capillaries formed are very fragile and can break very easily. An example procedure for pulling and scoring capillaries is described using a Sutter P-2000 (Sutter Instrument, Novato, CA) puller. Enter the pulling program into the instrument. It is good to experiment with different pulling parameters to find settings that pull very long gradual tips. An example single step program to generate long tapered tips has the settings: Heat: 450, Filament: 5, Velocity: 50, Delay: 225, Pull: 150. Place the pipette into puller securely and press “Pull”. Remove pipette from device and check for a good tip with axisymmetry. If the tip is not symmetric, place some tape in the holder to adjust the location of the laser on the capillaries. After tips are pulled, bring to microscope to check location of 100 micron diameter segment on tapered end of tip. Score and break the tips using the scoring stone (CTS, Sutter Instruments, Novato, CA) around the 100 micron diameter section. Score the glass gently then rinse the glass with methanol. Break the tip by pressing it down on to a glass slide while still wet with methanol. Check tips under microscope to be sure of a clean right angle break. Store tips on sticky wax with the not-pulled end in the wax.

Pulling can also be done by hand using a torch. Turn a butane torch on to the lowest setting that still produces a flame. Grasp two ends of a round glass capillary and hold the middle over the flame a few seconds until it is molten. Remove the molten center from the flame and
gradually and evenly pull outward to draw out the molten part of the capillary. If done carefully, a thick-walled axisymmetric pulled glass capillary with less probability of breaking can be formed. Typically the 300mm capillary will pull to 600mm in length if done properly. The pulled capillary will need to be scored in the center to create two tapered capillaries. These tapered capillaries can be scored and broken the same way as above to reach the appropriate orifice size.

For some applications it is also important to chemically functionalize the glass with silanes in order to avoid wetting issues during microfluidic droplet generation. A simple silanization treatment was developed for this purpose. In a vial, place 8 mL hexadecane, 1.98 mL chloroform, 0.02 mL silanizing agent (methyltrichlorosilane). Insert capillary tubes and make sure that the solution fills the insides. Allow tubes to sit for 20 min. Remove glass from solution and rinse with hexadecane. Finally rinse with acetone, ethanol, then with water.

A.3 Detailed Building of Microfluidic Device

In order to build a simple 'off the shelf' microfluidic device it is necessary to have the following pieces. Two 100 mm round capillaries, one 100 mm square capillary, two chromatography tee assemblies (P-713, IDEX Health & Science, Oak Harbor, WA), 1/16" ID and 1/32" ID Tygon tubing (E-3603, Saint-Cobain, Malvern, PA) and Intramedic® polyethylene medical tubing (BD, Franklin Lakes, NJ).

Each capillary will be fitted with Tygon tubing in order to seal it in to the tee assembly. Typically for the round capillaries you can use both 1/16" ID and 1/32" ID Tygon tubing to seal it in to the device. The 1/16" ID tubing will allow the capillary to slide after tightening the tee assembly, giving the operator the ability to adjust the orifice separation. The 1/32" ID tubing will
create a tighter seal but will fix the round capillary in place. Often I will fix the collection capillary in place and allow the injection capillary to be adjustable.

It is easiest to build the device in stages and clean each stage before full assembly. Starting with the center square capillary, we can place the connector on to it. First take two yellow ferrules and place them on the ends of two 1 cm pieces of 1/16" ID Tygon tubing. Slide one tubing/ferrule piece on to the end of the square capillary with the flat end of the ferrule flush with the tubing and the end of the square glass capillary. Next adjust the ends of the ferrule/tubing/capillary assembly so that the tubing is about 1mm away from the glass capillary edge and the flat end of the ferrule. This is shown in Figure A.1. From the other end of the capillary, feed on the screw along with the screw for the other side facing towards the other end of the capillary. Then insert the second tubing/ferrule component on the other end of the square capillary and perform the alignment step to move the tubing away from the edge of the glass capillary. The entire square capillary assembly can now be rinsed with ethanol and nitrogen dried to remove any dust.
Attach the tubing and ferrules to the round injection and collection tubes, then rinse the tubes with ethanol and dry with nitrogen. Attach the square capillary to the inside of the PEEK tees. Slide the round tubes in from the outside of the tees and through the square capillary. Secure them using screws as well. Finally attach 1/16" tubing to the top of the device in order to interface with the microfluidic polyethylene tubing.

The syringes are attached to the device using the Intramedics® polyethylene tubing fitted on 27 gauge needles. The tubing is fitted on to the device by sliding down inside the 1/16" Tygon tubing and being tightened in by the screws. The injection syringe tubing is slid inside 1/32" Tygon tubing. The other end of the 1/32" tubing is slide on to the injection capillary end sticking out of the tee. At this point, the syringes can be filled and the device can be run continuously.
Appendix B: Hydrophobic Dye Loading of Model PMMA Nanoparticles

B.1 Introduction

In order to track the production of gel microparticles and to image the particles in vivo, PMMA nanospheres were dyed with EtTP-5 and put into the aqueous phase. Approximately 250nm PMMA nanoparticles were provided by Arkema (King of Prussia, PA) as a stock solution at 50 % (w/w) particles. The EtTP-5 was obtained through collaboration with the Anthony group at Kentucky.1

B.2 Materials and Methods

The process for imbibing the fluorescent dye described here was adapted from a patent by Niechwiadowicz et al.2 A schematic of this process is shown in Figure B.1(a). A stock solution of PMMA nanoparticles was diluted down to a usable concentration, normally less than 25 % (w/w) solids. If the concentration is too high, the particles will dissolve and reform in a large clump during the dying process due to solvent swelling. EtTP-5 is dissolved in tetrahydrofuran (THF) (Sigma) at 8 mg/mL. This concentration is merely a theoretically calculated amount based on 2.3 % (w/w) dye loading of the nanoparticle core, assuming total loading, but a large portion of the EtTP-5 will end up crashing out of the solution. The THF dye solution is then rapidly mixed with the aqueous PMMA solution. It is important to mix the solutions well, as locally high solvent concentrations will cause the nanoparticles to clump into large aggregates. However, you must add the solvent quickly because if the THF/dye solution is added dropwise, the solvent quality drops very rapidly causing the EtTP-5 to crystallize out into the water and not partition into the nanoparticle core.
After the solutions have been mixed together, the vial is placed onto a rotary evaporator and the THF is pulled off over the course of a half hour at 100 torr. As the THF leaves the solution, some of the EtTP-5 will crystallize out into the water and the rest will partition into the nanoparticle cores and turn the solution a greenish color. The final step is a 5 micron filtration to remove large particles that could clog the microfluidic device. If we are attempting to dye high nanoparticle concentration solutions, the filtration step may be done while preparing the aqueous phase and after the PEG has been added to dilute the particles in order to avoid caking of the filter.

Figure B.1. a) Schematic of the dying process for the PMMA nanoparticles. b) Dynamic light scattering size graph for nanoparticles. c) Fluorescence measurements of nanoparticles. d) Fluorescent confocal image of nanoparticles in water.
B.3 Results and Discussion

To ensure that the size of the particles was not greatly affected by the addition and removal of THF, dynamic light scattering was done before and after the dying process. Figure B.1(b) shows a graph of the size of particles against the intensity of the signal. As can be seen in the graph, both the control particles that were taken from the stock solution and the dyed particles had essentially the same size distribution and intensity at the same concentration. To quantitatively show that there was EtTP-5 inside the particles, we compared the fluorescence intensity of the stock solution to that of the dyed particles. The solutions were diluted down to 0.005 % (w/w) nanoparticles and placed into quartz cuvettes. As well, the dyed particles were filtered to ensure no crystallized EtTP-5 was in the solution. The particles were excited at 460nm and the emission spectrum was collected between 500nm and 800nm. EtTP-5 is known to have two characteristic peaks at 635nm and 670nm.\(^1\) As can be seen in Figure B.1(c), there is no fluorescence coming from the control particles. However we see large characteristic peaks from the EtTP-5 within the dyed nanoparticles. We also noticed that the intensity of the particles increased with concentration. Finally, the nanoparticles were imaged under a confocal microscope and produced a fluorescence image that was detectable. This image is shown in Figure B.1(d).
Using the PMMA nanoparticles as a beacon due to their stability and resistance to free radicals, we developed a method to produce 20 micron GMP powders containing the fluorescent nanoparticles as shown in Figure B.2.

B.4 References


Appendix C: Supplementary Data for *In Vivo* Mouse Studies

C.1 Introduction

The purpose of this appendix section is to organize and supplement the thesis by including extra information regarding tests run on mice involving our cGMPs. This appendix is broken up into two different sections, one containing all of the preliminary data for determining the dosing conditions for the mouse experiments performed in Chapter 5 and the other is for the pathology reports generated during the studies.

