Microfluidics-Generated Biodegradable Polymeric Microparticles for Controlled Drug Delivery

by

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Fan Yuan

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University
2014
ABSTRACT

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Abstract

While drug-loaded biodegradable polymer microparticles have many therapeutic applications, bulk manufacturing methods produce heterogeneous populations of particles. A more highly controlled manufacturing method may provide the ability improve the microparticle characteristics such as the drug release profile. Microfluidic droplet-makers manipulate liquids on the scale of tens of microns and can produce highly regular and controlled emulsions. However, microfluidic droplet manufacturing is not typically designed for clinical translation and the chemicals used are often not biocompatible.

I developed a two-chip PDMS-based microfluidic device that can manufacture PLGA microparticle loaded with hydrophilic or hydrophobic drugs. I characterized protein-loaded microparticles made using this device and compared them with bulk-generated microparticles. The microfluidics-generated microparticles had similar release curves and encapsulation efficiencies as those of the bulk-generated microparticles but a much narrower size distribution. I generated peanut protein-loaded microparticles with this device and tested them in two mouse models of peanut allergy, improving the particles as the project evolved to have a higher loading level and a lower burst release. I also encapsulated hydrophilic and hydrophobic chemotherapeutic drugs for a brain cancer model.
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Above all, I would like to thank my husband, Dr. Kyle Eugene Roberts. Kyle, you have been not only my greatest supporter for the last six years but an inspiring colleague as well. You always give me the encouragement, commiseration, tough love, or distraction that I have needed to get me through my graduate career. Thank you for getting us started here at Duke and I can’t wait to see where our life together goes next.
Polymer microparticles have been employed as a vehicle to deliver hydrophilic and hydrophobic payloads in a variety of studies for many years. Poly(lactic-co-glycolic acid) (PLGA) microparticles, in particular, are poised for translation to medical applications because of the polymer’s FDA-approved status. Unfortunately, typical methods of generating emulsions result in heterogeneous populations of particles in terms of their size and drug distribution and the microparticles have uncontrolled drug release profiles. This limits their potential application in clinical settings, especially for hydrophilic payloads, which require double emulsions (DE).

Microfluidic technology has been widely applied in the precise manufacturing of designer droplets, including multiple emulsions and particles. However, the payloads and solvents used are not typically chosen with clinical translation as an end goal, so the resulting droplets and particles are not usually biocompatible and the payloads are almost always model drugs or contrast agents.

The overarching theme of my dissertation project is to create a microfluidic platform for generating hydrophilic drug-loaded PLGA microparticles and to use that platform in loading several different drugs that are on the pathway to translation.
In Aim 1, I compared protein-loaded microfluidics-generated PLGA microparticles with protein-loaded bulk-generated microparticles. In Aim 2, I manufactured peanut protein-loaded PLGA microparticles and used them in several \textit{in vitro} and \textit{in vivo} treatment models of peanut allergy. In Aim 3, I encapsulated hydrophilic and hydrophobic chemotherapeutics in PLGA microparticles. Ours is the first report of microfluidics-generated PLGA microparticles that encapsulate a drug intended for clinical translation.

1.1 Specific Aim 1: Development of a Microfluidic Platform for Generating Drug-Loaded PLGA Microparticles (Chapter 3)

Along with other Leong lab members, I developed a two-chip PDMS-based microfluidic platform that generates DE droplets. I used this device to manufacture protein-loaded microparticles, which I directly compared to bulk preparations through SEM, a release study, and the encapsulation efficiencies. We also increased the throughput capacity of the device and improved part of the device manufacturing protocol.

1.2 Specific Aim 2: Manufacture of Peanut Protein-Loaded Microparticles and Application in Animal Models of Peanut Allergy (Chapter 4)

I iteratively developed peanut protein-loaded PLGA microparticles using both bulk and microfluidic methods to modify two models of immunotherapy in mice used in Wesley Burks’s lab. In phase 1, we tested bulk-generated microparticles in a prophylactic model of oral immunotherapy. In phase 2, I increased the encapsulation efficiency of the microfluidics-generated microparticles from Aim 1 and our collaborators tested them \textit{in vitro} in basophils from peanut-allergic individuals. In phase 3, I optimized the microfluidics-generated microparticle formulation to produce particles with a loading level appropriate for use in the mouse model and our collaborators
tested the microparticles in a safety study in a subcutaneous immunotherapy (SIT) mouse model. In phase 4, I lowered the burst release of protein from the microfluidics-generated microparticles and we tested the microparticles in an efficacy study in an SIT mouse model.

1.3 Specific Aim 3: Encapsulation of Chemotherapeutic Drugs in Microparticles (Chapter 5)

I validated the increased toxicity of several versions of a metalloporphyrin compound against cancerous human cell lines in comparison with non-cancerous human cells as well as the combined action of porphyrin with ascorbate or doxorubicin. I encapsulated and characterized porphyrin, ascorbate, and doxorubicin in PLGA microparticles using our DE microfluidic platform and also generated doxorubicin-loaded PLGA microparticles using a single emulsion microfluidic method.
2.1 Biodegradable Microparticles

2.1.1 Introduction

Biodegradable polymer particles have proven to be a versatile, robust tool for drug delivery over the past several decades, having been studied and applied to numerous drug delivery problems. Poly (lactic-co-glycolic acid) (PLGA), in particular, has emerged as a popular polymer for drug delivery applications because it has a long history as a biocompatible, FDA-approved material and its degradation rate is strongly dependent on its molecular weight, lactide-to-glycolide ratio, and other characteristics. Researchers have studied PLGA particles for the delivery of vaccines [1, 2, 3], DNA [4, 5], protein [6, 7, 8, 9, 10, 11], chemotherapeutic drugs [12], and other hydrophilic and hydrophobic compounds. Patents have been issued for PLGA particle-based delivery of granulocyte-macrophage colony-stimulating factor [13], cancer therapeutics [14], and insulin [15].
2.1.2 **PLGA**

It has been established that PLGA degrades by simple hydrolysis of the ester bonds in the copolymer backbone when it is exposed to water. The degradation rate of PLGA is determined primarily by its lactide-to-glycolide ratio and then by its molecular weight, glass transition temperature, and crystallinity [16]. The release of drugs from PLGA particles first comes from a burst release from the surface, second from diffusion through the mass, and third from bulk degradation of the polymer [17, 18]. The drug release rate is dependent on the PLGA properties that control degradation as well as the drug type, loading level, particle size, and environmental pH [19].

2.1.3 **Bulk Methods**

Popular techniques for creating drug-loaded PLGA particles include emulsion/solvent evaporation, spray drying, and phase separation (coacervation) [20]. The DE/solvent evaporation process, which is necessary to encapsulate hydrophilic drugs, consists of 1) the formation of a primary water-in-oil emulsion, such as through vortexing, homogenizing, or sonication of a hydrophilic drug in an aqueous phase in an organic phase containing PLGA, 2) the emulsion of that primary emulsion in another aqueous phase with a surfactant, which forms a water-in-oil-in-water DE, and 3) the evaporation of the organic solvent, resulting in solid drug-loaded core-shell PLGA particles.

2.1.4 **Drawbacks**

The chief drawback to producing particles using this bulk method is that it affords poor control over the inner and outer droplet sizes [21], resulting in a heterogeneous population of particles, both in size and internal drug distribution. There is typically also a large burst release of the loaded drug due to its presence on the surface instead of the interior of the polymer mass. We intend to overcome these drawbacks by generating the emulsions with a microfluidic system.
2.2 Microfluidic Technology

2.2.1 Introduction

Microfluidics is the precise manipulation of fluids on a length scale of tens of microns, at which all fluid flow is essentially laminar [22]. The interesting and non-intuitive physics that occurs in laminar fluid flow combined with the creative applications of the technology have created a truly multidisciplinary field, drawing together physicists, mathematicians, biologists, chemists, engineers, healthcare practitioners, and computer scientists.

Since the field’s inception in the late 1990s, microfluidic technology has been used for diverse applications such as culturing a drosophila embryo at two temperatures simultaneously [23], producing sunlight-based fuel from photobioreactors [24], and detecting malaria-causing parasites in unprocessed blood [25]. In this dissertation, I will focus on droplet microfluidics.

2.2.2 Droplet Microfluidics

The first microfluidic droplets were published in 1997 and made using sunflower oil and water [26]. Now, droplet microfluidics is one of the most popular and versatile applications of microfluidic technology because it generates droplets of precisely controlled size at high frequency from immiscible phases. A single microfluidic device can make on the order of 1,000 to 10,000 droplets per second [27]. Macroscale shearing in bulk emulsification causes interface instability between phases [28]. In microfluidic devices, the process of breaking up the dispersed phase is highly controlled and monodisperse droplets can be guided by the choice of phases, surfactants, device geometry, and flow rates [28]. Working at the length scale of tens of microns causes interfacial forces to become very important because of the high surface area to volume ratio of the droplets [29], so the choices of appropriate phases and sur-
faces are even more vital than when working in bulk. Many groups have employed
droplet microfluidic techniques to increase control over processes that were originally
developed in bulk.

2.2.3 Applications of Droplet Microfluidics

Broadly, the applications of droplet microfluidics can be classified into chemical and
biological assays and particle production [30].

*Chemical and Biological Assays*

Microfluidic droplets can serve as microreactors and compartmentalizers for cells or
biochemical reactions. Droplets made in microfluidic devices can be manipulated
with a precision that is not possible with bulk methods. Droplets can be passively or
actively split, coalesced, mixed, sorted, and stopped [22, 28]. The parallel processing
naturally afforded by the microfluidic generation of identical microreactors can
generate large data sets with minimal reagent use [22]. A few examples of assays per-
formed using microfluidic chips are protein crystallization, advanced flow cytometry,
enzyme kinetics studies, and DNA and blood analysis [31].

*Particle Production*

Particles can be produced by complexation within picoliter droplets [32] or by con-
verting an entire droplet into a particle. A droplet can be converted to a particle
through solvent evaporation, gelation, and solidification [28], which can occur inside
or outside of the microfluidic device [22]. Microparticle production from microfluidic
droplets by solvent evaporation is covered more thoroughly in Section 2.3.

2.2.4 Droplet Formation and Stabilization

Droplets are formed by dispersing one fluid stream (the dispersed phase) within
another immiscible fluid (the continuous phase). The two immiscible phases are
commonly referred to as oil and water or organic and aqueous. The droplets are formed from surface-induced instability [28]. The smallest feature size of the device chiefly determines the size monodispersity [31]. Other factors influencing droplet size are the rest of the device geometry, flow rates, phase viscosity, interfacial tension, and channel wettability [28]. To aid droplet formation, the viscosity of the continuous phase should be higher than that of the discrete phase [22].

There are two regimes of droplet production: dripping and jetting. In the dripping regime, the viscous drag from the continuous phase pulls the emerging droplet downstream, while surface tension keeps the droplet connected to the rest of the discrete phase. When the force of the drag overcomes the force of surface tension, the droplet breaks off [30]. The dripping regime occurs at low Capillary number (Ca). Ca describes the balance between the continuous phase fluid drag and the surface tension of the discrete phase. As the flow rates and Ca increase, the finger of the emerging droplet is pushed further down stream before breaking off [31]. Shear forces are at work in this jetting regime, which results in higher polydispersity of droplet size because of the variation of the breakoff point [30].

Surfactants are commonly added to either the aqueous or organic phase to lower the interfacial energy between the discrete and continuous phases to prevent coalescence of droplets, thereby preserving the size monodispersity [28, 22].

The wettability of the channels also plays a vital role in stabilizing droplets as it all but determines which phase will become the continuous phase and which will become the discrete phase, no matter the flow rates [30, 31]. To form water-in-oil (W/O) droplets, the channel walls should be hydrophobic. To form oil-in-water (O/W) droplets, the channel walls should be hydrophilic. In theory, the dispersed phase never touches the channel walls as there is always a thin sheath of continuous phase between the droplets and the walls [31]. Proper wettability is even more important for double and higher order emulsions [31].
2.2.5 Device Materials

PDMS and glass capillaries are the dominant materials for making droplets that will become particles [30], of which PDMS is the more popular [28]. There are several advantages and disadvantages to using PDMS or glass capillaries that must be taken into consideration when choosing the appropriate device material.

PDMS-Based Microfluidic Devices

PDMS is an inexpensive silicon-based polymer that is quite easy to handle for molding designs [28, 22]. Prototype testing of designs can therefore be rapid [31]. The designs are reproducible and can be replicated in large numbers, which is advantageous for scaling up droplet generation [30]. Designs can also be tailored to create detailed new kinds of structures [30].

However, many liquids swell, degrade, or deform PDMS [22, 31], which limits the liquids that can be selected as the fluid phases, particularly when the exposure is for extended periods of time [28]. PDMS is only fully compatible with polar liquids and fluorinated oils [28].

The wettability properties of PDMS devices are even more crucial than those of glass capillary devices because the dispersed phase initially is in contact with the channel walls. [30]. During the droplet formation process, the continuous phase lifts the dispersed phase away from the two side channel walls, but the dispersed phase may remain in contact with the remaining two (the top and bottom). Channel walls that a preferentially compatible with the continuous phase helps the dispersed phase to lift away from the remaining surfaces to become fully surrounded by the continuous phase.
Glass Capillary Microfluidic Devices

Glass capillary devices are made by inserting a circular capillary inside a square capillary, where the outer diameter of the inner capillary matches the inner diameter of the outer capillary. This construction has the advantage of producing truly 3D flow that is ideally coaxial [30], so the dispersed phase never comes in contact with the channel walls. The wettability of the glass, while less crucial than in PDMS, is easily changed through surface modification to make the walls either more hydrophilic or more hydrophobic [30].

The main disadvantage of glass capillary devices is that they are time-consuming to produce so it is difficult to manufacture more than one at a time. There is also limited precision in the production process, so reproducibility is not very good [30].

2.2.6 Device Designs

The two device designs that are most commonly used for microfluidic droplet generation are the T-junction and the co-flow device. The co-flow and flow-focusing designs are sometimes conflated in the literature, but are distinct. Less common designs include step emulsification [28] and electrohydrodynamics [22].

T-Junction

The T-junction design was the first type of microfluidic droplet-maker [30]. It is a perpendicular device [22] wherein the dispersed phase is broken up by the cross-flow of the continuous phase [28] (Figure 2.1A). This design can be created using either PDMS or capillaries.

Co-Flow and Flow-Focusing Devices

The most frequently used design is the co-flow or flow-focusing device (FFD). In a co-flow device, the continuous phase surrounds the dispersed phase, breaking the
Figure 2.1: The basic designs of microfluidic droplet-makers, in both PDMS and glass capillary versions. (A) A T-junction (PDMS or glass capillary). (B) A typical PDMS co-flow design. (C) A typical PDMS flow-focusing design. (D) A co-flow design for a glass capillary device. (E) A flow-focusing design for a glass capillary device.
dispersed into droplets due to the Rayleigh-Plateau instability [28]. A flow-focusing device is a co-flow design that adds an orifice through which the two phases must flow, elongating the finger of the emerging droplet and creating smaller droplets [28].

In comparison with the T-junction, a co-flow of flow-focusing device is more controlled and stable [22] and produces better droplets at high flow rates and droplet generation frequencies [30, 28]. It also is able to produce droplets of a broader range of sizes just by varying the flow rates [28].

A frequently used co-flow design when made with PDMS brings the dispersed and continuous phases together at right angles (Figure 2.1B). By adding an orifice at the outlet side of the junction, the design becomes a flow-focusing device (Figure 2.1C). Co-flow devices made with glass capillaries use parallel flow of the continuous and dispersed phases (Figure 2.1D). To create a flow-focusing device, the tapered end of one capillary becomes the orifice through which the continuous and dispersed phases flow (Figure 2.1E). In this case, the continuous and dispersed phases flow into the same outer capillary from opposite ends.

2.2.7 Cutting-Edge Designer Droplets

The designs described in Section 2.2.6 are basic templates for creating single emulsion (SE) droplets. However, the field of droplet microfluidics has adapted these basic designs to generate very impressive and creative droplets. While it is common to generate single or double emulsion droplets, it is also possible to create higher-order droplets, up to quintuple emulsions, from both PDMS [33] and glass capillary devices [36]. The number of inner droplets in a double or higher-order emulsion droplet can be precisely controlled [36]. Parallelizing the inlet channel in a device can generate droplets with a double core of two separate phases [37]. Additionally, many groups have also published the microfluidic creation of Janus droplets and particles, which are single droplets composed of two hemispheres of different phases [38]. On top of
the droplet diameter control afforded by the co-flow design, group have developed techniques for creating sub-micron droplets through tipstreaming [39], electrohydrodynamic jetting [40], and sorting satellite droplets [41].

Finally, many groups have realized that it is necessary to design higher-throughput devices for scaled-up processing. Nisisako et al. designed a device with 128 T-junctions in a circle within a support holder to supply the phases to all the inlets.
This device can produce several kilograms per day of polymer microparticles. For Des, Nisisako et al. also designed a parallelized device within a support holder and Romanowsky et al. created a parallelized device from multilayer PDMS chip. This step toward scaled-up droplet production is a crucial one for the translation of microfluidic droplets and particles to industrial applications.

2.2.8 Industry Perspective on an Area for Growth

Though this chapter has focused on microfluidic droplet generation, one of the most compelling uses of this technology is to generate particles from the droplets, particularly drug-loaded particles for controlled release applications. Holtze acknowledges that microfluidic droplet generation can improve upon current bulk emulsification technologies to encapsulate hydrophilic drugs, which is the subject of Chapter 3.

2.3 Particles Made from Microfluidics-Generated Droplets

Droplets generated by microfluidic devices have been made into particles by solvent evaporation, solvent extraction, gelation, and solidification. The characteristics of these microparticles are often shown to be superior to those of microparticles made by the conventional method.

2.3.1 Microfluidics-Generated Microparticles Compared with Bulk-Generated Microparticles

Because microfluidic devices manipulate volumes of liquids that are orders of magnitude smaller than those used in bulk procedures, the particles that result from these droplets have characteristics that can be much more precisely controlled. The characteristics of particles that are commonly evaluated are the size, shape, and surface morphology, in both the absolute values and the variation among the particles.
When encapsulating a drug, the encapsulation efficiency, loading level, release curve, and internal drug distribution are also of great interest.

Several groups have substantiated the hypothesis that particles made from microfluidics-generated droplets are more regular and controllable than particles made from bulk-generated droplets, by direct comparison or by comparison with the literature. Xu et al. made SE droplets into PLGA microparticles containing bupivacaine by solvent evaporation and directly compared them with microparticles made by a conventional method [44]. The microfluidic particles were much more monodisperse and exhibited a lower drug burst release in comparison with with conventional microparticles. The size of the microparticles was tuned by changing the flow rates of the two phases.

2.3.2 Drug-Loaded Single Emulsion Particles

Contrast agents, model drugs, and particles have also been loaded into some of the microfluidic-generated particles. When a drug is soluble in the same phase as the polymer, it can be encapsulated in SE droplets. Examples of particles made from SE droplets include: quantum dot, nanoparticle, and tamoxifan-loaded polycaprolactone microparticles [45], 5-fluorouracil-loaded genipin-gelatin microparticles [46], and paclitaxel-loaded microparticles [12]. Hung et al. prepared SE PLGA microparticles loaded with fluorescein dye, evaporating or extracting the solvent on-chip. They demonstrated the effect of PLGA concentration in the dispersed phase on the ultimate size of the microparticles [47].

2.3.3 Drug-Loaded Double Emulsion Particles

Typically, DE droplets are necessary for drug-loaded microparticles when the drug is soluble in an aqueous phase while the polymer that generates the microparticles is soluble in an organic phase. In this case, the drug is in the aqueous inner phase, the polymer is in the organic middle phase, and the outer aqueous phase contains
a surfactant. There are only a few examples of microfluidics-generated DE particles encapsulating a hydrophilic payload in the inner phase, and in each case the encapsulated compound is either a dye or a model drug.

Choi et al. made PLGA microparticles loaded with dyes for better visualization [48]. The glass capillary microfluidic device consisted of a FFD and a T-junction. They added FITC or FITC-dextran to the inner phase and rhodamine 6D to the middle phase of DE droplets to visualize the pore interconnectivity of the resulting PLGA microparticles. They were able to control the diameters of the inner and middle phases of the droplets by modulating the flow rates of those phases. The PLGA microparticles they produced had hollow interiors and porous walls.

Similarly, Kim et al. used a glass capillary microfluidic device to create poly(lactic acid) microparticles with a green dye in the inner phase and a red dye in the middle phase to visualize the droplets and particles [49]. The capsules had an ultrathin polymer wall, which ruptured and released the green dye over the course of about two months.

Windbergs et al. created lipid core-shell particles using a glass capillary microfluidic device [50]. They encapsulated doxorubicin in the core and paclitaxel in the shell and chose a lipid that melts near body temperature. They tested the drug release efficacy with cell viability studies.

2.3.4 Conclusion

While several groups have explored manufacturing drug-loaded microparticles using microfluidics, not many have attempted encapsulating hydrophilic drugs. There is still great room for innovation in making microfluidics-generated polymer microparticles for drug delivery applications.
In this chapter, I detail how I developed a two-chip PDMS-based microfluidic device that generates double emulsion droplets. I used this device to make protein-loaded microparticles and compared bulk and microfluidic preparations of those microparticles according to their size uniformity, encapsulation efficiency, and sustained release. Our hypothesis was that the microfluidics-generated microparticles would have superior characteristics to the bulk-generated microparticles. I also increased the throughput capacity of the chips through their flow rates, chip design, and method of turning the channel walls hydrophilic. Finally, I present two ideas for further improvement of this microfluidic device.

3.1 Introduction

Our goal was to develop a microfluidic system to manufacture double emulsion droplets loaded with a hydrophilic drug in the inner phase. By evaporating the solvent from the polymer middle phase, we turned the droplets into degradable polymer
microparticles. We designed the microfluidic device and the microparticle formulation with the intention of ultimately translating the particles for clinical use. This objective informed our choices of platform material, solvent, drugs, flow rates, etc. Other groups have developed microfluidic systems for generating drug-loaded particles, but they generally use model drugs and materials that would not be appropriate for translation to clinical applications. Only a few groups have addressed manufacturing droplets or particles at a rate that is appropriate for real-world use. There has yet to be any published literature on microfluidic-generated poly(lactic-co-glycolic acid) (PLGA) particles that encapsulate therapeutically relevant drugs. Our focus on using only biocompatible materials for the formulation has provided the largest challenge in achieving our objective, yet it is also what makes this project uniquely impactful.

3.2 Platform Material Choices

The chief materials used for manufacturing microfluidic devices that make droplets are PDMS and glass. PDMS-based microfluidic devices are easy to design and manufacture and therefore are appropriate for applications that might require manufacturing many devices. By using PDMS, we were able to adjust the chip designs easily and alter the droplet characteristics. Glass capillary microfluidic devices take more time and skill to manufacture and there is more variation device-to-device, so they are not as appropriate for scaled-up droplet generation applications [33]. We chose to develop our platform using PDMS devices so that we could iterate the design process precisely and to leave open the possibility of scaled-up droplet generation for clinical applications. (See Section 2.2 for a more comprehensive discussion.)
3.3 Chip Design

I used an empirical chip design process in collaboration with other Leong lab members, chiefly Megan Ho, Ying Zhang, and Michael Keane, to create the microfluidic devices.

I chose a co-flow/flow-focusing device (FFDs) as my droplet-maker over a T-junction because of their superior droplet stability at higher flow rates [28].

Over the course of this project, I have used several variations of FFDs and surrounding chip geometry to successfully create double emulsion droplets using the two-chip system. As long as the primary droplet are stable and smaller than the secondary droplets, DEs form. However, my and Michael Keane’s efforts to design an integrated chip with two FFDs in series were not successful over multiple iterations. This was, possibly, due to our choice of solvents, in combination with imperfectly patterned wettability of the channel walls.

3.4 Formulation Choices

While the groups that drive innovation in microfluidic technology are free to use whatever solvents and drugs generate the most excellent droplets, we have limited our choices to only non-toxic, biocompatible options. Many of the commonly used solvents are therefore not available for our use. We also designed our device to encapsulate therapeutically relevant drugs. The key components that had to be chosen with the application in mind were the drug, the organic solvent, and the polymer.

3.4.1 Drug: Peanut Protein

Therapeutic protein is frequently administered naked as well as encapsulated in polymer microparticles in drug delivery applications. Our collaborators in the Burks lab
deliver naked peanut protein in an immunotherapy protocol. We decided to design microfluidics-generated microparticles to encapsulate peanut protein and release it in a sustained manner and compare them with bulk-generated microparticles.

In this chapter, I discuss the development of our microfluidic platform using peanut protein as the encapsulated drug. Chapter 4 details how we have applied the microparticles to peanut allergy treatment and there are more details about the proteins themselves in Appendix C. Chapter 5 details how we were also able to use this platform to encapsulate chemotherapeutic drugs.

3.4.2 Organic Solvent: Dimethyl Carbonate

I chose dimethyl carbonate (DMC) as the organic solvent for the middle phase. DMC was uniquely capable of fulfilling several requirements of our organic solvent:

- does not swell PDMS [51]
- has a boiling point lower than that of water
- dissolves PLGA
- has low toxicity/is environmentally friendly [47]

I had previously used dichloromethane (DCM) as the organic solvent in similar bulk-generated particles (4.2), but switched to DMC particularly to preserve biocompatibility and prevent PDMS swelling. DMC’s boiling point is 90°C. DMC has a low swelling ratio of 1.03 and low solubility parameter of 9.5 cal^{1/2}cm^{-3/2} in PDMS [51].

3.4.3 Polymer: PLGA

Because we want to focus on clinical translatability, we preferred a polymer that is already FDA-approved. I chose PLGA because it is a thoroughly studied polymer
that is available in many varieties of molecular weights and lactide-to-glycolide ratios, which are the key parameters to choose to tune the polymer degradation rate. Through the course of the project I tested multiple types of PLGA, and for most of it used one with a 50:50 lactide-to-glycolide ratio and a molecular weight of 38 to 54 kDa because should degrade comparatively quickly.

3.5 Manufacturing PDMS Chips

The following is a description of how we made PDMS chips for single and double droplet emulsions. We have tried an integrated double emulsion chip as well as two sequential SE chips for creating the DE droplets. All of the DE data presented in this dissertation has been collected using the two-chip system.

![PDMS chip manufacturing protocol](image)

**Figure 3.1:** The PDMS chip manufacturing protocol. A) The design is printed on a transparency. B) The transparent design is used as a mask to create a positive mold in photoresist on a silicon wafer. C) PDMS is cross-linked on the mold and becomes a block of PDMS with negative channels. D) The PDMS chip is bonded to a coverslip to create the fourth channel wall.
3.5.1 Photolithography

We used standard soft lithography techniques to create PDMS microfluidic chips [52]. We used a photolithography process to transfer 2-D chip designs to a mask, and then the mask became a positive mold onto which PDMS was cured. CAD/Art Services, Inc. printed our designs onto transparencies that were opaque except for the sections that became the negative channels in the PDMS (Figure 3.1A). We spread a photoresist, SU-8 3000, onto a silicon wafer and then exposed the wafer to UV light through the transparency pattern to cross-link the photoresist where the light was transmitted. We then baked the wafer and removed the uncrosslinked photoresist using a solvent (Figure 3.1B). The photoresist depths were 25 µm for the chips that generate the primary emulsions and 50 µm for the chips that generate the secondary emulsions.

3.5.2 PDMS Curing and Bonding

PDMS and curing agent (Sylgard 184 Silicone Elastomer) were mixed at a 9:1 mass ratio and degassed in a vacuum chamber before being poured onto a design template (Figure 3.1C). The PDMS was cured for 1 hour at 65-75°C. Inlet and outlet holes for the water and oil phases were punched through the PDMS. After being rinsed in ethanol and water and drying, the PDMS chips were bonded to glass coverslips using oxygen plasma at 40 W for 30 seconds. Immediately after the exposure to oxygen plasma, the PDMS chips were pressed onto glass coverslips so the three-sided PDMS microfluidic channels were sealed with glass on the fourth side (Figure 3.1D). The chips are baked for one hour at 80°C to complete the bonding process.

3.5.3 Coating to Turn Channel Walls Hydrophilic

Left untreated, the channels of the PDMS chip are hydrophobic and therefore only appropriate for W/O emulsions. For O/W or W/O/W emulsions to form properly,
the channel walls must be treated so that they are hydrophilic [53]. The droplets in this chapter and stages 1 and 2 of Chapter 4 were made with a PVA coating protocol. The droplets in stages 3 and 4 of Chapter 4 and Chapter 5 were made with the PEO-PDMS protocol, detailed in Section 3.10.

After bonding the cured PDMS chips to the coverslips, the chips were coated with 9-10 kDa PVA through a surface adsorption and heat immobilization process [47]. First, the chips were exposed to oxygen plasma at 20 W for 30 s. Second, the channels were filled with 1% PVA solution and left to adsorb for 10 min. Third, the chips were placed in a vacuum chamber for 10 min and after that any remaining PVA solution was displaced with air. Fourth, chips were placed on a 115°C hot plate for 15 min. This process starting with the PVA addition is repeated twice more and finally the chips were baked at 140°C for 20 min.

3.6 Single Emulsion PLGA Particles

We generated blank SE PLGA microparticles using a single FFD to test the development of our devices and formulation and begin to compare microfluidics-generated and bulk-generated particles.

3.6.1 Single Emulsion Droplets Method

I generated O/W droplets with a single FFD coated with PVA (Figure 3.2A). The dispersed inner phase consisted of 2% PLGA (38-54 kDa, 50:50 lactide-to-glycolide ratio) in DMC and the continuous outer phase consisted of 2% PVA in water. Flowing the inner phase at 7.5 µL/min and the outer phase at 25 µL/min resulted in O/W droplets (Figure 3.2B), which were collected for about 1 hour in a round-bottom flask containing 10 mL of the outer phase. I then transferred the droplets to a rotary evaporator, which evaporated the DMC at about 70 mBar while turning the flask at 100 rpm in a room temperature water bath. The resulting particles were collected.
by centrifugation for 5 minutes at 1 krpm, washed three times with deionized water by centrifugation, and lyophilized overnight.