C.2 Preliminary Data Regarding *In Vivo* Experiments

Below is a table documenting all of the preliminary *in vivo* experiments run on mice using our cGMPs in order to determine proper dosing and particle sizing to ensure survival of the studied groups.
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<th># mice injected</th>
<th>GMP Name</th>
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<th>PEGSH</th>
<th>PEG 400</th>
<th>PEG 600</th>
<th>DTT</th>
<th>TEA</th>
<th>Dye NP</th>
<th>Dry NP loading</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.2 B Group</td>
<td>2 GMP 28</td>
<td>27.5</td>
<td>15</td>
<td>12.5</td>
<td>10</td>
<td>10.2</td>
<td>0.271 LW2/ETTP5(20%)</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.4 B Group</td>
<td>2 GMP 7</td>
<td>31.5</td>
<td>20</td>
<td>11.5</td>
<td>16.6</td>
<td>10.1</td>
<td>0.243</td>
<td>LW2</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 5</td>
<td>Long Term 3</td>
<td>LT 3 Group</td>
<td>6 GMP 31</td>
<td>33</td>
<td>18</td>
<td>15</td>
<td>10</td>
<td>6.5</td>
<td>0.155 ETTP5</td>
<td>40-60</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Experiment 6</td>
<td>DTT Large Split</td>
<td>DTT Split Small</td>
<td>4 GMP 32 Small</td>
<td>32.3</td>
<td>32.3</td>
<td>6.9 AA</td>
<td>1.4</td>
<td>0.034 ETTP5</td>
<td>10-30</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>DTT Split Large</td>
<td>4 GMP 32 Large</td>
<td>32.3</td>
<td>32.3</td>
<td>6.9 AA</td>
<td>1.4</td>
<td>0.034 ETTP5</td>
<td>40-60</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Experiment 7</td>
<td>DTT Small GMPs</td>
<td>200UL Small</td>
<td>4 GMP 35</td>
<td>32.3</td>
<td>32.3</td>
<td>6.1 AA</td>
<td>1.4</td>
<td>0.035 ETTP5</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 UL Small</td>
<td>4 GMP 35</td>
<td>32.3</td>
<td>32.3</td>
<td>6.1 AA</td>
<td>1.4</td>
<td>0.035 ETTP5</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Experiment 8</td>
<td>Redosed GMP Inject</td>
<td>Control</td>
<td>7 GMP 30</td>
<td>26</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>10.2</td>
<td>0.282 ETTP5</td>
<td>30</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>DTT Processed</td>
<td>8 GMP 30</td>
<td>26</td>
<td>14</td>
<td>12</td>
<td>5mg(1:1)</td>
<td>10</td>
<td>10.2</td>
<td>0.282 ETTP5</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment Number</td>
<td>Injection Volume</td>
<td>Mouse Name</td>
<td>Injection Dry Mass (mg)</td>
<td>NP Dose dry (mg)</td>
<td>In lungs?</td>
<td>Intensity in the lungs</td>
<td>Survival Time</td>
<td>Survival percent</td>
<td>Euthanized</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td>200 µL</td>
<td>Mouse 4</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>low</td>
<td>lived</td>
<td>100 %</td>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>Mouse 5</td>
<td>4</td>
<td>0.204819277</td>
<td>yes</td>
<td>bright</td>
<td>24 hours</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td>100 µL</td>
<td>12 Micron 1</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Some</td>
<td>5 hours</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size effect</td>
<td>200 µL</td>
<td>12 Micron 2</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Some</td>
<td>5 hours</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>12 Micron double</td>
<td>4</td>
<td>0.204819277</td>
<td>yes</td>
<td>Some</td>
<td>2 min</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>100 µL</td>
<td>37 Micron 1</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Moderate</td>
<td>Lived</td>
<td>100 %</td>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>37 Micron 2</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Moderate</td>
<td>12 hours</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>66 micron 1</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Bright</td>
<td>90 min</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>100 µL</td>
<td>66 micron 2</td>
<td>2</td>
<td>0.102409639</td>
<td>yes</td>
<td>Bright</td>
<td>24 hours</td>
<td>0 %</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td>200 µL</td>
<td>LT Mouse 1</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>Lived</td>
<td>100 %</td>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long term 1</td>
<td>200 µL</td>
<td>LT Mouse 2</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>Lived</td>
<td>100 %</td>
<td>3 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>LT Mouse 3</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>Lived</td>
<td>100 %</td>
<td>4 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 4</strong></td>
<td>200 µL</td>
<td>1 mg Group</td>
<td>1</td>
<td>0.281767956</td>
<td>yes</td>
<td>Some</td>
<td>died in 4 hours</td>
<td>33 %</td>
<td>2 at 6 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size Study</td>
<td>100 µL</td>
<td>0.5 mg Group</td>
<td>0.5</td>
<td>0.140883979</td>
<td>yes</td>
<td>Some</td>
<td>died in 4 hours</td>
<td>55 %</td>
<td>1 at 24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>182 A Group</td>
<td>0</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>Lived</td>
<td>100 %</td>
<td>1 at 24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>384 A Group</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>Lived</td>
<td>100 %</td>
<td>1 at 24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>182 B Group</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Some</td>
<td>death at 12 hour</td>
<td>50 %</td>
<td>1 at 24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µL</td>
<td>384 B Group</td>
<td>1</td>
<td>0.242788462</td>
<td>yes</td>
<td>Bright</td>
<td>death at 12 hour</td>
<td>50 %</td>
<td>1 at 24 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 5</strong></td>
<td>200 µL</td>
<td>LT 3 Group</td>
<td>1.26</td>
<td>0.207341772</td>
<td>yes</td>
<td>Bright</td>
<td>died at 48 hour</td>
<td>83 %</td>
<td>1 at 6 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long Term 8</td>
<td>200 µL</td>
<td>DTT Split Small</td>
<td>1.34</td>
<td>0.046206697</td>
<td>yes</td>
<td>Low</td>
<td>died at 48 hours</td>
<td>75 %</td>
<td>1 at 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 7</strong></td>
<td>200 µL</td>
<td>DTT Split Large</td>
<td>1.08</td>
<td>0.037241379</td>
<td>yes</td>
<td>Low</td>
<td>Lived</td>
<td>100 %</td>
<td>1 at 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTT Small GMPs</td>
<td>200 µL</td>
<td>200 µL Small</td>
<td>1.16</td>
<td>0.04080402</td>
<td>n/a</td>
<td>n/a</td>
<td>1 death at 1 hour</td>
<td>75 %</td>
<td>1 at 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>100 µL Small</td>
<td>0.58</td>
<td>0.02040201</td>
<td>n/a</td>
<td>n/a</td>
<td>Lived</td>
<td>100 %</td>
<td>1 at 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 8</strong></td>
<td>125 µL</td>
<td>Control</td>
<td>0.5155</td>
<td>0.145251381</td>
<td>n/a</td>
<td>n/a</td>
<td>1 at 24 hr</td>
<td>86 %</td>
<td>50 N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reslosed GMP</td>
<td>125 µL</td>
<td>DTT Post Group</td>
<td>0.5155</td>
<td>0.145251381</td>
<td>n/a</td>
<td>n/a</td>
<td>2 died at injection 1</td>
<td>62.5 %</td>
<td>25 N</td>
<td></td>
<td></td>
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</table>
As shown in the tables, there were a large number of deaths associated with injection of hydrogel microparticles into mice at first. What was noticed is that given the same mass of dose, smaller particle sizes seemed to be causing more damage to the lungs of mice.

C.3 Pathology Reports on Mouse Injection Studies
Study Information:
Study object: Lung cancer research goal is to characterize/ find chemotherapeutic agent vehicle that will allow for prolonged, localized deposition of drug in pulmonary tissue using a degradable polyethylene glycol vehicle.
Experimental Design: 35 micron diameter PEG particles were injected intravenously at doses of 1.3 mg or 2.5 mg per 125 microliter dose (two mice at each dose).
History/clinical signs: Following dosing, rats had respiratory distress, and variable serosanguinous to hemorrhagic nasal discharge with recovery within several hours. During that time, imaging indicated drug deposition initially in thoracic cavity (lung) with some later cranial abdomen (possibly liver and/or spleen). In a prior experiment, dosed rats had similar respiratory and nasal signs with death within 90 minutes of compound administration.
Necropsy objective: Bryan is interested in learning pathophysiology of previous experiment deaths.
Necropsy processing plan: Surveillance; routine necropsy, all thoracic and visceral organs saved in 10% neutral buffered formalin; initial processing, examination, report on target tissues (lung, heart, liver, kidney spleen); process/examine additional tissues as needed.

Histologic Descriptions:
Rat #01
Lung: There were peripheral hemorrhagic and/or fibrinous foci in all lobes except one; which was completely filled with blood and fibrin. Hemorrhagic areas contain vessels and alveolar spaces plugged with irregular ovoid to round gray to amphophilic thrombi that had a moth-eaten, vacuolated or ground glass appearance. Affected vessels ranged from medium to small arteries down to small end-arteriolar pulmonary branches. Some vessels were lined by degenerate and/or necrotic endothelial cells and plugged with fibrin and neutrophils. Alveolar spaces in hemorrhagic foci contain alveolar macrophages with increased grey brown cytoplasmic material and alveoli variably filled with blood, fibrin, neutrophils and lymphocytes.
Heart: In the right ventricular free wall there were multifocal areas of myocardial inflammation and degeneration adjacent to small arterioles with transmural degeneration and multifocal cellular necrosis. Inflamed myocardial foci contain degenerate and necrotic myocytes with loss of striations,
cell swelling, cytoplasmic vacuoles and necrotic debris. The extracellular matrix of these areas contain macrophages, neutrophils, edema and small amounts of necrotic cell debris.