3.6.2 Single Emulsion Particle Characterization

I visualized the microfluidics-generated SE PLGA using SEM (Appendix A.1). The SE microparticles were uniform in diameter and spherical and had smooth surfaces (Figure 3.3A), similar to what had been reported in the literature [44, 47]. The diameter mean and standard deviation of these microparticles was $23 \pm 2 \mu m$ (Figure 3.3B).

3.7 Two-Chip System

To move from generating single emulsion O/W droplets to double emulsion W/O/W droplets, we chose to pursue a strategy of using two FFDs connected in series instead of an integrated chip (Figure 3.4).
Figure 3.3: Empty single emulsion microparticles from a single FFD. A) PLGA microparticles made from single-emulsion droplets. B) Histogram of SE microparticle diameters.

Working with the two-chip system has been advantageous during the formulation and chip design development because the two FFDs are easy to isolate from one another and operate independently as we have altered our methods to get proper droplet generation. The three reasons for this advantage are: 1) it is more straightforward to separately change the three flow rates going into the two FFDs than it is to change all three at once in an integrated chip as we found the appropriate flow rates to generate droplets; 2) it is easier to turn the channels of the second FFD...
Figure 3.4: Schematic of double emulsion droplet generation using a two-chip design. A) The primary FFD generates water (red)-in-oil (yellow) droplets. B) The secondary FFD generates water (red)-in-oil (yellow)-in-water (blue) droplets. C) The output of the first, hydrophobic FFD becomes the input to the second, hydrophilic FFD. D) Double emulsion droplets generated on-chip. E) The double emulsion droplets are collected in 10 mL of the aqueous phase in a round-bottom flask.

hydrophilic when the two FFDs are on separate chips [53]; 3) if a clog develops in either of the FFDs, that chip can be substituted without affecting droplet generation in the other chip.

3.8 Protein-Loaded DE PLGA Particle Development

I developed a method for generating protein-loaded double emulsion microparticles using the two-chip system and detailed a parallel protocol for making double emulsion droplets with a bulk method.
Figure 3.5: Images of DE droplets forming in the primary and secondary FFDs. The inner phase flow rate is 1 µL/min; the middle phase flow rate is 10 µL/min; the outer phase flow rate is 15 µL/min. Scale bars are 100 µm.

3.8.1 Making Microfluidic Droplets and Particles

The first FFD created the W/O emulsion (Figure 3.4A). The inner aqueous phase was 10 mg/mL peanut protein in PBS with 2% PVA as a surfactant. The middle phase was 2% PLGA (38-54 kDa, 50:50 lactide-to-glycolide ratio) in DMC. A short piece of tubing (approximately 5 cm), directly connected the outlet of the first FFD (Figure 3.4C) to the inner phase inlet of the second FFD, so the primary W/O emulsion became the dispersed phase in the second FFD (Figure 3.4B). The continuous phase in the second FFD was 3% PVA. The flow rates used to generate double emulsion droplets were 1 µL/min for the inner aqueous phase, 10 µL/min for the middle organic phase, and 15 µL/min for the outer aqueous phase (Figure 3.4D). Double emulsion droplets from the outlet of the second FFD were collected in 10 mL 3% PVA in a round-bottom flask (Figure 3.4E). Droplets were collected for at least one hour. When droplet collection concluded, the flask was rotary evaporated for 30 min at approximately 70 mBar rotating at 100 rpm in a room temperature water bath to evaporate the DMC. The particles were then collected by centrifugation, washed three times, and lyophilized overnight.
3.8.2 Bulk Protocol with DMC

To make the microparticles by the bulk method as close as possible to the microfluidic method, we used all the same aqueous and organic phases and surfactants in the same volume ratios. I vortexed 180 µL of 10 mg/mL peanut protein with 2% PVA in 1.8 mL of 2% PLGA in DMC. I added the primary emulsion to 30 mL 3% PVA and homogenized it at 2 krpm for 5 minutes using a Polytron 3100 homogenizer. I divided the double emulsion into three roughly equal portions so that the volume of liquid was about the same as what was generated by the microfluidic protocol. Evaporating the organic solvent from these three portions in sequence also simulated the amount of time the microfluidic droplets spend on the benchtop during the collection phase. Each portion of the double emulsion was rotary evaporated for 30 minutes at approximately 70 mBar. The particles were then collected by centrifugation, washed, and lyophilized.

3.9 Characterization of DE Particles

To evaluate our hypothesis that particles generated using microfluidic techniques are superior to those generated using bulk techniques, we performed side-by-side comparisons on the metrics of size distribution, shape, encapsulation efficiency, and release curve of the microparticles. We expected the microfluidics-generated particles to outperform the conventional particles in all metrics.

3.9.1 SEM and Histograms

The protein-loaded microfluidics-generated PLGA particles were more spherical and more uniform in size than bulk-generated particles (Figure 3.6). The microfluidic microparticle diameters mean and standard deviation was $23 \pm 4 \mu m$ and the bulk was $14 \pm 9 \mu m$ (Appendix A.1).
3.9.2 Encapsulation Efficiency

I measured the protein content of the microparticles using an extraction and digestion method [54] (Appendix A.3). Of the total amount of protein put in to the system, 2.3% of it was encapsulated in the microfluidics-generated microparticles and 24% was encapsulated in the bulk-generated microparticles.

We had expected a comparatively higher encapsulation efficiency from the microfluidic microparticles. I ran an experiment to determine where the steps of the microfluidic and conventional protocols differ and to identify which step may be responsible for the reduced encapsulation efficiency. Of the original amount of protein added to the microfluidic system, 70% is present in the supernatant of the collected particles post-rotary evaporation, whereas 57% of the protein is present for the conventional particles. There was no difference in concentration of protein present in the supernatant halfway through the microfluidic droplet collection and when collection was completed. Ten percent of protein fails to emerge from the tubing and chips in the microfluidic system and likely adsorbs onto the surfaces. The protein should theoretically not contact the sides of the channels after the primary droplets
are formed, but it does contact the tubing and the inlet to the first PDMS chip, and protein adsorbs to the uncoated PDMS [55].

From this set of experiments I was not able to identify what about the microfluidic device droplet generation procedure caused the encapsulation efficiency to be so much lower than that of the bulk procedure. I undertook another set of optimization experiments to raise the encapsulation efficiency of the microfluidics-generated microparticles, detailed in Section 4.3.1.
3.9.3 Release Study

I performed a release study to measure the burst and sustained release of protein from both the microfluidics-generated and bulk-generated microparticles (Appendix A.2). Microparticles resulting from at least 1 hour of collection time were resuspended in 1 mL PBS and shaken at 37°C for about one month. At each timepoint, microparticles were centrifuged and the supernatant was removed. Micro BCA assays were performed to evaluate the protein content of the supernatant. At the conclusion of the study, the remaining microparticles were digested and the protein was extracted to determine the amount of encapsulated protein that was still encapsulated (Section A.3).

A plot of the cumulative amount of protein released by each timepoint, normalized by the total amount of protein released and detected for each particle type, reveals a burst release for both particle types as well as sustained release extending over 30 days (Figure 3.7). The microfluidics-generated microparticles have a more sustained release profile than the bulk-generated microparticles. In percentage terms, the slopes of the linear portions of the release curves are 0.82%/day for the microfluidics-generated microparticles and 0.50%/day for the bulk-generated microparticles. The difference between these slopes was not significant at p<0.05 according to a t-test. The burst release from the microfluidics-generated microparticles of 26% of the total encapsulated protein is smaller than the 61% burst from the bulk-generated microparticles. At the end of the release period, 32% of the encapsulated protein remained in the microfluidics-generated microparticles and 7% remained in the bulk-generated microparticles.
3.10 Improvements to Chip Design and Manufacturing to Prolong Runtimes

One of the major objectives for our microfluidic device was to produce drug-loaded microparticles for animal studies. To that end, I wanted to improve the device design so that the devices would operate for longer periods of time without interruptions from clogging or channel wall wetting.

With Michael Keane, I investigated two changes to the secondary chip: the flow-focusing design and the method by which the channel walls were made hydrophilic. Neither of these changes made a discernible impact on the encapsulation efficiency, but they both improved day-to-day usage of the system because the manufacturing process was faster and the droplets were more consistent. This increased throughput because the microfluidic device remained operational for longer stretches of time without having to stop droplet generation.

**Chip Design**

Under my guidance, Michael Keane designed a new FFD for the secondary chip that had a more acute angle between the outer phase input channels and the inner input channels (Figure 4.17). The design we had been using was $90^\circ$ between the channels, but we then tested $45^\circ$ and $30^\circ$ angles and channel widths near the junction of between 30 and 75 µm. Though there was no appreciable difference between the encapsulation efficiencies of the microparticles produced by these various designs, it was apparent that design with 30 and 60 µm channel widths meeting at a $30^\circ$ angle produced the most stable and reproducible droplets.

**Hydrophilic Channel Walls Using PEO-PDMS**

To create hydrophilic PDMS channel walls without a coating procedure, we added an amphiphilic block copolymer surfactant, PDMS-b-PEO (Polysciences 09780-100,
molecular weight 600), to the PDMS along with the curing agent. When exposed to air, the PDMS moieties of the copolymer orient to the surface, and the surface remains hydrophobic. When exposed to water, the copolymer undergoes block re-orientation and the PEO moieties of the copolymer move to the surface [56], turning the channel wall hydrophilic.

To create PEO-PDMS secondary FFDs, we added 1% PEO-PDMS (w/w) to the PDMS base and curing agent before the curing step in the chip fabrication process [57]. After the PDMS was cured, the chips were swelled and de-swelled by subsequent overnight soakings in hexane, acetone, and water and finally air-dried overnight. The PDMS chips were then bonded to the glass coverslips by oxygen plasma ashing at 40 W for 30 s. Within a few hours before use, the bonded chips were exposed to oxygen plasma at 50 W for 60 s.
As the microparticles produced from these chips had the same qualities as the ones made in the PVA-coated chips, it was advantageous to switch. The chip manufacturing time is several times faster for the PEO-PDMS chips, there are fewer wasted chips because of clogging in the coating process, and the resulting channel walls do not have defects from a coating.

For a more detailed discussion of our exploration of PEO-PDMS, see Appendix B.

3.11 Next Steps

The members of the Leong lab and I have made great progress in developing DE PLGA droplets using our two-chip microfluidic system, yet there is still room for advancement of this technology. There are two chief ways I want to continue to innovate the device I use to make hydrophilic drug-loaded PLGA microparticles: on-chip solvent evaporation and using a glass capillary device instead of the two-chip PDMS device.

3.11.1 On-Chip Solvent Evaporation

I would like to transfer the organic solvent evaporation from off-chip (in a rotary evaporator) to on-chip. The solvent evaporation conditions have a great influence over the final characteristics of the microparticles, but they are currently not well-controlled. This would eliminate the last 'bulk' aspect of the protocol and transfer control to the small-volume environment of the microfluidic chip. DMC quickly evaporates when it comes in contact with air. The rotary evaporator is not ideal for solvent evaporation as there is often particle loss during the process. It would be beneficial to develop an on-chip option for solvent evaporation so that we could possibly improve the final microparticle characteristics and reduce loss. This would not preclude controlling the temperature of the evaporation step as the entire device
can be placed on a hotplate.

**Background**

Hung *et al.* evaporated DMC from SE PLGA microparticles on-chip. They added an outlet reservoir downstream of the FFD, from which they collected the microparticles. At the outlet reservoir, the DMC evaporates from the droplets in less than a minute. Included in their report is an image of droplets shrinking to particles as they move across the plane of the outlet reservoir [47].

**Preliminary Work**

First, I verified the rapid evaporation of DMC from SE droplets by pipetting the droplets onto a glass slide and observing the droplets shrinking on the slide (Figure 3.9 A and B).

Second, I attempted to repeat the on-chip solvent evaporation technique from Hung *et al.* I cut a rectangular reservoir downstream of the flow-focusing device on my secondary chips (Figure 3.9C). I generated O/W droplets; the DMC quickly evaporated from the droplets on the leading edge of the phase output (Figure 3.9D). However, the reservoir I created quickly filled up with the outer phase solution (Figure 3.9E). Since the PLGA/DMC droplets are more dense than the PVA outer phase, they stay at the bottom of the chip against the glass coverslip, and the PVA solution fills in on top of them. Therefore, only the leading edge of the droplet-containing phase comes in close enough contact with air to evaporate the DMC through the aqueous phase. As soon as the bottom of the reservoir is covered, there is too much aqueous phase between the droplets and air for the DMC to evaporate. The DE droplets behaved similarly, with those at the leading edge evaporating the organic solvent and those behind staying stable.
Figure 3.9: Preliminary on-chip solvent evaporation. A) SE droplets on a slide exposed to air during solvent evaporation. B) The same SE droplets after solvent evaporation. The images illustrate the droplets shrinking as the DMC evaporates. C) A secondary chip with a reservoir cut out. D) The leading edge of O/W droplets in the reservoir, depicting the DMC evaporation on the edge but not toward the bulk of the fluid. E) Stable droplets under a layer of aqueous solution from which the DMC does not evaporate on a short timescale.
Future Work

We plan to implement on-chip solvent evaporation by another method. Our lab has designed a few different droplet traps, which are PDMS structures that catch droplets as they flow past and allow the outer phase to pass through the chip to the outlet. After generating droplets and catching them in the droplet traps, we plan to replace the aqueous phase with air or alternate aqueous phase with air to evaporate the DMC. Then we can flow water in from the outlet to collect the solid microparticles at the inlet.

A potential drawback of this method is that a field of droplet traps can only hold so much mass of PLGA at a time, reducing overall throughput. Even though the DMC evaporation should take under a minute [47], it will take time to flow the droplets in and the particles out of the droplet trap chip. However, the possible improvements to the microparticle characteristics make this method worth pursuing as there may be other ways to address the throughput issue.

3.11.2 Glass Capillary Microfluidics

Glass capillary microfluidic devices require a great amount of skill and expertise to construct, so we established a collaboration with Nick Carroll at Duke University. Nick was trained in making these types of devices in one of the leading labs in the field. Together we are pursuing making protein-loaded PLGA DE droplets and particles.

Background

One of the advantages of switching our device material from PDMS to glass is that we will have a wider range of organic solvent to choose from. We could return to making microparticles with DCM or use chloroform or other solvents. Also, there is a smaller likelihood of protein adsorption to the glass surface than the PDMS as the
Preliminary Work

Nick Carroll constructed a combination co-flow and flow-focusing DE glass capillary device to test if it was possible to make protein-loaded PLGA droplets with our phases. We tested the device and found that the organic phase easily wet the walls of the glass capillary outlet even though they had been treated to be hydrophilic. We overcame this issue by adding 3.5% DMC to the outer PVA phase and keeping the organic phase flow rate quite low until the inner and outer phases co-flowed.