**Kidney:** No noteworthy findings

**Liver:** No noteworthy findings

**Spleen:** No noteworthy findings

**Rat #02**

**Lung:** There were variably severe peripheral hemorrhagic and/or fibrinous foci in all lobes with one more severe than the rest. Hemorrhagic areas contain vessels and alveolar spaces plugged with irregular ovoid to round gray to amphophilic particles that have a moth-eaten, vacuolated or ground glass appearance. Some vessels were lined by degenerate and/or necrotic endothelial cells and plugged with fibrin and neutrophils. Alveolar spaces in hemorrhagic foci contain alveolar macrophages with increased grey brown cytoplasmic material and alveoli variably filled with blood, fibrin, neutrophils and lymphocytes.

**Heart:** No noteworthy findings

**Kidney:** No noteworthy findings

**Liver:** No noteworthy findings

**Spleen:** There was minimal extramedullary hematopoiesis (incidental finding).

**Rat #S1**

**Lung:** There were findings similar to rats 01 and 02 with a greater incidence and severity of findings.

**Heart:** No noteworthy findings

**Kidney:** No noteworthy findings

**Liver:** No noteworthy findings

**Thymus:** No noteworthy findings

**Spleen:** There was minimal extramedullary hematopoiesis (incidental finding).

**Rat #S2**

**Lung:** There were findings similar to rats 01 and 02 with a greater incidence and severity of findings.

**Heart:** No noteworthy findings

**Kidney:** No noteworthy findings

**Liver:** There were several infarcts. Smaller ones had necrotic and or apoptotic centers and vacuolation of adjacent hepatocytes while a larger one had a center of coagulative necrosis.

**Spleen:** There was mild extramedullary hematopoiesis (incidental finding).

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**Diagnosis:**

**Lungs:** Multifocal, mild to severe, acute thrombosis and infarction with foreign body material

**Heart:** Minimal, multifocal, acute myocardial degeneration and necrosis and minimal focal vasculitis

**Conclusions:**

**Lung-**

Particulate matter caused thrombosis (obstruction) of medium to small arteries and arterioles with subsequent pulmonary hemorrhage. In this study, the incidence and severity of thrombosis were not severe enough to cause death as they usually occluded less than half of the vasculature. Because there were no deaths in this experiment, the cause of death in the prior similar experiment(s) can only be extrapolated. In cases where thrombi or emboli acutely occlude 60% or more of the pulmonary circulation, pure right-sided heart failure (cor pulmonale), occurs resulting in diminished blood flow and sudden death. In such cases, the right ventricle is variably dilated without hypertrophy.
Embolic obstruction of medium sized arteries with subsequent vascular rupture can cause pulmonary hemorrhage but usually does not cause pulmonary infarction (parenchymal necrosis due to loss of blood supply). This is because the lungs have dual blood supply with perfusion of tissue despite thrombi. Unlike medium sized arteries, embolic obstruction of small end-arteriolar pulmonary branches resulting in hemorrhage is likely to cause infarction and necrosis. In these four rats, the hemorrhage distribution patterns indicated that there was thrombosis and obstruction of medium to small arteries and/or terminal arterioles.

Heart-
The heart findings were short in duration and likely related to treatment. Though there were no overt histomorphological signs of foreign body material in cardiac arteries, the fact that fluorescent imaging analysis detected fluorescence first in the lung and then later in visceral organs suggests some component of the treatment passed from the right heart to the left. While pulmonary thrombosis is usually not a severe or life threatening event, thrombosis of cardiac or central nervous system vasculature can be.
Study Information:
Study object: Goal of previous study was to examine nanoparticle distribution. Particles were labeled with a fluorophore and a MRI contrast. This study objective was to document particle distribution/concentration and characterize treatment-related findings over 12 weeks.
Experimental Design: Unknown
Signalment: See study objective
Necropsy processing plan: Unknown

Histologic Descriptions:
15-1012-2 (1 week)
Lung: The vast majority of the lung parenchyma was collapsed. Tissues were of good quality and well preserved. There is diffuse thickening of alveolar walls by dull eosinophilic material, small poorly defined gray to amphophilic material (moth-eaten, vacuolated or ground glass appearance), type 2 pneumocytes, macrophages, lymphocytes and lesser numbers of neutrophils. There are increased numbers of macrophages with vacuolated gray to brown pigmented cytoplasm and the above-mentioned material. The number and shape of endothelial lining pulmonary arteries and the thickness of the tunica media are within the range of normal for wild type C57 mice.
Heart: There were no noteworthy findings.

15-1012-1 (2 weeks)
Lung: Findings were similar to 1-week lung with the exception of slightly more foamy alveolar macrophages and a slight difference in the thickened alveolar wall tinctorial qualities. Instead of dull eosinophilic, more tan-purple.
Heart: There were no noteworthy findings.

15-1012-4 (4 weeks)
Lung: Findings were similar to 15-1012-1 lungs.
Heart: There were no noteworthy findings.
15-1012-6 (6 weeks)
Lung: Findings were similar to above animals with the exception of more pigmented macrophages. There were few scattered clumps of swollen macrophages containing clumps and particles of brown pigment.
Heart: There were no noteworthy findings.

15-1012-3 (8 weeks)
Lung: Findings were similar to 15-1012-6 with the exception of larger, more numerous pigmented macrophage clumps.
Heart: There were no noteworthy findings.

15-1012-5 (12 weeks)
Lung: Findings were similar to 15-1912-3 and 15-1012-6.
Heart: There were no noteworthy findings.

Diagnosis: Mild to moderate, diffuse alveolar wall thickening with multifocal inflammation, pigment deposits and small amounts of interstitial particulate matter.

Conclusions: While histopathology was not a sensitive indicator of persistence of particulate matter in this study, it was effective at detecting chronic findings associated with the initial pulmonary response to the foreign body emboli. There were no clear duration-related trends in the amount of persistent particulate matter or the severity of alveolar wall findings. This is could be due to variability in individual rodent response. While no cardiac findings were detected, it does not necessarily mean there wasn’t an initial cardiac effect. After four or more weeks, acute cardiac lesions may have resolved and been undetectable. There were no appreciable differences in vascular characteristics.

Future plans: Including control group mice for histopathology would greatly increase the strength of the analysis. The impact of the wide variability in individual mouse responses could be better understood with power analysis. Though histopathology did not detect morphologic cardiovascular changes, pulmonary changes similar to those seen here can be associated with changes in cardiovascular function (i.e. hypertension). If there is a sensitive modality to monitor blood pressure in a mouse, such data may better characterize the functional effects related to this treatment. To better characterize the incidence and severity of pulmonary fibrosis, trichrome lung staining on 3 or 4 of the treated mice would be informative.
Study Information:

**Study Objective:** The goals of the study were to assess particle deposition/concentration and evaluate histopathological findings in mouse lung and heart following intravenous administration of three different formulations of polyethylene glycol microparticles. The three particles included 90 μm uniform particles and 35 or 97 μm polydisperse microparticles labeled with a fluorophore. Live animal imaging was performed to compliment histopathological analysis.

**Experimental Design:** Studies were conducted in young, sexually mature male CD-1 mice. Animals were assigned to one of 4 dose groups (vehicle, 35, 90, 95 μm) and evaluated at 4 time points (30 min, 1, 3, or 7 days). Mice were administered vehicle or 0.9 mg particles intravenously and then underwent CT scan evaluation under anesthesia prior to necropsy. Lung and heart were collected at necropsy; fixed in 10% buffered formalin, embedded, sectioned, and stained with H&E. Heart tissues were evaluated only at the 3 day time point. Lung tissues were evaluated at 30 min, 1, 3, and 7 days for the incidence and severity of histopathological changes and the extent of particle deposition.

Histopathology:

**Scoring Criteria:**

**Heart:** Surveillance of multiple heart sections collected on day 3 following particle administration demonstrated no signs of pathological changes. As a result, no cardiac scoring criteria were established.

**Lung:** Scoring criteria were developed for pulmonary neutrophil and alveolar macrophage accumulation, hemorrhage, necrosis and particle size. Pulmonary scoring criteria were as follows:

- Hemorrhage: 0 = none, 1 = less than 10 small foci or a medium sized focus, 2 = 10 or more small, multiple medium foci or a large focus, 3 = up to but not including hemorrhage of entire lobe or 10 or more large foci, 4 = 10 or more large foci or entire lobes, 5 = multiple, entirely hemorrhagic lobes.

- Necrosis: 0 = none to background, 1 = minimal amounts, 2 = mild amounts, 3 = moderate amounts of necrosis.

- Neutrophils: 0 = none to background number, 1 = rare neutrophils around vessels forming 1-2 cell layer cuffs, 2 =
vessels rimmed by 4 to 6 layers of neutrophils, 3= 7 or more layers of perivascular neutrophils, 4= perivascular neutrophilic accumulations forming intra-aggregate bridges, 5= multi-lobar hemorrhage.

Macrophages: none to normal background, 1= approximately 5 or less macrophages per 600x field, 2= approximately 6-15 macrophages per 600x field, 3= approximately 16-40 macrophages per 600x field.

Particle size: 0= no particles or inflammation (necrosis, edema, fibrin, macrophages, neutrophils), 1= no free, non-cell bound particles but small amounts in macrophages, 2= minimal numbers of particles in macrophages less than 50 μm in size, 3= few free particles and mild numbers of particles ~50 to 80 μm in size, 4= when in large vessels 30 μm particles, and/or 80 to 200 μm microparticle aggregates in tissue, 5= amorphous, uniform sized, non-clumped intravascular particles ~100-150 μm

Lung Scores:

<table>
<thead>
<tr>
<th>Slide</th>
<th>Particle Treatment</th>
<th>Time</th>
<th>Hemorrhage Description</th>
<th>Hemorrhage Score</th>
<th>Neutrophil Description</th>
<th>Neutrophil Score</th>
<th>Alveolar Macrophage Description</th>
<th>Mac Score</th>
<th>Necrosis Description</th>
<th>Necrosis Score</th>
<th>Particle Size Description</th>
<th>Particle Size Score</th>
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<tbody>
<tr>
<td>1</td>
<td>35 micron poly</td>
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<td>2 layers around particles</td>
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<td>0</td>
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<td>when in large vessels, 30 microns, in tissues, up to 80 to 100s, 5/400x power filed</td>
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<td>min fibrin aggs</td>
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<td>min fibrin aggs</td>
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<td>2 large, 6 medium, diff small</td>
<td>4-7 layers</td>
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<td>mild in fibrin and neuts</td>
<td>vacuolated, moth-eaten ghosts of particles rimmed by fibrin, neuts, necrotic cell debris</td>
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<td>2 large, 6 medium</td>
<td>4-7 layers, + in caps</td>
<td>20+600</td>
<td>mild-mod in fibrin and away from vessels</td>
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<td>4l, 5 m, modify</td>
<td>4-7 layers, + in caps</td>
<td>15/600</td>
<td>mild near vessels</td>
<td>like 21 but some mixed cell aggs (func of cut)</td>
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<td>few in perivas histiocytic aggs</td>
<td>25/600</td>
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<td>inflamm, no particles, foamy alv mac</td>
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<td>inflamm, no particles, foamy alv mac</td>
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<td>few in eprivas histiocytic aggs</td>
<td>25/600</td>
<td>erythro</td>
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<td>histiocytic ags w/ few neuts</td>
<td>20/600</td>
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<td>no particles, smaller foci inflam, mostly macs, lymphos, scattered alv macs with clear vacuoles</td>
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<td>histiocytic ags w/ few neuts</td>
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<td>no particles, smaller foci inflam, mostly macs, lymphos, scattered alv macs with clear vacuoles</td>
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<td>histiocytic ags w/ few neuts</td>
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<td>slightly smaller than uniform, has vacuoles, not as uniform, 15 per 100x</td>
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<td>3-4 layers, ++vessels,</td>
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<td>slightly smaller than uniform, has vacuoles, not as uniform, 15 per 100x</td>
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<td>1 lobe, 9 large, 10+m, diff</td>
<td>7 layers</td>
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<td>In nonhemorrhagic areas, can not see particles, only aggregates of inflammation, alveolar mac = erythropago. In hemoragia areas, only inflam</td>
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<tr>
<td>56</td>
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</table>
Histopathologic Findings: Control tissues had no noteworthy findings.

- **30 min:** The most notable finding at this early time point was the presence of particles in the lungs of treated animals. While most all particles in the 90 and 97 μm groups were thromboemboli, a substantial number of 35 μm particle emboli were in patent (nonclotted) vessels and still ~35 μm. Inflammation, as assessed by neutrophil and macrophage accumulation, was generally mild and similar between the three particle groups. No necrosis was observed in any treatment groups. Pulmonary hemorrhage in the 35 μm and 90 μm groups had low incidence (one of four mice) and severity while the 97 μm group had high incidence (four of four mice) and variable severity (scores of 1-3).

- **Day 1:** By day 1, the most notable findings were the appearance of necrosis and enhanced neutrophil levels. There were perivascular neutrophilic cuffs 2 to 10 cell layers thick and increased numbers of enlarged, vacuolated alveolar and interstitial macrophages. The extent of the necrosis was slightly greater in the 90 and 97 μm groups compared to the 35 μm group. Minimal-to-mild multifocal necrosis (hypereosinophilia, karyorrhexis, karyolysis, cell debris) was centered on thromboemboli. Though no groups had detectable free microparticles in vessels or extracellular spaces, most macrophages in particle-treated groups contained increased amounts of cytoplasm and/or cytoplasmic vacuoles that resembled microparticle material.

- **Day 3:** By this time point, necrosis has largely resolved in particle-treated groups. Hemorrhage in 35 and 90 μm dose groups was less than at Day 1 but did continue to persist. Neutrophil numbers in the 35 and 90 μm dose groups were comparable to Day 1. Particulate matter was no longer discernable. Alveolar macrophages contained variable numbers of cytoplasmic vacuoles.

  Day three heart: There were no signs of particle treatment-related infarction, inflammation or necrosis.

- **Day 7:** Particle-treated mice no longer exhibited hemorrhage or necrosis. All dosed groups had similar, mildly increased macrophage scores. The neutrophil scores for the 35 μm group were similar to control levels while the scores for the 90 and 97 μm group were minimal. There was no discernable particulate matter in lung tissue.

**Discussion Points:**

- **Summary of results:**
  - There were no cardiac findings.
  - At 30 minutes, the larger particles (90 and 97 μm) caused more lung pathology (venous thromboemboli, hemorrhage, inflammation) than the smaller particles (35 μm) because they had greater potential to occlude microvasculature. The lower damage scores for the smallest particle emboli (35 μm) correlated with unclotted, patent vessels still containing free microparticles.
  - Treatment-related pulmonary changes scores were greatest at Day 1. While macrophage, neutrophil, necrosis and particle scores were comparable between the three treatment groups at this time, the 97 μm group has slightly higher hemorrhage scores. Day 3 was the last timepoint that microparticles could be detected with light microscopy.
  - By Day 7, most treatment-related damage (necrosis and hemorrhage) had resolved.
The above described results represent the following events:

**Heart:** No groups had signs of cardiac thromboemboli, infarcts, vasculitis or necrosis. Particulate matter was retained in the lung and did not pass to the heart.

**Lung:** At 30 minutes, microparticles from venous blood flowed from large pulmonary arteries to progressively smaller vessels until they plugged arterioles/capillaries smaller than themselves (thromboemboli). After the formation of thromboemboli, endothelial cells undergoing hypoxia/degeneration along with pneumocytes initiated inflammation, the formation of fibrin clots, leakage of blood (hemorrhage), chemotaxis of inflammatory cells (neutrophils, macrophages), and small amounts of necrosis. By Day 1 post-exposure, maximal hemorrhage, inflammation and necrosis caused by thromboemboli were observed. By Day 3, the incidence and severity of hemorrhage, neutrophilic inflammation, necrosis and particle scores were less than Day 1 (declining). By Day 7, most of the consequences of treatment had resolved and particle scores for all three dosed groups were comparably low and similar to the Day 3 particle scores.

**Conclusions:**
- While any thrombi going to the heart can cause occlusion of the vasculature, myocardial hypoxia and death, thrombi to the lung are not generally considered a life threatening condition. This is because the lung has dual blood supplies carrying variable levels of oxygen and nutrients; the pulmonary and bronchial artery systems. If pulmonary arteries become occluded, oxygen and nutrients are still supplied via the bronchial arteries. This is why the Day 7 lung findings appeared to be reversible and the rodents were well on their way to recovery.
- The use of particles larger than lung capillaries precluded any particles from passing through the lung to the heart where they could cause life threatening and irreversible cardiac damage. The larger the particle, the less likely to pass to the heart.
- Light microscopy allowed detection of particles through Day 1.
- Small sample sizes, intragroup variability and the acute nature of the study precluded robust statistical analysis or toxicity assessment. That said, it appears as though treatment-related changes in all groups were reversible with 35 micron particle causing slightly less pulmonary damage.

Conclusions: The presence of microparticle-related thromboemboli in all of the treated lung sections, but none of the treated heart sections, suggests that all microparticle emboli were captured in the pulmonary microvasculature. While there was variability in lung hemorrhage, inflammation and necrosis between the three particle-dosed groups, there were no treatment related deaths, pulmonary infarcts or substantial irreversible changes. By Day 7, most treatment related changes had resolved. Though histopathological surveillance is the gold standard for detection of treatment-related toxicity, it was not a sensitive indicator of persistent pulmonary microparticulate material beyond 1 day post-treatment. Detecting persistent particles may be achieved by other means (i.e. fluorescent imaging).
Appendix D: Gelation of PVA Microparticles by Freeze-Thaw Cycling

D.1 Introduction

Polymeric drug delivery systems are of significant interest due to the therapeutic advantages that they provide over conventional treatment methods.\textsuperscript{1,2} Once administered, pharmacologically active drugs face bioavailability and specificity challenges. Poor drug absorption and off-target side effects lead to unnecessarily high doses of expensive drugs, and morbidity due to organ toxicity.

An example of a drug delivery system is poly(ethylene glycol) (PEG) gel microparticles for the encapsulation of nanoparticles for lung imaging and drug delivery.\textsuperscript{3} The PEG microparticles were carriers for nanoparticles that were loaded with therapeutic and imaging agents. The microparticles, because of their size (10 – 40 µm), would filter out of circulation and lodge in the microcapillary spaces in the lungs, allowing for their slow degradation and the prolonged release of drugs directly into the lungs. Formation of the PEG matrix through two different chemical reactions (UV radical polymerization and Michael addition polymerization) was studied. UV radical polymerization led to detrimental damage of encapsulated material, while Michael addition polymerization did not.