The flow rates we used to generate the droplets were 1,000 µL/hr for the inner phase, 1,350 µL/hr for the middle phase, and 15,000 µL/hr for the outer phase (Figure 3.10A). The phases were 1 mg/mL peanut protein, 4% PLGA in DMC, and 1% PVA with 3.5% DMC. We collected the droplets and I rotary evaporated them at 100 mBar for 30 minutes. We successfully made microparticles using this method, though they were partially collapsed and rather heterogeneous (Figure 3.10B). This is likely the result of not using optimized phases and solvent evaporation conditions.
Future Work

These results are a promising start; we will continue to optimize protein encapsulation in PLGA microparticles using a DE glass capillary microfluidic device. While the glass capillary microfluidic system does not offer the scale-up potential that PDMS microfluidic devices do, this device in particular has a higher throughput than our current two-chip system (1,000 \( \mu \text{L/hr} \) vs. 240 \( \mu \text{L/hr} \)). It is also likely that after the formulation is optimized, the particles may be more homogeneous than those we make in the two-chip system because of the more increased control we have over the number of inner droplets.

3.12 Conclusion

I was successful in making drug-loaded PLGA microparticles using a two-chip PDMS microfluidic device to generate the double emulsion droplets. The resulting particles are much more uniform in size and shape than bulk-generated microparticles. Though we expected the encapsulation efficiency and burst release to be superior in the microfluidic method, they were comparable between the two methods. I also increased the throughput capacity of the chips by altering the design and manufacturing protocols and increasing the flow rates of the phases.
I have applied the microfluidic technology we developed in Chapter 3 to manufacturing microparticles to modify an immunotherapy protocol in mouse model. I initially manufactured peanut protein-loaded microparticles using a bulk method. Later, I switched to using a microfluidic method so that we would have more control over the particle characteristics and further optimized those microparticles. Mike Kulis, Ben Wright, and Kelly Gewain from the Wesley Burks lab at the University of North Carolina at Chapel Hill provided the peanut protein, conducted the \textit{in vitro} and \textit{in vivo} experiments, and provided feedback on what the optimized microparticle characteristics should be.

This project moved through four stages of microparticle development and application as we iterated our goals with our collaborators in the Burks lab.

In stage 1, we made chitosan-coated poly(lactic-\textit{co}-glycolic acid) (PLGA) microparticles, with and without CpG as a co-encapsulant, using a bulk method and tested them in a prophylactic mouse model of oral immunotherapy (OIT).
In stage 2, we made microfluidics-generated microparticles and tested them using a basophil activation assay.

In stage 3, we increased the loading level of peanut protein in the microfluidics-generated microparticles and completed a safety study of subcutaneous immunotherapy (SIT) in a therapeutic mouse model.

In stage 4, we decreased the burst release of protein from the microfluidics-generated microparticles and completed an efficacy study of SIT in a therapeutic mouse model.

4.1 Background

Food allergy, and in particular peanut allergy, is a growing affliction in the United States, especially among children. While there are protocols in place to desensitize peanut-allergic children to accidental exposure, inducing tolerance to the peanut allergen has only been achieved in about half of the patients who completed one recent study [58]. Several groups have explored using PLGA particles to assist in the treatment of food allergy, but none have yet established a protocol for treating patients with PLGA microparticles encapsulating peanut protein.

4.1.1 Disease Burden

Food allergy is the most common source of anaphylaxis treated in emergency rooms; 90% of US fatalities resulting from anaphylaxis are due to allergic reactions to peanuts or tree nuts [59]. Approximately 6% of children and 3-4% of adults in western countries suffer from some form of food allergy. In the United States, the most common food allergies among children are to milk, egg, and peanut. Peanut allergy is typically lifelong, as only about 20% of children outgrow the allergy, and more recently diagnosed patients are less likely to outgrow the allergy than those diagnosed earlier [60, 61]. There was an 18% increase in food allergy reported in US
children between 1997 and 2007 [62] and the prevalence of peanut allergy has tripled from 1997 to 2008 [59].

The current standard of care for the allergic individual is to avoid exposure to the antigen and to carry an antihistamine and epinephrine, but accidental ingestion remains common [62]. The morbidity of food allergy is significant for the patients, families, schools, and communities [63]. Children on restricted diets for their food allergies suffer from nutritional deficiencies and growth delays. Food-allergic individuals report poorer overall health, more limited social activities, and less vitality than the non-allergic population. Even insulin-dependent diabetics report a higher quality of health-related life than food-allergic patients [59]. The avoidance prescription is not very effective; accidental exposures occur about once every three to five years per patient [64].

4.1.2 Immunology of Peanut Allergy

The immune system actively suppresses the natural immune response to antigens detected through the gastrointestinal (GI) tract [63]. Food allergy is a failure to develop tolerance or a breakdown of tolerance. The allergic reaction is Th2-dominant; Th2 cells secrete IL-4, IL-5, IL-9, and IL-13, which induce B cells to produce food-specific IgE antibodies. IgE binds to receptors on mast cells and upon subsequent exposure to the allergen the mast cells degranulate and an allergic reaction commences [62, 64], typically involving hives, swelling, vomiting, abdominal pain, wheezing, dyspnea, and shock [60].

4.1.3 Peanut Proteins

Researchers have identified thirteen peanut allergens, named Ara h 1 to Ara h 13, of which Ara h 1, 2, 3, and 6 are the most common allergens [65]. The proteins are classified as albumins and globulins. Albumins are water-soluble and include the
congluitin family. Globulins are saline-soluble and include the legumin, vicilin, and glycinin families.

Ara h 1, 2, and 3 are seed storage proteins [66]. Ara h 1 is a vicilin protein that both interacts with the adaptive immune system and promotes Th2-type cell development through adjuvant activity [65]. Ara h 2 is a congluitin protein and Ara h 6 is a homolog of Ara h 2 [65]. Clinical diagnosis of peanut allergy is correlated to the presence of Ara h 2-specific IgE [66] and Ara h 2 and 6 are necessary to induce anaphylaxis in allergic mice [65]. Ara h 3 is a glycinin protein [65]. Further details on the arah proteins can be found in Appendix C.

4.1.4 Immunotherapy

Several immunotherapy (IT) protocols have been developed for a variety of allergies and are widely used, but different allergens and routes of administration present different challenges.

Previous Work

The goal of IT is to desensitize the patient to the allergenic food and ultimately induce a state of tolerance so that accidental exposure does not result in an anaphylactic reaction. While successful immunotherapy protocols have been established for patients with allergies to aeroallergens (pollen, grass, and pet dander) and bee venom, food allergy immunotherapy has only recently shown that one protocols has achieve tolerated in a fraction of patients that completed the course [59, 67, 58].

In IT, increasing amounts of the allergen are administered to the allergic individual until she can be exposed to a substantial amount of the allergen without an adverse reaction, indicating that the effector cells have become nonreactive to the allergen (desensitization) [63]. The protein is administered regularly in a clinical setting, although for the maintenance doses in OIT they can be administered at
home. After ceasing treatment for some time, the patient undergoes a challenge. If there is no allergic reaction, tolerance has been achieved and the nonreactive state is permanent.

While the mechanism is not fully understood, observations of the immune response of patients who achieve desensitization show a decrease in IgE and an increase in food-specific IgG4. Oral tolerance is mediated by T cells [68]; the Th2 allergenic response becomes skewed more toward a Th1 response [62]. One study also found a suppression of mast cells and basophils, an increase in regulator T cells (Tregs) followed by a decrease after 12 months, and changes in cytokine levels [59].

Routes of Administration

Routes of administration of immunotherapy include oral, subcutaneous, epicutaneous, and sublingual. In this project, we used the oral and subcutaneous routes.

Subcutaneous SIT is the traditional approach to allergy treatments. It was first explored in the 1930s and is now commonly known as allergy shots [59]. SIT has long been effective with certain allergens, such as inhalant [68]. A typical response observed with SIT is an increase and then decrease in allergen-specific IgE, an increase in allergen-specific IgG4, and a change from T-helper type 2 phenotype to a type 1 phenotype [60]. However, previous studies attempting SIT for peanut allergy have been discouraging because they achieved only partial efficacy and the patients experienced serious anaphylactic side effects [59].

The SIT protocol in mice involves subcutaneous injections of the antigen at regular intervals in increasing doses. If the mice are being treated with particles, the particles form a depot from which the antigen is released and taken up by dendritic cells.
The GI tract is the largest immunologic organ in the body. It strikes a balance between processing and absorbing useful food while preventing pathogens from reaching the rest of the body. The innate immune system induces mucosal tolerance and regulatory responses for the former and initiates a robust inflammatory immune response for the latter [59]. Oral routes of administration are being investigated because of the risk of severe anaphylaxis through subcutaneous immunotherapy [60].

Several studies out of the Burks lab have demonstrated that OIT is effective at inducing desensitization to peanuts over months and years of treatment. Our collaborators this year were the first to report sustained unresponsiveness or tolerance to peanut protein as a result of an OIT protocol. After OIT for up to five years, 50% of patients who completed the study achieved desensitization, passing a challenge one month after ceasing OIT [58].

The OIT protocol in mice involves oral gavage of the antigen at regular intervals in increasing doses. Some fraction of the antigen is taken up by the M cells, while the rest passes through the GI tract.

The uptake of particles by the M cells in Peyer patches has been studied by several groups. Particles 5 to 10 µm in diameter remain in the Peyer patches, while particles smaller than 5 µm in diameter migrate to the mesenteric lymph node and then the spleen and have the potential to effect immunological changes [69].

4.1.5 PLGA Microparticles as Protein Delivery Vehicle

Protein-loaded microparticles and nanoparticles have been explored for use as vaccines [1, 6, 70, 71], for hormone delivery [11], and for the treatment of allergies [9, 72]. To encapsulate protein in a PLGA microparticle, the most common method is to use a double emulsion (DE)/solvent evaporation process.
PLGA Particles as Antigen Delivery Vehicles

Several groups have used PLGA microparticles or nanoparticles to deliver antigen either subcutaneously or by the oral route, manufacturing the particles using bulk methods. PLGA particles have been used to deliver model antigen, such as ova [71, 73] and BSA [74], and allergens, such as bee venom [75, 76] and olive pollen [72]. These studies have demonstrated that PLGA particles can effect an increased antibody response after exposure to the antigens, even at much lower doses than required by naked antigens. Encapsulating the protein also preserved its immunogenicity [76].

Immunological Impact of PLGA Microparticle Delivery

Antigen delivery through PLGA microparticles reduces the Th2 and IgE responses in favor of a higher Th1 response [72, 76]. This profile reduces the allergic response. It has been shown that PLGA itself can play the role of an adjuvant in promoting a Th1 response, whereas other adjuvants such as aluminum salts promote a Th2 response [76].

Co-Encapsulating Adjuvant

To increase the effectiveness of vaccines, several groups co-encapsulated CpG with the delivered antigens to lower the dose threshold needed to induce an allergen-specific immune response [76]. CpG, a pathogen-associated molecular pattern (PAMP), has been shown to shift the immune response toward Th1 by binding to TLR9 and activating NFκB [76]. The groups that co-encapsulated CpG with antigen stabilized the CpG using protamine [76] or DOTAP [73]. The encapsulation efficiency of the antigen actually increased in the presence of CpG [73]. Encapsulating the CpG along with the antigen was also shown to be more effective at inducing an antibody response than simply admixing the CpG with naked antigen [76].

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4.2 Stage 1

Based on previous work by other groups, we hypothesized that encapsulated peanut protein would be more effective than naked peanut protein at inducing desensitization and tolerance to peanut allergy and that co-encapsulating CpG would enhance effectiveness. We manufactured peanut protein-loaded microparticles using a bulk method to test the efficacy of feeding mice peanut protein-loaded microparticles against feeding naked protein in a prophylactic model of peanut allergy. We also tested the co-encapsulation of CpG as an adjuvant.

We chose the oral route for our initial attempt at desensitizing peanut-allergic mice using encapsulated protein.

We hypothesized that encapsulating the peanut protein in microparticles would:

- protect the protein from degradation in the GI tract
- present the peanut antigens to the immune cells in a different and possibly more effective manner
- provide an adjuvant effect (from the particles themselves as well as the CpG)
- reduce the frequency of administration necessary
- reduce the time to the induction of tolerance

Additionally, we hypothesized that adding a chitosan coating to the PLGA microparticles would make the particles more mucoadhesive [77], therefore prolonging the residence time in the gut and increasing the amount of antigen passed through to the Peyer patches. Because particles below 5 µm in diameter can be taken up by the M cells in the gut, which would then pass the antigen to the antigen-presenting cells in the Peyer patches, our goal was to produce microparticles in that size range [78].
4.2.1 *Peanut Protein-Loaded Microparticles by a Bulk Method*

We made peanut protein-loaded PLGA microparticles using a bulk DE/solvent evaporation technique. We modified the protocol so that our engineered microparticles encapsulated CpG and bore chitosan on their surfaces.

*Development of PLGA Microparticles Double Emulsion Protocol*

We developed the PLGA microparticles by optimizing the components of the three phases of the DE and the emulsifying methods themselves. The inner phase was 10 mg/mL peanut protein, the middle phase was PLGA in dichloromethane (DCM), and the outer phase contained PVA as a surfactant.

*Optimization* To optimize the conventional DE PLGA microparticles protocol for our purpose of delivering peanut protein, I tested various phase formulations and emulsifying methods. The basic protocol of emulsifying the protein in an organic phase containing PLGA and then emulsifying the product in an aqueous phase containing 1% PVA was kept throughout, but I attempted several modifications to make the particles more spherical, to give them a smooth surface, to increase the encapsulation efficiency, and to decrease the burst release of protein. The parameters I tested were:

- PLGA: lactide-to-glycolide ratio, molecular weight, concentration
- outer phase properties: volume, pH, additives (chitosan, NaCl, trehalose)
- emulsifying method: vortexing, homogenizing, vigorous pipetting
- method for evaporating the organic phase: stirring, rotary evaporation

The PLGA varieties I tested had molecular weights and lactide-to-glycolide ratios of: 38-54 kDa 50:50, 24-38 kDa 65:35, 4-15 kDa 75:25, 76-115 kDa 75:25. The PLGA
concentrations I tested were between 1 and 5%. I used outer phase volumes between 4 and 50 mL. I varied the pHs between 4.4 and 7.0. The chitosans I tested were purchased from Vanson, Fluka, Aldrich, Wako, and Kimica. The primary emulsion was always formed by vortexing, but I tested forming the secondary emulsion by vigorous pipetting and homogenizing between 0.5 and 3 krpm. To evaporate the organic phase, I either stirred the emulsion overnight or rotary evaporated at 70 mBar for 15 minutes over a room temperature water bath rotating at 100 rpm.

**Co-Encapsulating CpG** CpG oligodeoxynucleotides are short, unmethylated single strands of DNA that contain a ”CG” pair of nucleotides. CpG is a PAMP; TLR9 receptors bind CpG and activate the innate immune system. We used class B CpGs as an adjuvant to stimulate B cell and monocyte maturation. We co-encapsulated CpG with the peanut protein by simply combining the CpG solution with the peanut protein solution. We calculated the amount of CpG and peanut protein to add by using the encapsulation efficiency measurement and solution concentrations to obtain a mass ratio of 10:1 peanut protein:CpG in the CpG-loaded microparticles. In the CpG group, 50% of the particles we delivered had only peanut protein and 50% had peanut protein and CpG, for an overall peanut protein:CpG ratio of 20:1.

**Microparticle Final Formulation**

We generated optimized protein-loaded microparticles by a bulk DE/solvent evaporation process and then characterized them for use in an animal model of peanut allergy.

**Method** The inner phase for the peanut protein-loaded microparticles was 10 mg/mL peanut protein. The inner phase for the peanut protein and CpG-loaded microparticles was a 1:1.15 volume ratio of peanut protein solution to CpG solution.
The middle phase was 5% PLGA (75:25 lactide-to-glycolide ratio, 56-118 kDa) in DCM.

To make the outer phase, I suspended 0.5% Vanson chitosan in 2% acetic acid and filtered overnight. I added 0.9% NaCl and 1% PVA to the chitosan solution and adjusted the pH to 4.4.

To form the primary emulsion, I vortexed 400 µL of the inner phase in 2 mL of the middle phase. To form the secondary emulsion, I transferred the primary emulsion into 50 mL of the outer phase as it was being homogenized at 2,000 rpm. I homogenized the secondary emulsion for 5 minutes, then transferred the DE to a round-bottom flask. I rotary evaporated the solution for 15 minutes at about 70 mBar and 100 rpm over a room temperature water bath. I collected, washed, and lyophilized the resulting microparticles overnight.

Characterization I characterized the microparticles using SEM (Appendix A.1), a release study (Appendix A.2), and a total protein assay (Appendix A.3).

**Particle Size and Shape** The microparticles were spherical and had a smooth surface, but where heterogeneous in size. The diameter of the particles, measured from SEM images, was 6 ± 7 µm (Figure 4.1).

**Release Study** The release study showed a burst release of protein, followed by a roughly linear release from day 2 to 7, and finally the release trailed off between 10 and 30 days (Figure 4.2). The burst release was approximately 3% of the total encapsulated protein and only 18% of the encapsulated protein was released within one month. I determined the amount of encapsulated protein by a total protein assay (Appendix A.2).
Figure 4.1: SEM of peanut protein-loaded microparticles made using the bulk method. Scale bar is 20 µm.

Figure 4.2: Release curve of peanut protein-microparticles made using the bulk method.


**Encapsulation Efficiency**  The microparticles had an encapsulation efficiency of 66% for peanut protein and 57% for CpG. The measurement was performed by digesting the particles and measuring the protein loading with a micro BCA assay (Appendix ??) and the CpG loading using a NanoDrop.

**Loading Level**  The protein loading level of the peanut protein-loaded microparticles was 295 $\mu$g/mg and the protein loading level of the peanut protein and CpG-loaded microparticles was 117 $\mu$g/mg.

4.2.2 **Preliminary Animal Study**

Using the microparticles I manufactured, our collaborators conducted a pilot prophylactic animal study to evaluate the possibility of delivering peanut protein using PLGA microparticles with and without a co-encapsulated adjuvant.

**Methods for Animal Study**

Our collaborators in the Burks lab conducted a pilot animal study to evaluate the efficacy of delivering peanut protein encapsulated in microparticles in comparison with naked peanut protein. They performed a prophylactic study, wherein mice were treated with peanut protein to tolerize their immune systems to the antigen once per day for three days before a peanut allergy was induced with a combination of naked peanut protein and cholera toxin (Figure 4.3). Following sensitization, the mice were bled to evaluate blood IgE and IgG2a antibody levels. The mice were then challenged with peanut protein intraperitoneally and their body temperature and allergic symptom scores were recorded. The anaphylaxis scores are: no response (0), scratching (1), puffiness (2), wheezing (3), inactivity (4), and death (5). The mice were sacrificed and their splenic cells cultured for cytokine and Treg measurements.

There were seven experimental groups of five mice each:
Figure 4.3: The stages of the prophylactic animal study and the days of various interventions. Treatments (naked protein, protein-loaded microparticles, protein-and CpG-loaded microparticles, and sham) were administered on days 1, 2, and 3. All groups save naïve were sensitized to peanut protein by co-administration of peanut protein and cholera toxin orally over three weeks. Mice were bled to measure Ig levels and then challenged with peanut protein and their symptoms were recorded. Finally, mice were sacrificed and the splenic cells were cultured for an evaluation of T cells.

- naïve (no treatment, no sensitization to peanut protein)
- sham-tolerized
- 1 mg naked peanut protein
- 5 mg naked peanut protein
- 1 mg encapsulated peanut protein
- 5 mg encapsulated peanut protein
- 1 mg encapsulated peanut protein and 50 µg CpG

The masses listed indicate the treatment dosage administered on each of days 1, 2, and 3.

Results of Animal Study

Based on some of the results, the microparticle encapsulation strategy outperformed the naked protein delivery strategy. However, to fully confirm our results another control could is necessary to clarify the mechanism with respect to the adjuvant. The additional group would be 1 mg of naked peanut protein along with 50 µg of CpG
Figure 4.4: Selected results from the prophylactic animal study. A) Symptom scores post-challenge. B) Body temperature drop post-challenge. C) Peanut-specific IgE level after sensitization. D) Peanut IgG2a level after sensitization. CD4+, CD25+, and Foxp3+ regulator T cells cultured in E) RPMI medium and F) CPE medium. Results and plots prepared by Michael Kulis.
delivered either orally or intraperitoneally to properly control for the microparticles that encapsulated both.

Upon challenge with peanut protein, the only groups with symptom scores and drops in body temperature as low as the naïve (non-allergic) mice in response to the challenge were the peanut protein and CpG microparticles group (both symptom score and body temperature) and the 5 mg dose of naked protein group (symptom score alone) (Figure 4.4 A and B). The desensitization of the groups dosed with microparticles with 1 and 5 mg peanut protein was comparable to that of the 1 and 5 mg of naked protein groups. The antibody, cytokine, and Treg results indicated that the microparticles had helped to shift the immune response from Th2 toward Th1, though ideally both Th1 and Th2 responses would be suppressed in favor of a Treg response (Figure 4.4 C, D, E, and F). The peanut protein and CpG group was most effective in bringing the mice toward desensitization. However, an unencapsulated control group in parallel to that group is needed to confirm the efficacy of the microparticles (Figure 4.4).

Since the M cells in the hypothesized delivery route only take up microparticles under 5 µm in diameter, it is likely that only a fraction of the antigen fed to the mice was eligible for uptake. In that case, the effective dosage of encapsulated antigen would be much smaller than if the microparticles had all been in the correct size range. If a smaller dosage of encapsulated microparticles could have the same efficacy as a full dose of naked antigen, that would mean that the encapsulated antigen is likely more effective in desensitizing the mice, even without co-encapsulating CpG.

4.2.3 Conclusions

Based on the results of the pilot prophylactic animal study, we decided to continue studying the treatment of peanut protein allergy with a modified OIT protocol using peanut protein-loaded microparticles. To give the microparticles a better chance of
being taken up by the M cells of the gut, we decided to switch from manufacturing the microparticles using a bulk method to making them with a microfluidic method (Chapter 3). The microfluidic method can produce microparticles with a much narrower size range, and our goal was to reduce the average particle diameter to 1 to 5 \( \mu m \).

4.3 Stage 2

Because of the promise of the microparticles from stage 1, we developed a method for manufacturing peanut protein-loaded microparticles over which we had better control over the droplet characteristics that translate to the particle diameter, size distribution, and drug release profile. Our goal was to slowly release protein over several days to two weeks. The requirement of our proposed delivery route of M cell uptake of the microparticles restricts the microparticle diameter to below 5 \( \mu m \). We pursued designing a microfluidic platform for microparticle generation to that end (Chapter 3).

For the microfluidics-generated microparticles developed in Chapter 3 to be used in animal studies, I needed to increase the encapsulation efficiency, which I did through a series of experiments. My goal was to increase the encapsulation efficiency above 50%.

Before resuming animal experiments, we decided to test the microparticles and released peanut protein in vitro. We wanted to verify that the particles alone did not activate basophils of allergic and healthy people, that the released protein was bioactive, and that the released protein activated basophils of allergic individuals at a similar level to naked peanut protein. Our collaborators in the Burks lab adapted a basophil activation assay for this purpose.
4.3.1 Particle Development

The microparticles developed in Chapter 3 had a low encapsulation efficiency of 2.3%. I increased the encapsulation efficiency above 50% and characterized the resulting microparticles.

Increasing Encapsulation Efficiency

Increasing the encapsulation efficiency was necessary for any clinical translation so that the system would have a high enough throughput to be practically useful and to not waste drug material. In a series of experiments, I tested many ideas for increasing encapsulation efficiency. Experiment 1 showed me which ideas were most promising, which I further explored in Experiments 2 through 4.

Ideas [79] provides a detailed review of the parameters than influence the encapsulation efficiency of a drug in a polymer microparticle. The attributes that contribute to fast solidification of the polymer matrix and therefore a high encapsulation efficiency are: low solubility of the polymer in the organic solvent, high solubility of the organic solvent in the aqueous phase, high concentration of polymer, low continuous phase to dispersed phase volume ratio, and fast solvent removal rate. Other factors identified by [79] that increase encapsulation efficiency are high stability of the primary emulsion and when the inner phase has a small volume or high viscosity. PLGA that has a lower lactide-to-glycolide ratio, higher molecular weight, hydrophilic end caps, and is in higher concentration will also increase encapsulation efficiency. Altering the pH of the outer phase can also dramatically alter the encapsulation efficiency [80].

Experiment 1 I made a number of small, independent changes to the microfluidics-generated microparticles protocol to test which ones would have the greatest effect on
the encapsulation efficiency. I measured the amount of protein encapsulated in the particles using a total protein assay and in the supernatant of the collection fluid by microBCA assays. I took the previous protocol as the control for this experiment so all changes and differences in encapsulation efficiency are relative to that particular formulation.

Parameters Altered I decreased the mass ratio of protein to PLGA both by decreasing the peanut protein concentration (from 10 mg/mL to 5 mg/mL and 1 mg/mL) and by increasing the PLGA concentration (from 2% to 4%).

I tried to increase the stability of the primary emulsion by using 5% Tween 80 as the surfactant instead of 2% 25 kDa PVA.

I also increased the pH of the outer phase to pH 9.

To increase the ratio of the continuous phase to the dispersed phase, I increased the outer phase flow rate from 15 µL/min to 50 µL/min. I also decreased the tubing length from the outlet of the second FFD to the collection from about 50 cm to 10 cm (Figure 3.4) so that the droplets would be exposed to the greater volume of collection fluid as quickly as possible, going from about 2 minutes down to about 20 seconds.

I switched out my previously used chip design pair for one that generated one-to-one droplets (Figure 4.5). The flow rates I used to generate these droplets were 1 µL/min for the inner phase, 3 µL/min for the middle phase, and 12.5 µL/min for the outer phase.

By using different round-bottom flasks, I achieved higher and lower pressures in the flask during rotary evaporation; this should have a direct effect on the solvent evaporation speed. The pressure I compared against was about 70 mBar; the lower pressure was about 55 mBar and the higher pressure about 100 mBar.
Figure 4.5: Achieving a one-to-one ratio of inner to outer droplets in the two-chip DE device. A) Droplet generation in the primary chip. B) Droplet generation in the secondary chip. Scale bars are 100 µm.

Figure 4.6: Experiment 1; the encapsulation efficiencies of a variety of formulation parameters, normalized to the encapsulation efficiency of the control particles.
Figure 4.7: FFDs in primary chips. The 15 µm vs. 35 µm inlet channel widths were tested in encapsulation efficiency optimization experiment 2.

Results  The results showed that the most dramatic increases in protein encapsulation were from doubling the PLGA concentration (3.3-fold increase), lowering the rotary evaporation pressure from approximately 70 to 55 mBar (2.5-fold increase), decreasing the peanut protein concentration from 10 mg/mL to 1 mg/mL (3.5-fold increase), and increasing the pH of the outer phase to pH 9 (1.7-fold increase) (Figure 4.6).

Combining these improved conditions together resulted in a new method, which raised the encapsulation efficiency of the microfluidic microparticles to 35 ± 4%.

The updated method was: The inner aqueous phase was 1 mg/mL peanut protein in PBS with 2% PVA as a surfactant. The middle phase was 4% PLGA (38-54 kDa, 50:50 lactide-to-glycolide ratio) in DMC. The outer phase was 3% PVA adjusted to pH 9. The flow rates used to generate DE droplets were 1 µL/min for the inner aqueous phase, 10 µL/min for the middle organic phase, and 15 µL/min for the outer aqueous phase. DE droplets from the outlet of the second FFD were collected in 10 mL 3% PVA in a round-bottom flask for at least one hour. When droplet collection concluded, the flask was rotary evaporated for 30 minutes at approximately 55 mBar rotating at 100 rpm over a room temperature water bath to evaporate the DMC. The particles were then collected by centrifugation, washed three times, and lyophilized overnight.
Experiment 2 I examined the microfluidic chip-specific parameters in a second round of experiments.

I increased the width of the inner phase inlet on the first FFD from 15 µm to 35 µm (Figure 4.7). This produced larger primary droplets; without changing the flow rates, this increased the encapsulation efficiency 3.3-fold. Additionally the chips clogged less frequently as the smallest feature on the chips had just doubled in size.

Decreasing the inner phase flow rate from 1 µL/min to 0.5 µL/min increased the encapsulation efficiency 2.8-fold while increasing the flow rate from 1 µL/min to 2 µL/min decreased the encapsulation efficiency by more than a factor of 2, indicating that the lower inner phase flow rate had an additional encapsulation efficiency improvement beyond what could be accounted for by the decreased mass ratio of protein to PLGA (Figure 4.8). As I kept the flow rate of the middle phase constant at 10 µL/min, this was likely because of the lower volume ratio of the inner to middle phases.

Figure 4.8: Experiment 2; the encapsulation efficiencies of the microfluidic chip-specific parameters, normalized to the encapsulation efficiency of the optimized formulation from Experiment 1.
Experiment 3  A study of outer phase pHs from 4.0 to 8.0 at 0.5 increments showed that encapsulation efficiencies did not vary dramatically (Figure 4.9). pH 6.0 had the highest encapsulation efficiency (123% of the control) while pH 8.0 was the lowest (77% of the control).

Experiment 4  Further experimentation with rotary evaporation conditions explored pressure setpoints from 50 to 100 mBar and water bath temperatures from room temperature up to 50°C. This series of experiments yielded the best encapsulation efficiency with a 100 mBar pressure setting and a room temperature water bath, but the overall improvements were not dramatic (Figure 4.10). However, the pH also affected the macro presentation of the microparticles, as some microparticles clumped more or created a foam that was less dense than water upon resuspension.
After incorporating the results from Experiments 1 through 4 into a new microparticle formulation, I characterized the resulting microparticles with SEM images followed by a release study from which I calculated the encapsulation efficiency and loading level.