Although the Michael reaction was successful, the use of chemical agents to crosslink polymers into a matrix is not ideal as such methods always have the possibility of leaving behind residual materials that can cause toxicity, such as acrylated end-groups. Likewise, as seen in the UV radical polymerization case, chemical agents can alter or destroy the substances to be released. Therefore, there is an interest in crosslinking polymers without the use of chemical
agents or any other potentially harmful method. A solution to this problem is the physical crosslinking of poly(vinyl alcohol) (PVA) polymers through cycles of freezing and thawing.\(^4\)

PVA is a suitable choice for a polymer drug carrier. It is FDA approved for injection, displays good biocompatibility, and has desirable properties such as an elastic nature and high degree of swelling in aqueous solutions. The physical crosslinking method for forming PVA hydrogels through cyclic freezing and thawing was first developed by Peppas in 1975.\(^4\) Physically linked PVA hydrogels were first proposed for use in biomedical applications, such as soft contact lenses, implants, artificial cartilage, and artificial organs, in 1984.\(^5\) To prepare PVA gel particles, several groups have employed an emulsion technique combined with the cyclic freezing-thawing process.\(^6\)\(^-\)\(^8\) An aqueous PVA solution is emulsified in oil and the emulsion is then frozen and thawed for long duration of times. Li and Lee reported that freezing at -20°C for 20 h and thawing at room temperature for 4 h for three and four cycles respectively were needed before particles became hard enough to be filtered through a membrane for washing away the oil phase.\(^6\)\(^,\)\(^8\) Ficek and Peppas reported that freezing at -4°C for 18 h and thawing at room temperature for 8 h for two cycles were necessary before particles became hard enough to be filtered and washed.\(^7\)

Although these previous methods were successful at making PVA particles, there is an interest in making them faster as the current process is very time consuming. A review compiled by Hassan and Peppas summarized the parameters controlling PVA crystallization during freeze-thaw cycling, such as PVA molecular weight, PVA solution concentration, freezing time, freezing rate, thawing time, and number of freeze-thaw cycles.\(^9\)
Using a higher molecular weight of PVA led to an increase in the size of crystallites, but a decrease in the number of crystallites.\textsuperscript{10} Even though the number of crystallites decreased, there was an increase in lamellar thickness, indicating that the crystallites formed were more stable.\textsuperscript{10,11} Higher molecular weight PVA also correlated with a larger gel fraction of PVA, indicating that gels formed quicker and easier using a higher molecular weight rather than a lower one.\textsuperscript{12}

The degree of crystallinity was found to increase with increasing PVA solution concentration.\textsuperscript{4,11} In addition, using a higher PVA solution concentration led to forming gels with larger compressive modulus, indicating the gels formed were more resistant to compression and harder.\textsuperscript{13}

Crystallinity was also found to increase with increasing freezing time.\textsuperscript{4} At low temperatures, there was a restriction in molecular motion. This promoted crystalline formation through hydrogen bonding between PVA molecules. Crystallization proceeded further as the solution remained at low temperatures for longer times due to PVA polymers having more time to hydrogen bond.\textsuperscript{14} Even though crystallinity, strength, stability, and swelling ratio of PVA hydrogels initially increased with freezing time, they eventually leveled off, indicating that there was an optimal freezing time after which the PVA hydrogel experienced little change in its properties.

Freezing rate affected the size of crystallites formed and the number of crosslinks between PVA polymers. A faster freezing rate led to the formation of larger crystallites.\textsuperscript{15} Similarly, faster freezing rates caused crystallite growth rates to be slower than crystallite nucleation rates.\textsuperscript{16} During thawing, the size of crystallites initially increased and then decreased
afterwards before leveling off. The crystallites reach their peak crystallinity around an hour. Likewise, the crystallinity, strength, stability, and swelling ratio of PVA hydrogels all initially increased with thawing time before they leveled off.\textsuperscript{11,17}

As was seen with freezing time and thawing time, properties of PVA hydrogels, such as crystallinity, strength, stability, and swelling ratio, initially increased with number of freezing-thawing cycles before they leveled off.\textsuperscript{13,18,19} Several studies have indicated different points at which the leveling off occurred. Holloway’s work indicated that this occurred around six cycles.\textsuperscript{13} Ricciardi and Fukumori’s works indicated that this occurred around seven cycles.\textsuperscript{19,20} In addition, the number of freezing-thawing cycles had little effect on the drug release profiles of oxprenolol and theophylline, again suggesting that after a certain number of cycles, the properties of PVA hydrogels had reached a final state.

A fast freezing-thawing technique for forming PVA gel microparticles was investigated by increasing freezing rate (lowering the freezing temperature to -80°C) over a previously attempted value (-4°C) for PVA crystallization.\textsuperscript{7} Here, 5%, 10%, and 20% (w/w) solutions of PVA were made into PVA hydrogel solutions and PVA solution in oil emulsions. These were frozen at -4°C and -80°C for three to six cycles at long (24 hr) and short (30 min) time amounts per cycle. The number of cycles was capped at six because previous studies showed little improvement in PVA properties beyond that point.\textsuperscript{13,18-20} The thawing time per cycle was set at one hour because a previous study showed that peak PVA crystallinity occurred at that time.\textsuperscript{4}
All together, the parameters of study here were PVA solution concentration, freezing time, freezing rate, and number of cycles. Figure D.1 above shows an overview of the experimental setup. The overall objective was to determine a robust freezing-thawing method for producing PVA gel microparticles for use in drug delivery applications.
D.2 Materials and Methods

D.2.1 Materials

Poly(vinyl alcohol) (average MW: 11,000 – 31,000, 98% hydrolyzed) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Silicone oils (100 cSt and 500 cSt) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Silicone oil surfactant was obtained from Dow Corning Corp. (Midland, MI). Poly(methyl methacrylate) was purchased from Polysciences, Inc. (Warrington, PA). 2,2,10,10-Tetraethyl-6,14-bis(triisopropylsilyl)ethynyl)-1,3,9,11-tetraoxadicyclopenta[b,m]-pentacene (EtTP-5) fluorescent dye was synthesized by protocols previously published. Sodium lauryl sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). Tetrahydrofuran (THF; HPLC grade), methanol (MeOH; 99.8%), hexanes (98.5%), and isopropyl alcohol (IPA; 99.5%) solvents were obtained from Sigma-Aldrich Co. (St. Louis, MO). Deionized water (DI water; 18.2 MΩ•cm) was generated by a NANOpure Diamond UV ultrapure water system (Barnstead International, Germany).

D.2.2 Preparation of PMMA/EtTP-5 nanoparticles

PMMA nanoparticles with fluorescent EtTP-5 dye were injected into PVA solutions to act as models of encapsulated nanoparticle drugs in PVA hydrogels and PVA microparticles. PMMA/EtTP-5 nanoparticles were created via Flash NanoPrecipitation using a two-inlet vortex mixer. A THF stream with dissolved EtTP-5 (10 mg/mL) was rapidly mixed against a PMMA stream (50 mg/mL) in a 1:2 volume ratio and collected in a DI water bath to reduce the final THF concentration to 20% by volume. To remove THF, the nanoparticle solution was dialyzed against a large volume of water in the dark for 6 hr, changing the water every 2 hr. The dialyzed
nanoparticle solution was then filtered through a 1 μm filter to remove larger PMMA/EtTP-5 particles.

D.2.3 Preparation of PVA solutions

At the desired weight percentage, PVA was dissolved in DI water by heating and stirring on a hot plate with a magnetic stirrer for 24 hr. After dissolution was complete, the solution was cooled at room temperature for 1 hr. Higher concentrated PVA solutions are more difficult to make and dissolution takes longer.

D.2.4 Preparation of PVA hydrogel solutions

A PVA solution was injected with a PMMA/EtTP-5 nanoparticle solution in a 20:1 volume ratio. The resulting solution was homogenized using a vortex mixer, mixing for approximately three minutes. Afterwards, the solution was subjected to the cyclic freezing-thawing process described earlier. PVA hydrogel solutions were used to determine at which point pure PVA solidified. By testing the hardness of PVA hydrogel solutions after three, four, five and six cycles, a conjecture could be made as to whether or not PVA microparticles in oil phase also solidified at those points.

D.2.5 Preparation of PVA microparticles

A PVA solution was injected with a PMMA/EtTP-5 nanoparticle solution in a 20:1 volume ratio. The resulting solution was homogenized using a vortex mixer, mixing for approximately three minutes. The solution was then added to 100 cSt silicone oil with 3% (w/w) 749 Fluid® (Dow Corning, Midland, MI) in a 1:10 volume ratio. The resulting emulsion was
homogenized using a vortex mixer, again mixing for approximately three minutes. Then the
emulsion was subjected to the cyclic freezing-thawing process described earlier.

At the end of three, four, five, and six cycles, to filter out the PVA microparticles,
vacuum filtration through a 1 µm SEFAR NITEX (Depew, NY) nylon mesh fabric was
performed. The fabric was wetted with hexanes before approximately 250 µL of emulsion was
placed on. The oil phase was then rinsed with hexanes until it was completely gone. Successive
rinsing with MeOH and IPA dried the remaining PVA microparticles. Once dried, the
microparticles were scraped off and collected with the use of a microspatula.