**Encapsulation Efficiency-Optimized Formulation**  The final formulation for the microfluidic microparticles used the larger-width inlet channel in the first FFD with a rotary evaporator pressure setting of 100 mBar over a room temperature water bath. The inner phase was 1 mg/mL peanut protein with 2% PVA 25 kDa flowed at 0.5 µL/min. The middle phase was 4% PLGA 50:50 38-54 kDa in DMC flowed at 10 µL/min. The outer phase was 3% PVA 25 kDa adjusted to pH 7.0 flowed at 15 µL/min (Figure 4.11).
Figure 4.11: Images of droplets forming in the primary and secondary FFDs for the encapsulation efficiency-optimized formulation. The inner phase flow rate is 0.5 \( \mu \text{L/min} \); the middle phase flow rate is 10 \( \mu \text{L/min} \); the outer phase flow rate is 15 \( \mu \text{L/min} \). Scale bars are 100 \( \mu \text{m} \).

Figure 4.12: Encapsulation efficiency-optimized microparticles from stage 1. Scale bar is 200 \( \mu \text{m} \).

Particle Visualization  The microfluidic microparticles are very uniform in size and shape and spherical and smooth (Figure 4.12). The diameter mean and standard deviation was 33 \( \pm \) 11 \( \mu \text{m} \).

Release Study  There was about a 25\% burst seen on day 1 and ultimately about 90\% of the encapsulated protein was released (Figure 4.13).
Figure 4.13: The cumulative release of peanut protein from the encapsulation efficiency-optimized microparticles. The microfluidic particles (blue) released 90±4% of encapsulated protein and the conventional particles (red) released 89±3% of encapsulated protein.

**Encapsulation Efficiency** The encapsulation efficiency was 52±3%, which met our goal. This value was calculated by adding the cumulative protein released in the release study to the protein remaining in the microparticles at the end of the release study, as measure by a total protein assay (Section A.3). This increase in encapsulation efficiency came from both changes to the microfluidic device and changes to the formulation phases and solvent evaporation process.

**Loading Level** The loading level was 1.8 μg/mg.

**Conclusion** Through a series of optimization experiments, I was successful in increasing the encapsulation efficiency of the microfluidics-generated microparticles by
over 20 times. A few of the formulation adjustments were to the phases or the solvent evaporation parameters and therefore would directly translate to a bulk protocol as well, but some of them were specific to the microfluidics method of droplet generation such as chip design parameters and flow rates. The microparticles were very uniform in size and shape. The resulting release curve was quite sustained and linear, with a low burst release and a high overall fraction of protein released over two weeks.

4.3.2 In Vitro Experiments

We applied these encapsulation efficiency-optimized microparticles to an in vitro basophil activation experiment. Whole blood samples were taken from peanut-allergic and non-allergic individuals. Basophils from allergic individuals have antigen-specific IgE expressed on their surfaces. When the antigen binds to the IgE, CD63 and CD203c are upregulated. anti-CD63-FITC and anti-CD203c-PE antibodies were applied to the whole blood and the cells were analyzed using flow cytometry.

Methods

Basophils were dosed with naked peanut protein and protein-loaded microparticles at 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, and 0.001 µg/mL. Other basophils were dosed with an equivalent mass of empty microparticles as the most massive loaded particle dose. The negative control was basophil media alone and the positive control was anti-IgE. CD63 and CD203c expression on the basophil surface was measured using flow cytometry to assess the activation levels.

The peanut protein-loaded microparticles and controls were administered to the basophils of four allergic individuals and one non-allergic control.

Experimental Results

The CD63 and CD203c expression, indicating activation, from each of the patients varied, but an overall pattern emerged. (Experiment was conducted and figures were
Subject 1 showed activation from the empty microparticles, which was intended to be a negative control. The activation was higher than that of the protein-loaded microparticles. However, none of the other patients showed high activation from the empty microparticles, so we regarded the result from that group as spurious.

The controls for subject 2 were consistent with expectations, and the activation from the protein-loaded microparticles was less than that from naked protein at 0.001, 0.01, and 0.1 µg/mL but more at 1 µg/mL (Figure 4.14).

Subject 3 and subject 4 were both autoreactive and subject 4 showed low CD63 expression across all conditions.

Across all four subjects, the CD63 expression was slightly lower at 0.1 µg/mL for the peanut protein-loaded microparticles than for the naked peanut protein (Figure 4.14).
Figure 4.15: Fraction of basophils expressing CD63 at 0.1 µg/mL dosages of naked peanut protein (PN, darker green) and peanut protein microparticles (PNMP, lighter green).

4.15). This indicates that the protein released from the microparticles was bioactive, but also likely that not all of the protein had been released from the microparticles.

4.3.3 Conclusions

From these \textit{in vitro} experiments, we analyzed the bioactivity of the protein and clarified our next set of goals for the microparticles before continuing animal experiments in stages 3 and 4.

Peanut Protein Release and Bioactivity

The encapsulated peanut protein appears to be bioactive because the basophils were activated by the microparticles at about the same level as the naked protein. Also,
the microparticles themselves did not activate the basophils because the empty microparticles produced similar activation to the basophil media (the negative control) for subjects 2, 3, and 4 and the non-allergic control. We also confirmed that the basophil activation was generally lower from the peanut protein-loaded microparticles than the naked peanut protein, which is consistent with the hypothesis that the peanut protein is just as bioactive but is more slowly released from the particles.

**Increase Loading Level**

Because of the peanut protein loading level of the microparticles of 1.8 µg/mg, we decided to change the particle formulation before going forward with stage 3. To administer the desired dose of peanut protein to a mouse with the current microparticles, we would need to use almost one gram of microparticles. It is not feasible to dilute that mass of particles in 1 to 2 mLs to administer to a mouse.

Based on a review of protein delivery in the literature, we set a goal to raise the particle loading level to 20 to 40 µg/mg [81, 82, 83].

**Increase Throughput**

To complete a safety study in mice, we would need about 22.5 mg of peanut protein encapsulated in microparticles. To estimate the amount of time it would take for our microfluidic system to generate this mass of encapsulated protein, I used

\[
    t = \frac{m}{Q \times c \times E}
\]

where \( t \) = time, \( m \) = mass of protein encapsulated, \( Q \) = flow rate, \( c \) = protein concentration, and \( E \) = encapsulation efficiency. In the formulation used to generate these high-encapsulation efficiency particles (Section 4.3.1), \( Q = 0.5 \) uL/min, \( c = 1 \) mg/mL, and \( E = 0.52 \). Encapsulating 22.5 mg of peanut protein in the encapsulation efficiency-optimized microparticles would therefore take approximately one year of working days to produce, which is too long to be practical.
Since that generation time is too long to be useful for a clinical setting, I sought to increase the throughput of our system, defined as the mass of encapsulated protein generated per minute running the system. Since $E$ had already been optimized, I explored how to increase $Q$ and $c$. $Q$ can be increased either by adjusting the flow rate setting on the pumps or by parallelizing the microfluidic chip pairs.

4.3.4 Change Route of Administration

Together with our collaborators in the Burks lab, we decided to test the peanut protein-loaded microparticles against naked protein delivery using the SIT model instead of the OIT model. Our collaborators suggested that the difference between the naked and encapsulated protein delivery methods would be easier to distinguish in the SIT model as opposed to the OIT model because the oral route of delivery has many factors that might obscure the comparison such as cell uptake. Because of this change, we did not have to reduce the diameter of the microparticles to under 5 $\mu$m or add a chitosan coating, which left other parameters such as the drug release profile more free to be optimized.

4.4 Stage 3

In stage 3, we increased the loading level of our microfluidic peanut protein-loaded microparticles, decreased the generation time of the microparticles, and characterized the resulting microparticles. Our collaborators tested the SIT model by completing a safety test in a mouse model of peanut allergy.

4.4.1 Particle Development

After optimizing the encapsulation efficiency of the peanut protein-loaded microfluidics-generated microparticles (Section 4.3.1), I had three objectives:

- raise the loading level of the microparticles to 20 to 40 $\mu$g/mg
- decrease the generation time so that the particles for the animal study could be generated within two work weeks
- keep the encapsulation efficiency above 20%

Increasing Loading Level

To increase the theoretical loading level of the particles - the ratio of the mass of peanut protein added to the microfluidic system to the mass of PLGA added to the system - I made two simple changes: 1) I increased the concentration of peanut protein in the inner phase from 1 mg/mL to 12 mg/mL (the maximum concentration provided by the Burks lab). 2) I increased the flow rate ratio of the inner (peanut protein) phase to the middle (PLGA) phase from 0.5:10 to 2:10. The 2:10 ratio was the highest possible for primary droplet formation in our chips. However, while these changes increased the theoretical loading level, they also decreased the encapsulation efficiency to about 10%, also due to the higher ratio of protein mass to polymer mass. Therefore, I had to further optimize the encapsulation efficiency.

The biggest jump in encapsulation efficiency and loading level came from adding NaCl to the outer phase; I tested 1, 2, and 5%. The encapsulation efficiency increased 3.5-fold when 2% NaCl was added, in comparison with 0% NaCl.

Increasing Throughput

The changes I made to increase the theoretical loading level, increasing the inner phase flow rate to 2 µL/min while keeping the middle phase flow rate at 10 µL/min, also served to increase the throughput, defined as mass of protein encapsulated per time. The increased concentration of the peanut protein and increased inner phase flow rate each contributed to increasing the throughput. To further increase the inner phase flow rate, I doubled both the inner and middle phase flow rates and nearly doubled the outer phase flow rate. Increasing the inner phase flow rates increased
the overall encapsulation efficiency (Figure 4.16).

The final higher-throughput flow rates were: 4 µL/min for the inner phase, 20 µL/min for the middle phase, and 35 µL/min for the outer phase. Our primary chips would not support a higher inner phase flow rate than 4 µL/min while still forming regular droplets no matter what the middle phase flow rate. In the updated throughput variables, $c$ increased from 1 to 12 mg/mL and $Q$ increased from 0.5 to 4 µL/min. The encapsulation efficiency decreased the Phase 2 levels, but the change did not counteract the other increases.

**Characterization**

I characterized the loading level-optimized microparticles through SEM, a release study, and calculating the encapsulation efficiency and loading level.
Figure 4.17: Images of droplets forming in the primary and secondary FFDs for the loading level-optimized formulation. The inner phase flow rate is 4 $\mu$L/min; the middle phase flow rate is 20 $\mu$L/min; the outer phase flow rate is 35 $\mu$L/min. Scale bars are 100 $\mu$m.

**Formulation Parameters** The loading level-optimized formulation phases were 12 mg/mL peanut protein for the inner phase, 4% PLGA (50:50 lactide-to-glycolide ratio, 38-54 kDa) for the middle phase, and 3% PVA 25 kDa 5% NaCl pH 7.0 for the outer phase. The flow rates were 4 $\mu$L/min for the inner phase, 20 $\mu$L/min for the middle phase, and 35 $\mu$L/min for the outer phase (Figure 4.17). The rotary evaporation pressure was sent to 100 mBar and rotated at 100 rpm over a room temperature water bath. The microparticles were collected by centrifugation, washed three times, and lyophilized overnight.

**Particle Shape and Size** The microfluidic microparticles have a smooth surface and are regular in size (Figure 4.18). This formulation of microfluidic-generated microparticles was not as regular and spherical as the microparticles made in Chapter 3, perhaps because of the higher flow rates and protein concentration.

**Release Study** The release curves show a reverse result of what we obtained in Chapter 3. The microparticles had a high burst release and an overall high fraction of
Figure 4.18: SEM of loading level-optimized microparticles. Scale bar is 100 µm.

Figure 4.19: Release profile of loading level-optimized microfluidic microparticles.
encapsulated protein released. The release on day 1 was 84% of the total encapsu-
lated protein and the overall fraction of protein released from these microparticles
was 100.0%.

*Encapsulation Efficiency* We measured the protein encapsulation efficiency by sum-
mimg all the protein accounted for during the release study with the result of a
total protein assay on the particles remaining after two weeks (Appendix A.3). This
method showed a 31 +/- 3% encapsulation efficiency for the microparticles.

*Loading Level* I measured the loading level by assessing the total protein content of
an accurately measured mass of microparticles through a release study and a total
protein assay (Section A.3). The microparticle protein loading level was 37 ± 4
µg/mg assessed after the two-week release study.

### 4.4.2 Mouse Experiments

Our collaborators in the Burks lab conducted a safety study using a therapeutic
mouse model, administering the protein subcutaneously. The study tested whether
the microparticle formulation was as safe as the naked formulation with a single
administration of the drug.

*Methods*

In the therapeutic mouse model, the mice are made allergic to peanut protein by
sensitization with repeated high doses of peanut protein with cholera toxin. The
allergy is confirmed by the presence of anti-peanut IgE, IgG2a, IgG1, and total IgE.
The therapeutic model is more similar than the prophylactic model to the ultimate
clinical goal of treating peanut allergy in allergic patients.

The maximum tolerated dose of naked peanut protein and peanut protein-loaded
microparticles was determined in two groups of mice. Each other group of mice
was then subcutaneously challenged with a fraction of the maximum tolerated dose. Naïve (non-allergic) mice, mice administered PBS instead of protein, and mice administered empty microparticles were the control groups. The drop in mouse body temperature and the anaphylaxis scores, both indications of acute allergic response, were recorded.

Results

Temperature Response  The control groups showed no change in temperature as expected. The temperature drop for the 10 µg and 50 µg groups were approximately the same between the naked and microparticle formulations. At the higher protein doses of 100 and 200 µg, the microparticle formulation appeared to be protective against larger temperature drops in comparison with the naked formulation (Figure 4.21A). However, the differences between the temperatures of the dose-matched encapsulated and naked protein groups at 60 minutes were non-significant at \( p < .05 \) according to a t-test.

In the 100 and 200 µg dose groups there was another distinction between the naked and microparticle administrations. While each mouse in the naked formulation group responded similarly to one another (Figure 4.21B), the mice in the
Figure 4.21: A) Average temperature drops of mice post-challenge with naked or encapsulated peanut protein. The naïve and placebo groups are challenged with PBS only. The other groups are challenged with 10, 50, 100, or 200 µg of naked or encapsulated peanut protein. B) The response of individual mice administered 200 µg of naked peanut protein. C) The response of individual mice administered 200 µg of encapsulated peanut protein. Figure prepared by Ben Wright.
microparticle formulation group had much more variation. In the 200 μg group, there was a correlation between the magnitude of the temperature drop and the time between particle resuspension and administration. When resuspending all the particles at the same time, the first mouse administered those particles (2 minutes after resuspension) had the smallest temperature drop and the last mouse (8 minutes after resuspension) had the largest temperature drop (Figure 4.21C). In the 100 μg group the pattern was similar except the first and last administered mouse experienced larger temperature drops than the second and third administered mouse.

Anaphylactic Response  The anaphylaxis scores had a similar pattern to the temperature drop scores. None of the control groups (naïve mice, mice administered a placebo, and mice administered empty microparticles) registered any anaphylactic reaction. At each dosage, the mice administered the protein-loaded microparticles had less of an anaphylactic reaction than the mice administered the naked protein, but according to a t-test the differences were non-significant between the dose-matched groups at \( p < .05 \). However, at the 200 μg dosage the variation among the microparticle group was much greater than that of the naked protein group. The more time that had passed from the resuspension of the microparticles to their administration, the stronger the anaphylactic reaction of the mice.

Conclusions

The apparent correlation of temperature drop and anaphylaxis with the magnitude of time passed since the microparticle resuspension in the 200 μg group indicates that there was a high burst release of peanut protein from the surface of the microparticles. The release study showed 84% of the protein was released in the first 24 hours, but we did not know what fraction of that was released in the first few minutes after resuspension. This correlation also indicates that protein burst from
Figure 4.22: (A) Anaphylaxis scores of mice post-challenge with naked or encapsulated peanut protein. 0 is no sign of anaphylactic shock and 5 is death. The naïve and placebo groups were challenged with PBS only. The other groups were challenged with 10, 50, 100, or 200 µg of naked or encapsulated peanut protein. (B) The response of individual mice administered 200 µg of naked peanut protein. (C) The response of individual mice administered 200 µg of encapsulated peanut protein. Figure prepared by Ben Wright.
the microparticles in solution *ex vivo* is faster than the burst *in vivo*. The *in vitro* release study is a perfect sink condition, which is not the case *in vivo* unless there is a great amount of edema.

4.4.3 Conclusions

Due to the correlation between the time since resuspension and the severity of allergic response, we decided to investigate how to reduce the burst release of protein from the microparticles. The higher the burst release, the more similar the microparticle formulation is to the naked formulation. In the case of a longer time period between resuspension and administration, what is actually administered is microparticles with some encapsulated protein and some protein in solution.

We also decided to alter the administration protocol so that the dose of microparticles for each mouse is individually resuspended to minimize and standardize the time between resuspension and administration.

4.5 Stage 4

In stage 4, we optimized the microfluidic microparticle formulation to reduce the burst release and continued the animal safety study from stage 3 into an efficacy study.

4.5.1 Particle Development

Because of our observation about the time dependence of anaphylactic response following microparticle resuspension, we set a goal of reducing the burst release of protein from the microparticles to under 50%, where the burst release is defined as the percentage of total encapsulated protein that is released within the first 15 min after resuspension. We need to maintain a high overall release of the protein from the microparticles so that we can properly match the amount of protein released
with a naked protein delivery group. However, our previous objectives of keeping the encapsulation efficiency and loading level high enough for practical use were still necessary. Our goal was for the encapsulation efficiency to be at least 20% and the loading level at least 20 $\mu$g/mg. Additionally, the particles should be a fine powder so that they could be easily resuspended without clogging the needle used for injection.

Reduction of Burst Release

I tried many alterations to the loading level-optimized formulation (listed below). Our goal was to lower the burst release to 50% of the total encapsulated protein. I defined burst release as the mass protein released in the first 15 minutes after resuspension compared to the overall amount of protein encapsulated.

Methods To test the burst release of the formulations, I followed a general protocol that modified the release study protocol (Section A.2). I divided a batch of particles into three groups of three accurately weighed portions. I resuspended the particles in 1 mL of PBS and placed them in a shaker at 37°C. To collect the supernatants after the desired amount of time, I centrifuged the particles for 5 minutes at 1 krpm and pipetted off the supernatants. I let one group of three replicates release protein for 15 minutes, one group for 60 minutes, and one group for 24 hours. I replaced 1 mL of PBS for the 24-hour release group and resuspended them, repeating the timepoint daily for 3-7 days. I assessed the protein content of the supernatants using a microBCA assay. At the conclusion of some of the release studies I conducted a total protein assay (Section A.3).

Observations Through the course of the optimization experiments, I refined the methods I used to find the optimized protocol based on three observations regarding the reproducibility of bulk particles, mass loss while taking timepoints, and the macroscale presentation of the microparticles.
Microfluidic vs. Bulk  Because of the reduced time it takes to generate bulk microparticles in comparison with microfluidic particles, one might expect that the optimization process would be faster when using the bulk method. However, when I tried manufacturing microparticles in bulk, I found that they were not very reproducible. Therefore, I continued to manufacture microparticles using the microfluidic technique for the remainder of the optimization process.

Timepoints  The microparticles were divided into three groups to minimize the loss of protein mass during the crucial early timepoints of 15 minutes, 60 minutes, and 24 hours. However, more protein was often measured to have been released for a given mass of particles at 15 minutes than at 1 hour or 24 hours. This occurred to a lesser degree with the microfluidics-generated particles than the bulk-generated particles, which reinforced that the heterogeneity of the bulk particles hindered the ability to draw conclusions from the experiments. To ensure that the protein release was accurately measured and to minimize loss, each batch of particles was divided into only three portions and a 15 minute time point, 24 hour time point, and subsequent timepoints at 24-hour intervals were all taken from the same mass of particles. This also approximately tripled the mass available for each replicate, which likely improved the precision of the measurements.

Qualitative Observations  As I processed through the formulation alterations, the encapsulation efficiency and loading level calculations and qualitative observations proved to be just as valuable input as the measurement of our primary focus of the burst release. Many of the formulations I tested throughout this process met the singular goal of a burst release under 50%. However, the microparticles that provided that burst release often had very low encapsulation efficiencies, loading levels, or throughput times, which made them unsuitable for our upcoming animal
experiments. Many of the formulations also did not resuspend well, producing large clumps that could not be broken mechanically or by pipetting.

Conditions Examined  I tested many conditions through the course of this optimization experiment, in both bulk and microfluidic preparations of microparticles.

- volume ratio of inner phase to outer phase, from 1:5 to 1:60
- concentration of protein in the inner phase, 6 to 12 mg/mL
- volume ratio of middle phase to outer phase, from 3:50 to 3:10
- concentration of PLGA in the middle phase, from 2 to 16%
- molecular weight of PLGA, 38-54 kDa or 40-75 kDa
- lactide-to-glycolide ratio of PLGA, 50:50 or 75:25
- dimethyl carbonate vs. dichloromethane in the middle phase
- concentration of chitosan in the outer phase, 0, 0.1, and 0.5%
- concentration of NaCl in the outer phase, 0 to 5%
- concentration of PVA in the outer phase, 1 to 10%
- concentration of DMC in the outer phase, 0 or 3.5%
- pH of the outer phase 3.0 to 7.0
- microfluidic vs. bulk emulsification
- vortexing time for the primary bulk emulsion, 60 or 150 seconds
- homogenization speed for the secondary bulk emulsion, 0.5 to 7.5 krpm
• rotary evaporation for 30 minutes vs. stirring overnight

• rotary evaporation pressure, 100 mBar or about 70 mBar

• temperature of water bath during rotary evaporation, room temperature or on ice

• addition of trehalose before lyophilization

• freezing before lyophilization

Characterization Results  The formulation parameters that most consistently impacted the burst release of the encapsulated protein were:

• the mass ratio of protein to PLGA

• the pH of the outer phase

• the concentration of NaCl in the outer phase

• the water bath temperature (0°C vs. room temperature)

Key Parameters Compared  Several of these parameters are compared in the final optimization experiment I conducted, which measured protein released after 15 minutes and at 24-hour intervals for seven days, followed by a total protein assay.

All of the microparticles in this experiment were made with the microfluidic method. The inner phase was 12 mg/mL peanut protein flowed at 4 μL/min; the middle phase was 4% PLGA in DMC flowed at 40 μL/min; the outer phase was a PVA/NaCl solution flowed at 40-50 μL/min. I increased the middle phase flow rate to decrease the burst release and better stabilize the primary droplet formation. The distinctions between the microparticle generation protocols are listed in Table 4.1. The temperature in Table 4.1 indicates the temperature over which the droplets were
Table 4.1: Burst release optimization formulation parameters for a selected experiment.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PVA (%)</th>
<th>NaCl (%)</th>
<th>pH</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>7.0</td>
<td>0°C</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>4.0</td>
<td>0°C</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.5</td>
<td>7.0</td>
<td>RT</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>5</td>
<td>7.0</td>
<td>RT</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>5</td>
<td>4.0</td>
<td>0°C</td>
</tr>
</tbody>
</table>

Table 4.2: The burst releases and encapsulation efficiencies for five formulations in a selected experiment.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Burst Release (%)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.8</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>71.9</td>
<td>22.9</td>
</tr>
<tr>
<td>3</td>
<td>35.1</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>77.1</td>
<td>53.9</td>
</tr>
<tr>
<td>5</td>
<td>67.7</td>
<td>32.1</td>
</tr>
</tbody>
</table>

collected and the temperature of the bath under the rotary evaporator, either on ice (0°C) or at room temperature (RT).

The burst release percentages and encapsulation efficiencies for each microparticle formulation are listed in Table 4.2.

While formulation 3 achieved the 50% burst release objective, its encapsulation efficiency and loading level did not meet the minimums needed to efficiently generate particles suitable for animal work. Formulation 1 was excluded for the same reason. Of the three remaining formulations, formulation 5 had the lowest burst release (Figure 4.23). Because formulation 5’s particles are a fine powder, it was chosen for the animal studies. Formulation 4, which had the highest loading level and encapsulation efficiency, had clumps that were difficult to resuspend.

SEM images of the particles from formulations 2, 4, and 5 confirm that formulation 5 produces the most uniform, spherical, and smooth microparticles (Figure 4.24).
Figure 4.23: Cumulative release profile of protein from microparticles, normalized to the mass of microparticles. Formulation 1 (blue squares), formulation 2 (red diamonds), formulation 3 (green triangles), formulation 4 (purple X), and formulation 5 (teal circles).

*Theoretical Loading Level* The most important parameter for altering the burst release was the mass ratio of protein to PLGA, the theoretical loading level (TLL), whether the alteration that was achieved by changing the concentrations of the inner and middle phases or the volume ratio between the two. The lower the TLL, the lower the burst release. However, there was a practical limitation as to how high the concentration of PLGA in the middle phase could be raised (not above 4%) and how much the volume ratio of the middle phase to the outer phase could be increased.
Figure 4.24: SEM images of A) formulation 2, B) formulation 4, and C) formulation 5 show that formulation 5 produces the most uniform microparticles with the smoothest surface.

This meant that lowering the TLL past a certain point was dependent on lowering the concentration and volume of the peanut protein inner phase. While lowering the TLL raised the encapsulation efficiency, those formulations still resulted in long generation times for the amount of protein we needed to use in the animal studies because of the lower $Q$ and $c$, and were therefore not eligible for consideration as the final formulation.

**Conclusion**  I chose to move forward with formulation 5 microparticles from the experiment outlined above, which is further characterized in the next section. This formulation had the best combination of a low burst release, high encapsulation efficiency, high loading level, smooth and regular particle morphology, and easy re-suspension.

**Characterization**

While formulation 5’s burst release did not reach the objective of being below 50%, the burst release was successfully reduced to 70% without compromising our other objectives of high encapsulation efficiency, high loading level, and powdery microparticles.
Figure 4.25: Images of the primary (A) and secondary (B) droplet formation on-chip for the burst release-optimized particles. The flow rates were 4 $\mu$L/min for the inner phase, 40 $\mu$L/min for the middle phase, and 50 $\mu$L/min for the outer phase. Scale bars are 100 $\mu$m.

Figure 4.26: Burst release optimized microparticles A) under SEM and B) macroscopically. The particles are fairly monodisperse and present as a fine powder.

Droplet Generation  The final phases used for the optimized burst release particles were 12 mg/mL peanut protein in the inner phase, 4% 50:50 38-54 kDa PLGA in the middle phase, and 3% PVA 5% NaCl at pH 4.0 in the outer phase. The flow rates were 4 $\mu$L/min for the inner phase, 40 $\mu$L/min for the middle phase, and 40 to 50 $\mu$L/min for the outer phase (Figure 4.25).
Particle Size, Shape, and Consistency  The microparticles have smooth surfaces but are less spherical and monodisperse than particles made with a lower mass of PLGA (Figure 4.26A). Macroscopically, the microparticles presented as a fine powder and any clumps were easily broken apart (Figure 4.26B). They resuspended well, with most microparticles going into solution and the remaining fraction forming a foam on top.

The mean diameter of the microparticles was 29 $\mu$m with a standard deviation of 10 $\mu$m (Figure 4.27).

Release Study  The microparticles released 68% of their total encapsulated protein within the first 15 minutes of resuspension, indicating that most of the encapsulated protein was still surface-associated. By the end of 24 hours, another 23% had been

**Figure 4.27**: A histogram of the frequency of diameters of the burst release optimized microparticles.
released. 3% of the total encapsulated protein remained in the microparticles after seven days of release, which met our objective of having a high overall release percentage (Figure 4.23).

While we would prefer if the release profile were more sustained (Figure 4.23), as the administered doses in the mouse model are three times per week, the amount of protein released between 15 minutes and 48 hours should provide some sustained release until the next dosage of microparticles.

**Encapsulation Efficiency**  The encapsulation efficiency was $32 \pm 3\%$, which was high enough to encapsulate the desired mass of protein within a working week.

**Loading Level**  The loading level of the microparticles was $29 \pm 3 \mu g/mg$, which was within our original target loading level range of 20 to 40 $\mu g/mg$.

**Generation Time**  Before accounting for handling waste, we needed 9.28 mg of encapsulated protein for the efficacy portion of the mouse experiment. With an inner phase flow rate of 4 $\mu L/min$, a peanut protein concentration in the inner phase of 12 mg/mL, and a 32% encapsulation efficiency, the desired mass of protein can be encapsulated in 10.1 hours of droplet generation time, well within the two working weeks goal even after accounting for loss.

### 4.5.2 Mouse Experiments

The efficacy portion of the animal study we planned in stage 3 was resumed with our newly optimized microparticles, with some adjustments for a smaller number of available animals. Following a challenge to verify the continued allergy of the mice, our collaborators in the Burks lab began treatments with naked and encapsulated peanut protein.
Table 4.3: The dosage schedule for the therapeutic animal study. The low dosage and high dosage groups are for both naked and microparticle formulations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
<th>Dose 4</th>
<th>Dose 5</th>
<th>Dose 6</th>
<th>Doses 7-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve and Placebo</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Low Dosage</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>High Dosage</td>
<td>10</td>
<td>15</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>250</td>
</tr>
</tbody>
</table>

Methods

The 24 mice were divided into six groups of four each: naïve, placebo, and low and high dosages of both naked and encapsulated peanut protein. Each group was dosed three times per week for 4 weeks (Table 4.3). The first six dosages in the low dosage group were 5, 10, 15, 20, 30, and 40 µg each and the last six were 50 µg. The first six dosages in the low dosage group were 10, 15, 25, 50, 100, and 200 µg each and the last six were 250 µg. These dosages are lower than the ones used in the OIT protocol to reduce the risk of anaphylaxis.

Results

Temperature Response The temperature response data showed that all groups of mice that had been made allergic experienced temperature drops after a challenge with peanut protein, with the largest drop for the mice treated with only PBS (Figure 4.28). The differences between the dosage-matched groups of naked and encapsulated peanut protein were not significant at $p < .05$ according to a t-test.