PVA microparticles with SLS were prepared by adding 1.25% (w/w) SLS to the PVA
solution before emulsifying. The rest of the procedure remained the same. SLS was added in
order to study its effects on particle formation. Ficek and Peppas proposed it as a way to improve
particle stability, particle size, and phase separation between the polymer matrix and
encapsulated material during the freezing-thawing process. PVA sheared microparticles were
prepared by shearing the PVA in oil emulsion, after it had been homogenized, with an Anton
Paar MCR 501 rheometer. PVA solutions here were emulsified with the 500 cSt silicone oil in
order to facilitate the shearing process. The rest of the procedure stayed the same. PVA in oil
emulsions were sheared in an attempt to create more uniformly sized PVA microparticles.

An ordinary household freezer was used to freeze PVA samples at - 4°C. A Thermo
Scientific Ultra-Low Temperature chest freezer (Waltham, MA) was used to freeze PVA
samples at - 80°C.
D.2.6 Microscopy

Microscopic images were taken with a Nikon Eclipse E200 microscope (Tokyo, Japan). Confocal images were taken with a Leica TCS SP5 confocal microscope (Wetzlar, Germany). Images were analyzed with NIH ImageJ software.

Table D.1. Solidification results of PVA hydrogel solutions

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<tr>
<th>Freezing Rate and Time</th>
<th>5% (w/w) PVA</th>
<th>10% (w/w) PVA</th>
<th>20% (w/w) PVA</th>
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<tr>
<td>- 4°C &amp; 30 min</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>- 4°C &amp; 24 h</td>
<td>No</td>
<td>No</td>
<td>Yes after five cycles</td>
</tr>
<tr>
<td>- 80°C &amp; 30 min</td>
<td>No</td>
<td>No</td>
<td>Yes after three cycles</td>
</tr>
<tr>
<td>- 80°C &amp; 24 h</td>
<td>No</td>
<td>No</td>
<td>Yes after three cycles</td>
</tr>
</tbody>
</table>

D.3 Results and Discussion

D.3.1 Generating initial PVA hydrogels

PVA hydrogel solutions were prepared and frozen at -4°C and -80°C for 30 min and 24hr and thawed at room temperature for 1 hr. This was done to determine if 20% (w/w) PVA became a solid within six freeze-thaw cycles. If so, then 20% (w/w) PVA solution in oil emulsions would then be made and cycled accordingly in hopes of forming solid PVA microparticles.

For the 20% (w/w) PVA hydrogel solution frozen at -4°C for 30 min, solidification was not seen after six cycles. For 20% (w/w) PVA hydrogel solution frozen at -80°C for 30 min,
Figure D.2. Image A shows the dry, congealed PVA microparticles that were collected after vortex emulsification. Image B shows that the PVA microparticles separated from each other once they were hydrated, confirming that distinct PVA microparticles were formed. Scale bar is 100 μm. (Images taken after three cycles)

solidification was seen after three cycles. For 20% (w/w) PVA hydrogel solution frozen at -4°C for 24hr, solidification was seen after five cycles. For 20% (w/w) PVA hydrogel solution frozen at -80°C for 24hr, solidification was seen after three cycles. Table D.1 summarizes the results from all the PVA hydrogel solutions.

From cycling the PVA hydrogel solutions, the most significant result was that 20% (w/w) PVA hydrogel solutions solidified after three cycles of freezing at -80°C for 30 min freezing and 1hr thawing at room temperature. If PVA solution droplets in oil emulsions also solidified after three cycles (following this same cyclic processing), then there would be a great reduction in the amount of time, when compared to previous methods, needed to produce PVA microparticles. To investigate this, 20% (w/w) PVA in oil emulsions were prepared and frozen at -80°C for 30 min and thawed at room temperature for 1hr for three, four, five, and six cycles. In addition, SLS was added to observe its effect on particle stability, particle size, and phase separation. Emulsions were also sheared in a rheometer in an attempt to create uniformly sized PVA microparticles and
D.3 Images A and B, taken after six cycles, show that PMMA/EtTP-5 nanoparticles are not well dispersed within the PVA gel microparticles, suggesting phase separation. Image A is the optical overlay and image B is just the fluorescence.

Figure D.3. Images A and B, taken after six cycles, show that PMMA/EtTP-5 nanoparticles are not well dispersed within the PVA gel microparticles, suggesting phase separation. Image A is the optical overlay and image B is just the fluorescence.

To study the effect of uniform shearing. The results for 20% (w/w) PVA microparticles are presented in the next few sections.

D.3.2 Generation of PVA microparticles

After three cycles, 20% (w/w) PVA microparticles were hard enough to be filtered. PVA microparticles were subsequently collected at four, five, and six cycles. Microscopic images (Figure D.2) showed that during vacuum filtration and drying, PVA microparticles clumped and congealed together when collected. Upon hydration with DI water, the clumps dissociated and some individual PVA microparticles were visible.

Confocal images (see Figure D.3) showed that phase separation between the PVA matrix and PMMA/EtTP-5 nanoparticles was still seen after six cycles. ImageJ size analysis determined that the average size of PVA microparticles was 4.0 \( \mu \text{m} \) in radius with a standard deviation of 1.3 \( \mu \text{m} \) after six cycles. This was due to the large number of small satellites generated by vortexing.
D.3.2.1 PVA microparticles with SLS generated by vortex mixing

After three cycles, 20% (w/w) PVA microparticles with SLS were also hard enough to be filtered. PVA microparticles were subsequently collected at four, five, and six cycles.

Microscopic images (see Figure D.4) showed that during vacuum filtration and drying, the PVA microparticles with SLS had also congealed together into clumps. Similarly, upon hydration with DI water, some of the clumps dissociated and individual PVA microparticles were visible.

Likewise, confocal images (see Figure D.5) showed that phase separation between the PVA matrix and PMMA/EtTP-5 nanoparticles was still seen after six cycles. ImageJ size analysis determined that the average size of PVA microparticles was 3.4 µm in radius with a standard deviation of 1.0 µm after six cycles, due to many small satellite particles.

The addition of SLS did not improve phase separation, as the PVA microparticles without SLS showed similar phase separation as those with SLS. In this sense, SLS was not needed in
order to create PVA microparticles with well-dispersed PMMA/EtTP-5 nanoparticles inside. However, the addition of SLS did lead to slightly more uniform PVA microparticles, as evident by the lower standard deviation of particle size for PVA microparticles compared to those without SLS. Likewise, the addition of SLS also led to a smaller average particle size, indicating that SLS had a slight effect on particle size as well, likely due to decreasing interfacial tension.

D.3.2.2 PVA microparticles with SLS produced by shear emulsification

Since SLS was determined to have a slight effect on increasing particle uniformity and controlling particle size, it was kept in the formulation for PVA sheared microparticles. Controlled shearing of the PVA in oil emulsion was done in order to create more uniform PVA microparticles. As seen by the microscopic images (see Figure D.6) of the non-sheared PVA in oil emulsion and the sheared PVA in oil emulsion, there is a huge difference in the size distribution of PVA droplets in the emulsions. After shearing, the size of the droplets became more uniform.
Starting with a uniform PVA emulsion was hypothesized to lead to uniform PVA microparticles after freezing and thawing. Like before, three cycles was enough to create hard 20% (w/w) PVA sheared microparticles with SLS to be filtered. PVA microparticles were subsequently collected at four, five, and six cycles. The average particle sizes and standard deviations of the particle sizes at three, four, five, and six cycles are shown in Table D.2.

Shearing the PVA in oil emulsion did not greatly affect the average PVA particle size, which is dominated by satellite droplets. Although emulsion droplets were very different in uniformity, results showed after freezing and thawing that the sheared PVA microparticles became approximately the same size as the non-sheared PVA microparticles. Likewise, standard deviations of particle sizes were also similar to those of non-sheared PVA microparticles, suggesting no improvement in particle stability was achieved by shearing. These results indicate that using a rheometer to shear PVA solution in oil emulsions before freezing and thawing might not be a reasonable method to control particle size or particle stability for PVA microparticles formed through physical crosslinking.
Table D.2. Average particle size for controlled shear generated PVA microparticles with SLS

<table>
<thead>
<tr>
<th>Number of Cycles</th>
<th>Avg. Particle Radius (µm)</th>
<th>Standard Deviation (µm)</th>
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</thead>
<tbody>
<tr>
<td>Three</td>
<td>3.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Four</td>
<td>3.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Five</td>
<td>4.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Six</td>
<td>3.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Confocal images (see Figure D.7) showed that phase separation between the PVA matrix and PMMA/EtTP-5 nanoparticles was seen after six cycles.

D.4 Conclusion

PVA gel microparticles were made by using a fast freezing-thawing technique that consisted of freezing a 20% (w/w) PVA solution in oil emulsion (1:10 volume ratio) at -80°C for 30 min and thawing at room temperature for 1 h. Solid PVA gel microparticles were able to be filtered after three cycles of freezing and thawing. PVA gel microparticles encapsulated poly(methyl methacrylate) (PMMA) nanoparticles. A fluorescent dye in the nanoparticle core was used to monitor phase separation between the PVA gel matrix and the nanoparticles. The effect of sodium lauryl sulfate (SLS) addition on PVA microparticle formation was investigated. Adding SLS led to similar phase separation, but produced slightly more stable and smaller PVA gel microparticles. The effect of shearing emulsions before cyclic freezing and thawing on PVA microparticle formation was also investigated. Sheared samples led to similar phase separation and produced PVA microparticles with similar size and stability.
Figure D.7. Images A and B, taken after six cycles, show that PMMA/EtTP-5 nanoparticles are not dispersed within the sheared PVA gel microparticles with SLS. Image A is just the fluorescence and image B is the optical overlay. Some particles in the overlay appear to have no fluorescence due to being out of focus.

D.5 References


Appendix E: Dual Imaging Boscalid Nanoparticles for Enhanced Pesticide Delivery to Plant Roots

Abstract

The high cost of the damage caused by pathogens and the inefficiencies in the delivery of pesticides to plants create the need for a better delivery system of biocontrol agents to plants. The main goal of this project was to improve the delivery of boscalid, a fungicide made for use with food crops, to grow plant roots. Moreover, it was desired to have the ability to track the movement and transport of boscalid to enhance understanding of the complex delivery pathway within the rhizosphere and into the roots. Nanoparticles of tunable sizes encapsulating boscalid, LD 688 dye and a co-encapsulate, either vitamin E or polystyrene (PS), were packaged with the block copolymer PS-PEG and synthesized. These nanoparticles were in the range of 65 nm to 254 nm. The success encapsulating boscalid suggests that the same may be done for other chemically similar organic actives.
E.1 Introduction

The ecosystem made up of plant roots and the immediate environment, known as the rhizosphere, encompasses many complex interactions between microorganisms that inhabit the surrounding soil and the root itself.\textsuperscript{1} The rhizosphere has been shown to be a crucial component of the plant’s survival, as carbon exudates given off from the plant to nourish the microbial community could otherwise be directed toward the production of protective structures or collection of light above ground.\textsuperscript{2} The multitude of reactions and transport of nutrients that occur here make the rhizosphere a “central commodities exchange,” where plants and the surrounding microbial community trade carbon, nitrogen and other nutrients that are essential to not only maintaining the health of the plant and the microbiome, but also to sustaining the carbon and nitrogen cycles.\textsuperscript{1}

Further, the interaction of microorganisms and microbivores that feed on these microorganisms drives the cycling of nutrients in the rhizosphere. Protozoa have been shown to be especially beneficial to the rhizosphere as they strongly regulate its composition by means of selectively grazing bacteria, consumption of which is central to the release of sequestered nutrients back into the soil and to the plant.\textsuperscript{2} In addition, it has been shown that bacteria are able to live in protozoa by means of defense mechanisms such as secretion of toxins or production of protective membrane structures, and can therefore be transported by protozoa and deposited through ingestion and egestion around the root.\textsuperscript{3,4} In this way, bacteria may be transported to different parts of the rhizosphere, such as to growing roots, where the highest concentration of exudates from the plant are found, and where control of microorganisms is therefore most crucial.
A major impediment to effective delivery of bacteria and other forms of biocontrol is the inability of the biocontrols to keep up with the rapidly elongating root, a process that occurs much faster than bacterial reproduction. Because of this, the soil surrounding nascent roots is often uninhabited by crucial biocontrol bacteria.\textsuperscript{3} Thus, the functionality of protozoa in transporting nutrients and biocontrol agents throughout the rhizosphere is of great interest, especially in the agriculture industry where millions of dollars are lost because of damage by pests. It is estimated that farmers in New Jersey alone lose $290 million annually to pests or the control of pests.\textsuperscript{5,6}

To address the problems surrounding biocontrol delivery, a number of approaches have been taken. Shipp \textit{et al.} investigated a method of using bees to deliver biopesticides to crops damaged by or infected with pests. The goal was to design a hive-like dispenser that would deposit biocontrol agents on the legs and body hairs of the bees whenever they entered and exited. The bees would then transport these biocontrols to plants and deposit them while foraging or grooming. They found that using bees to deposit a specific biocontrol agent, \textit{Beauveria bassiana}, was a promising defense against pests on greenhouse tomatoes and sweet peppers.\textsuperscript{7}

Russo \textit{et al.} investigated the efficacy of alginate, a highly hydrophilic linear copolymer, as a carrier for inoculation of seeds with a biocontrol strain of \textit{Pseudomonas}.\textsuperscript{8} To enhance biocontrol delivery, two methods were used. Alginate beads inoculated with the biocontrol were placed in the soil next to the seeds, or an alginate coating was deposited onto the seed. They found that such methods were effective in enhancing colonization when compared to a control system in which free cells were applied to the seed.\textsuperscript{8}
Another way to approach the biocontrol delivery problem is to utilize the high swimming speeds of protozoa, which is about 400 um/s, a speed that is able to match the rapid growth of root tip growth. Protozoa thus offer a means of transport to root tips within the rhizosphere, one that is already utilized by bacteria. The ultimate goal of this project was to explore this approach and to evaluate the functionality of protozoa as a delivery system for pesticide-encapsulated nanoparticles to growing root tips as an alternate form of biocontrol.

In this research project, nanoparticles were synthesized using Flash NanoPrecipitation (FNP) a technique used to encapsulate hydrophobic actives within the cores of nanoparticles. The process involves dissolving an organic active and a di-block copolymer in an organic phase and rapidly mixing the solution with a miscible anti-solvent using a confined impinging jet (CIJ) mixer to yield nanoparticles of a narrow size distribution. Several key features characterize the process. The first is the rapid mixing time, which is shorter than the formation time of a nanoparticle. Additionally, the block copolymer can promote nucleation, afford steric stability, and provide a functional surface to better suit the nanoparticle application, whether it be as a mucoadherent for improved drug retention, or as a surface with properties similar to Low Density Lipoproteins (LDL) that allow the nanoparticles to interact with LDL receptors and therefore cross the blood brain barrier. In addition, FNP yields nanoparticles that have a narrow size distribution, but simultaneously allows for tuning of sizes by changing relative concentrations of the organic active and the block copolymer.

The characteristics of FNP-processed nanoparticles are desirable in the case of boscalid nanoparticles, especially the flexibility in tuning the size of the nanoparticles. This will allow for knowledge of an optimal size of nanoparticle for uptake by the protozoa, or an understanding of how different sized nanoparticles may differ in transport and delivery to the plant.
For the nanoparticles discussed, the organic active was either vitamin E or boscalid, and the di-block copolymer was poly (styrene)-b-poly (-ethylene glycol) (PS_{1500}-b-PEG_{5000}); the organic phase was tetrahydrofuran (THF); the anti-solvent was MilliQ water.

E.2 Materials and Methods

E.2.1 Materials

Poly(styrene-b-ethylene oxide) (PS(1600)-PEO(5000)) and Polystyrene (molecular weight 1900 g/mol) were purchased from Polymer Source, Inc. LD 688 dye was purchased from Photonic Solutions. Boscalid was purchased from BASF. Vitamin E, 97% was purchased from Sigma Aldrich. Compritol 888 was purchased from Gattefosse. And soybean oil was purchased from Target. Poly vinyl alcohol (PVA) was purchased from Aldrich.

E.2.2 Nanoparticle production

Nanoparticles encapsulating Vitamin E and LD 688 with PS-PEG were made with varying concentrations of each of the three components. All three components were dissolved in THF. A stock solution of 10 mg/mL LD 688 was made and stored in a scintillating vial. This and all other solutions containing LD 688 were wrapped in foil to limit the exposure of the solution to light and to minimize the photodegradation of LD 688. Required masses of vitamin E and PS-PEG were measured out and mixed with the appropriate amount of LD 688 stock solution. If necessary, the vial containing the solution was placed in an ultrasonic bath for thirty second increments to promote thorough dissolving. The CIJ mixer was placed above a vial containing 8 mL of MilliQ water on a stir plate and the FNP process was carried out by rapidly mixing 1 mL of the organic phase solution with 1 mL of MilliQ water in the CIJ and allowing this to mix into the 8 mL of MilliQ water.
Dialysis of the solution with MilliQ water was performed to remove the THF from the solution. The MilliQ water was replaced once every hour for four hours and then left dialyzing overnight to remove any remaining THF. The solutions were then stored in foil-wrapped scintillating vials in a refrigerator.

\(E.2.3\) LD 688 microparticle production

Compritol and soybean oil were heated to 90°C and mixed at a 1 to 3 ratio. LD 688 was added to oil and the whole solution was added to 90°C water containing 0.5% (w/w) PVA. The emulsion was homogenized hot at 15,000 rpm using an Ultraturrex®(IKA) mixer for 20 minutes. The suspension was removed from the mixer and cooled on ice quickly. The suspension was then centrifuged and the particles of sizes 1-5 microns were decanted.

\(E.2.4\) Nanoparticle fluorescence

Fluorescence of nanoparticles that encapsulated LD 688 was measured by making a 20-fold dilution in a plastic cuvette. Excitation and emission slit widths were set to 5 nm and the PMT voltage was set to 400 V.

\(E.2.5\) Light microscopy

Microscopy with a light microscope set to 10 and 40 times magnification was implemented to monitor the stability of the solutions—that no crystallization or other phenomena occurred in the final nanoparticle solutions.
E.2.6 Confocal microscopy

Microscopy with a confocal microscope (Broadband Confocal Leica TCS SP5) was also used to investigate the fluorescent properties of nanoparticles made with LD 688.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Vitamin E (mg/mL)</th>
<th>PS PEG (mg/mL)</th>
<th>LD 688 (mg/mL)</th>
<th>Core Loading of LD 688</th>
<th>PS (mg/mL)</th>
<th>NP Size (nm)</th>
<th>Max Fluorescence Intensity</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>70</td>
<td>0.75</td>
<td>1.29%</td>
<td>-</td>
<td>342</td>
<td></td>
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<tr>
<td>2</td>
<td>17.5</td>
<td>50</td>
<td>0.75</td>
<td>2.19%</td>
<td>-</td>
<td>122.4</td>
<td>14.43</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>70</td>
<td>0.75</td>
<td>1.29%</td>
<td>-</td>
<td>164.2</td>
<td>53.3</td>
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<td>4</td>
<td>4.40</td>
<td>8.8</td>
<td>0.19</td>
<td>2.54%</td>
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<td>68</td>
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<tr>
<td>5</td>
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<td>17.8</td>
<td>0.19</td>
<td>1.29%</td>
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<td>6</td>
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<td>70</td>
<td>0.90</td>
<td>1.70%</td>
<td>35</td>
<td>255</td>
<td>196</td>
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</table>

Table E.1. Formulations of LD 688 NPs. Except for solution 2, the ratio of PS-PEG concentration to the active compound (either vitamin E or PS) was roughly 2:1.

E.3 Results and Discussion

E.3.1 LD 688/vitamin E nanoparticles

Formulations of the LD 688 nanoparticle solutions are given in Table E.1. Nanoparticle size increased roughly linearly with total mass, as shown in Figure E.1. A range of nanoparticle sizes, from 68 nm to 255 nm, was achieved, as shown in Figure E.2.

Maximum fluorescence intensity is plotted as a function of LD688 core loading for vitamin E encapsulating nanoparticles in Figure E.3. Corresponding formulations are shown in Table E.1. There is a slight trend of decreasing maximum fluorescence intensity with increasing core loading.
E.3.2 LD688 microparticles

Microparticles were produced and sent out for protozoa pilot studies. The size of the particles ranged from 1 to 5 microns and they were all fluorescently active as shown by confocal microscopy. Figure E.4 shows the LD 688 microparticles produced by shear mixing.

E.3.3 Boscalid nanoparticles

The first goal in synthesizing boscalid nanoparticles was to determine the maximum concentration of boscalid that could be encapsulated. This was found by making different nanoparticle solutions with increasing concentrations of boscalid until the solution became unstable when crystallization was observed in the pre-FNP solution. This was observed at 2
mg/mL boscalid. No crystallization was observed at 1 mg/mL boscalid, therefore all subsequent solutions were made with 1mg/mL boscalid and varying concentrations of the other components in THF.

Boscalid nanoparticles of different sizes were desired to allow for flexibility in nanoparticle delivery to the protozoa in later stages. This was achieved by tuning the relative

Figure E.2. LD 688 nanoparticle diameter increases roughly linearly with total solids concentration. Data shown corresponds to solutions 2 through 6, which all encapsulated vitamin E. The solution made with PS was not included in this graph because of its dissimilar encapsulation properties.

Figure E.3. Fluorescence of LD 668 nanoparticles as a function of core loading.
concentrations of boscalid, vitamin E, and PS-PEG. This was the reasoning for the formulations of solutions 1, 2, and 3, shown in Table E.2.

A comparison of solutions 6 and 7 of Table E.2 and the size of the resulting nanoparticles indicates that modest changes in vitamin E concentration, ±10 mg/mL, do not have a large effect on the size of the nanoparticles. In contrast, solution 8 has four times as much vitamin E as solution 6, and was roughly twice the size of solution 6 nanoparticles. Solutions 19 and 20 suggest that the addition of LD 688 affects encapsulation and that even very high concentrations of vitamin E yield smaller nanoparticles than solutions made without LD 688.

In order to obtain a range of sizes, the concentration of vitamin E was increased. However, increasing vitamin E can only increase the particle size to a defined amount before emulsion droplets begin to form, an unfavorable phenomenon. As can be seen in Figure E.5, high
<table>
<thead>
<tr>
<th>Solution</th>
<th>Boscalid (mg/mL)</th>
<th>Vitamin E (mg/mL)</th>
<th>PS PEG (mg/mL)</th>
<th>LD 688 (mg/mL)</th>
<th>NP Size (nm) before dialysis</th>
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</thead>
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</tr>
<tr>
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<td>1</td>
<td>100</td>
<td>100</td>
<td>0.35</td>
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<td>1</td>
<td>120</td>
<td>120</td>
<td>0.35</td>
<td>126</td>
</tr>
</tbody>
</table>

Table E.2. Boscalid nanoparticle formulations.

concentrations of vitamin E and relatively low concentrations of PS-PEG caused the excess vitamin E to form droplets.

Because it was not possible to make larger boscalid nanoparticles by co-encapsulating with vitamin E, PS was tested as another possible co-encapsulate. Two formulations were made, as shown in Table E.3. Comparing solution 8 of Table E.2 with solution B, which had similar formulations except for the co-encapsulate and the addition of LD 688 in solution B, it is shown that larger boscalid nanoparticle sizes can be obtained with PS instead of vitamin E.

The final segment of the project involved encapsulation of LD 688 within the boscalid nanoparticles with the goal of making dual-functioning nanoparticles that could serve as both a delivery system for boscalid and that could also be imaged to afford a better understanding of the transport and pathways of the nanoparticles. Solutions 19 and 20, shown in Table E.2, and solutions A and B, shown in Table E.3, achieved this goal. Solution 19 yielded a clear pre-FNP solution of boscalid, vitamin E, PS-PEG, and LD 688, while solution 20 yielded a cloudy pre-
FNP solution. This suggests that the maximum solids loading falls somewhere between these two solutions.

Figure E.6 shows a comparison of Solutions 19, A, and B under confocal microscopy. The smaller particles shown in E.6a are more monodisperse and uniform in size. As the particle size increases, the monodispersity decreases, as evidenced by the presence of the large, bright spots against the background of smaller spots in Figure E.6c.

Different compounds encapsulate in different ways: as shown in Table E.1, solutions 3 and 7 were made with the same formulation, with the exception that solution 3 encapsulated vitamin E and solution 7 encapsulated PS. Comparison of the nanoparticle sizes of solutions 3 and 7 indicates that encapsulating PS instead of vitamin E results in larger nanoparticles.
Table E.3. Boscalid nanoparticle formulations with co-encapsulated polystyrene.

PS-encapsulated nanoparticles were much larger and brighter than their vitamin E counterparts, as shown in Figure E.3, indicating that PS encapsulates differently than does vitamin E. Data shown in Figure E.3 suggests that maximum fluorescence intensity reaches a maximum, beyond which quenching occurs. This is confirmed by experiments that found the optimal core loading of LD 688 to be 1.8%. More solutions with LD 688 core loading in between 1.29% and 2.19% are needed to show this maximum at 1.8%.

The formation of vitamin E emulsion droplets can be explained by considering the characteristics and interaction of the components of the nanoparticle solution, which included vitamin E, boscalid, and PS-PEG dissolved in THF. Because the goal at this point of the experiment was to make larger nanoparticles, it was desired to increase the concentration of the vitamin E, as it was believed that this would increase the size of the nanoparticle. At the same time, it was also desired to limit the concentration of PS-PEG, as solubility of total solids had become a concern. In previous solutions, higher concentrations of PS-PEG caused instability and crystallization. Further, vitamin E is made of non-polar carbon and hydrogen groups, and is therefore not completely soluble in THF, a moderately polar solvent. Thus, high concentrations of an insoluble compound without sufficient concentrations of PS-PEG to
encapsulate it led to emulsion-like droplets similar to droplets that would be found in an oil-in-water emulsion.

The larger particles made by replacing vitamin E with PS indicate that the two actives have dissimilar encapsulation properties. This likely arises because of the differences in molecular structure between vitamin E and PS: vitamin E is made of aliphatic chains, while PS is an aromatic molecule. These different structures interact differently with boscalid.

Nanoparticle formulations and microparticles were sent off to be tested by Leslie Shor's group at UConn for uptake and efficacy studies. They showed that the nanoparticles were taken up and transported by the protozoa while the microparticles were not. Boscalid was not a suitable material for their studies, however there is promise to find another more suitable molecule to transport in the protozoa as we know the nanoparticles will be engulfed.
E.4 Conclusions

Boscalid fungicide was successfully encapsulated with LD 688 dye in nanoparticles of a range of different sizes, for what is hoped will constitute an enhanced delivery system via protozoa ingestion, transport, and egestion. The inclusion of the fluorescent dye LD 688 within the nanoparticle also enables imaging of the particles during transport within the protozoa, through the rhizosphere, and into the roots and allows for an improved understanding of the mechanisms and pathways of biocontrol agent delivery. Different co-encapsulates lend to different types of encapsulation and ultimately yield different sized nanoparticles. PS yields larger nanoparticles than vitamin E. Inclusion of LD 688 gives smaller particles than solutions without LD 688.

Further studies will investigate the formulation of other biological agents, such as mesotrine, to see if the material can be encapsulated by FNP. The same types of characterization can be performed for other pesticides which would enlarge the applicability of this technology to a wider variety of crops.

E.5 References