Anaphylactic Response The anaphylactic response data showed that all groups of mice that had been made allergic had an anaphylactic response after a challenge with peanut protein, with the highest average score for the mice treated with only PBS. The differences between the dosage-matched groups of naked and encapsulated peanut protein were not significant at $p < .05$ according to a t-test.
Figure 4.28: Plot of the average temperature drops of mice after a challenge with naked peanut protein. The mice had been treated with dosages of naked and encapsulated peanut protein. The naïve mice had never been made allergic to peanut and the PBS group had been treated with only PBS. The other mice were treated with a low dosage schedule of naked peanut protein (PN (low)), a low dosage schedule of encapsulated peanut protein (PNMP (low)), a high dosage schedule of naked peanut protein (PN (high)), and a high dosage schedule of encapsulated peanut protein (PNMP (high)), according to Table 4.3. Figure prepared by Ben Wright.

4.5.3 Conclusions

The efficacy study did not support our hypothesis that delivering encapsulated peanut protein would be more effective in desensitizing peanut-allergic mice than delivering naked peanut protein. The differences between the decrease in body temperature and the anaphylactic scores of the dose-matched groups will all nonsignificant.

There were a few issues with this study that may make it worth repeating if they are addressed. 1) The burst release of the particles was still quite high; if it can
Figure 4.29: Plot of the anaphylaxis scores of mice after a challenge with naked peanut protein. The mice had been treated with dosages of naked and encapsulated peanut protein. The naïve mice had never been made allergic to peanut and the PBS group had been treated with only PBS. The other mice were treated with a low dosage schedule of naked peanut protein (PN (low)), a low dosage schedule of encapsulated peanut protein (PNMP (low)), a high dosage schedule of naked peanut protein (PN (high)), and a high dosage schedule of encapsulated peanut protein (PNMP (high)), according to Table 4.3. Figure prepared by Ben Wright.

be reduced below 50% of the total encapsulated protein there would be a more sustained release profile, which may differentiate the encapsulated protein groups from the naked protein groups. 2) By the time our collaborators performed the efficacy study, the mice were about four months past their sensitization period. Though they were challenged to verify that their allergy had been maintained, the serological markers measured post-challenge (IgG1, IgG2a, IgE) did not exhibit the usual pattern; our collaborators typically observe larger differences among the levels of the various antibodies, but the treated groups in this study were very similar to the PBS-treated control. This indicates that the mice may have been sensitized for too long or challenged too many times.
4.6 Future Directions

The results we have observed through the OIT and SIT mouse studies have prompted additional questions about how to apply peanut protein-loaded microparticles to IT.

4.6.1 Co-Encapsulation of Peanut Protein with CpG

One of the most promising results from this study was in the co-encapsulation of peanut protein and an adjuvant in PLGA microparticles. The next step is to create these microparticles using the microfluidic method. I do not anticipate that adding CpG to the inner phase alongside peanut protein would disturb the primary or secondary emulsion formations as the droplets are stabilized by the protein itself. As an alternative we may form CpG-polymer nanoparticles to further stabilize the DNA, which we could add in to the primary phase with the peanut protein. We would test this formulation in the SIT model and move onto the OIT model if the results are promising.

4.6.2 Encapsulate Major Allergens Alone

One of the possible reasons why the optimization of the peanut protein-loaded microparticles has been so challenging is that the protein we are encapsulating is not a single protein but a heterogeneous mixture of thirteen proteins (Appendix C). Each of those proteins has a different molecular weight, solubility, and isoelectric point. While we know the overall encapsulation efficiency and release of the protein mix, it is possible that certain proteins are over- or under-represented in the encapsulated mass of protein vs. the starting mass of protein. To investigate this, we would like to try encapsulating only the major peanut allergens, Ara h 1, 2, 3, and 6 separately. These particles should still be effective in treating peanut allergy and their characteristics may also prove to be more controllable because of the single type of encapsulated protein.
4.7 Conclusions

I made peanut protein-loaded microparticles using both bulk and microfluidic methods. Through four phases of experimentation with our collaborators in the Burks lab, we refined the peanut protein-loaded microparticle formulation to have a high encapsulation efficiency, high loading level, and moderate burst release. We tested these microparticles in a modified OIT and SIT protocol in both prophylactic and therapeutic mouse models. These results suggest that further study of the co-delivery of encapsulated peanut protein with an adjuvant and encapsulated single peanut proteins may be worthwhile.
Encapsulation of Porphyrin, Ascorbate, and Doxorubicin in Microparticles

In this chapter, I further demonstrate the flexibility of our microfluidic platform by generating poly(lactic-co-glycolic acid) (PLGA) microparticles separately loaded with a porphyrin molecule (a hydrophilic drug) and doxorubicin (dox) (either a hydrophilic or a hydrophobic drug). I conducted viability studies in several human cell types to explore the selective action of porphyrins against cancerous cells and the combined action of porphyrin with ascorbate or dox. I encapsulated porphyrin, ascorbate, and dox in microfluidics-generated microparticles and characterized the microparticles by their particle morphology, drug release profile, drug encapsulation efficiency, and drug loading level.

5.1 Introduction

I review our development of microfluidics-generated microparticles for the sustained delivery of a superoxide dismutase (SOD) mimic and a bioreductant to treat cancer. The SOD mimic was a novel compound studied by our collaborators in the
Batinic-Haberle lab and the bioreductant was either ascorbate or dox. Porphyrin and ascorbate together are selectively toxic to cancer cells while porphyrin prevents dox toxicity in healthy cells while maintaining toxicity in cancerous cells. I encapsulated porphyrin, ascorbate, and dox in separate PLGA microparticles using our double emulsion (DE) microfluidic platform.

Intraperitoneal or subcutaneous injection of Mn porphyrins over a month only results in nanomolar concentrations in the brain, which may not be sufficient for anticancer efficacy [84]. The bioavailability of the porphyrins to a brain tumor could be increased by the direct injection of a particulate delivery system. Porphyrin with ascorbate or dox can be slowly released from the microparticles and produce cytotoxic peroxide extracellularly. The peroxide can enter the cell to further disrupt the redox state [85]. We showcased the combined action of porphyrin with ascorbate or dox in selectively killing cancerous cells and demonstrated their potential as payloads in PLGA microparticles.

5.2 Background

5.2.1 SOD Mimics

Cancerous cells have a different redox state than normal cells as are constitutively subjected to higher oxidative stress; this oxidative stress is both causative of carcinogenesis and contributes to tumor progression and metastasis [86]. The SOD family of enzymes regulates levels of reactive oxygen species (ROS) in cells by the dismutation reaction with superoxide, leading to formation of water and hydrogen peroxide. Mitochondrial SOD (MnSOD) has both tumor suppressor/antioxidant and oncogene/pro-oxidant effects. A cell that has low levels of MnSOD expression may experience an altered redox state that transforms it into a cancerous cell. As the cancerous cell proliferates, oxidative stress increases and the cell may upregulate MnSOD, resulting in increased hydrogen peroxide levels and downstream effects on
signaling pathways that promote cancer growth [84].

One promising therapeutic approach is to use small molecule drugs to alter the oxidative stress of cancerous cells. Normal cells would tolerate a similar amount of oxidative stress because of their different ratio of superoxide- to hydrogen peroxide-removing enzymes [86, 85]. A perturbation toward increased hydrogen peroxide in the cancerous cell will eventually cause apoptosis through mitochondrial permeability transition pore opening [87].

A class of SOD mimics that has been studied in the Batinic-Haberle lab as a therapeutic is metalloporphyrins. The first was MnTE-2-PyP\(^{5+}\) (MnTE), then leading to MnTnHex-2- PyP\(^{5+}\) (MnTnHex), and finally MnTnBuOE-2- PyP\(^{5+}\) (MnTnBuOE). This sequence of porphyrins has increasing lipophilicity and decreasing systemic toxicity. A similar group of metalloporphyrins with Fe in the place of Mn was also developed (Figure 5.1).

5.2.2 Porphyrin Co-Delivery with Ascorbate

The mechanism of action of Mn porphyrin-based SOD mimics is either to directly remove ROS and lower oxidative stress or to increase ROS levels and increase oxidative stress, depending on cell environmental and dosing factors. However, in the presence of cellular reductants such as ascorbate, porphyrins act as a superoxide and oxygen reductase; its superoxide removal is coupled to redox cycling of ascorbate or glutathione, furthering the production of hydrogen peroxide [85, 88]. When ascorbate and Mn porphyrin are delivered to the body, Mn porphyrin catalyzes ascorbate oxidation, producing hydrogen peroxide. If peroxide-removing enzymes have been downregulated, such as in a cancer cell, the oxidative stress will become too high and the cells will undergo apoptosis [84].
Figure 5.1: A) The core of metalloporphyrins, around either Mn or Fe. B) The R groups that generate the varieties of porphyrins: TE, TnHex, and TnBuOE. Adapted from [84].

5.2.3 Porphyrin Co-Delivery with Doxorubicin

Dox is frequently used to treat several types of cancer. Patients undergoing treatment with dox report a mental fogginess known as 'chemobrain.' Dox-induced oxidative stress at plasma level causes elevated levels of TNF-α, which crosses the blood-brain barrier to damage healthy tissue [89]. Porphyrin compounds can protect the healthy
brain tissue when co-administered with dox [90]; their redox-based effect suppresses NF-κB, activation and, downstream, TNF-α production [91]. We hypothesize that the co-delivery of dox and porphyrin, for instance in the case of breast cancer treatment, would maintain a strong anti-cancerous effect while suppressing damage to the brain and heart.

5.2.4 Microparticle Encapsulation

By encapsulating porphyrin, ascorbate, and dox in microparticles and co-delivering them, we expect that ROS will be produced more continuously than if we simply apply a bolus of naked drugs, because the slow release of the compounds from the microparticles will prevent the molecules from beginning to cycle all at once upon delivery. This type of encapsulation is also a technical challenge because it is difficult to form stable primary droplets with only biocompatible surfactants and no protein.

5.3 Cell Experiments

I worked with our collaborators to establish the toxicity of four types of porphyrins in cancerous and non-cancerous human cells using MTT and clonogenic assays. We then chose to move forward with the brain cancer model and studied porphyrin toxicity in D-245 MG cells. I calculated the MnTE, ascorbate, and dox half maximal inhibitory concentrations (IC50s) and tested their combined effect.

5.3.1 Methods

After screening seeding conditions, we settled on a protocol for measuring the toxicity of the porphyrins using an MTT assay on all of the cell types. Twenty-five thousand cells/well were seeded in a 96-well plate and left to attach overnight. Drugs at a range of concentrations in medium and controls (medium only and untreated cells for the negative controls, 1 mg/mL polyethylenimine (PEI) for the positive control)
were added to the cells and incubated for 48 hours. Wells were then rinsed twice with PBS and 100 uL of medium with 10 uL of MTT reagent was added to each well. After four hours, 85 uL was removed from each well and replaced with 50 µL DMSO. The plates were briefly shaken and then incubated for 10 mins at 37°C. The absorbance of the plates was read at 544 nm using a plate reader. The absorbance corresponds to cell viability, so the untreated cells will produce high absorbances and the PEI-dosed cells will produce low absorbances. The data were analyzed by subtracting the absorbance of a well containing only medium and MTT reagent from the absorbance of the drugged cells and then normalizing to the absorbance of the no treatment control.

The IC50s were calculated by fitting a sigmoid curve to the data using Origin. The viabilities of porphyrin in combination with ascorbate or dox was compared to the no-treatment control using an unpaired two-tailed T-test with significance level p<.05.

5.3.2 Selective Porphyrin Activity Against Cancerous Cells

Our collaborators had previously tested the efficacy/toxicity of their porphyrins on bacterial cells. We hypothesized that the porphyrins would be more toxic against cancerous cell lines than non-cancerous and that their toxicity would be enhanced by the presence of ascorbate.

IC50s of Porphyrins in Various Cell Types

We determined the IC50s of four metalloporphyrins in four cell types: two Mn porphyrins, MnTE and MnTnHex, and two Fe porphyrins, FeTE and FeTnHex.

The four cell types tested were NHDF (primary fibroblasts), HeLa (cervical cancer), MCF-7 (breast cancer), and MCF-10A (normal breast). The IC50s of the four types of porphyrins across the four cell types ranged from 44 µM to 4.3 mM (Figure
Figure 5.2: IC50 results of four types of porphyrins in four cell types. A) Table of all results. Selected plots from which IC50s were calculated for B) FeTE in MCF-7, C) MnTE in MCF-10A, D) FeTnHex in HeLa, E) in MnTnHex in NHDF.

5.2. The IC50s broadly supported three conclusions: 1) cancerous cell lines are more sensitive than non-cancerous cells to porphyrins, 2) cells are more sensitive to Fe porphyrins than Mn porphyrins, and 3) the compounds with higher lipophilicity, MnTnHex and FeTnHex, were more toxic than the compounds with low lipophilicity. We were not able to determine the IC50 of FeTnHex in NHDF, most likely because
the high concentration of FeTnHex adsorbed to the plastic wells and prevented an accurate absorbance from being measured.

**Viability Tests of Nontoxic Concentrations of Porphyrins with Ascorbate**

After determining the IC50s of the four porphyrins in HeLa, NHDF, MCF-7, and MCF-10A, we investigated how the addition of ascorbate would impact the toxicity of the porphyrins. We chose two porphyrin concentrations that were non-toxic in all cell types, 3 and 15 µM. We tested MnTE, FeTE, MnTnHex, and FeTnHex at those two concentrations with and without 1 mM ascorbate (also a nontoxic concentration) using the same positive and negative controls as the IC50 experiments. Broadly, the experiments supported the hypothesis that the combination of nontoxic levels of porphyrins and ascorbate is toxic to cancer cells but less so to normal cells (Figure 5.3A-D, selected results). For example, the percent viability of HeLa with 1 mM is 95% and with 15 µM MnTnHex is above 100%, but when applied together the viability drops significantly to 36%.

**Clonogenic Assay of Porphyrin with Ascorbate for Confirmation**

As an additional confirmation of our results from the MTT experiments, I performed a clonogenic assay of MnTnHex applied to HeLa cells. HeLa cells were seeded at 250,000 cells/mL in a 12-well plate and allowed to attach over 2 nights. MnTnHex and ascorbate were added and allowed to incubate for 48 hours. Cells were then replated at a 1:1000 dilution and colonies were grown for 14 days. Colonies were fixed, stained with crystal violet, and dried overnight before they were counted.

The colony counts showed that the 15 µM MnTnHex and 1 mM ascorbate control groups had as many or more colonies than the untreated control group, but the MnTnHex and ascorbate experimental group had only 62% of the colonies of the untreated control (Figure 5.3E). This assay independently confirmed that the addition
Figure 5.3: Selected viability results for porphyrin plus ascorbate, $p < .05$ comparison to no treatment control. A-D) MTT assay results. Striped bars represent no ascorbate added and solid bars represent 1 mM ascorbate added. The MnTnHex and ascorbate combination (both at nontoxic levels) was tested on four cell types: A) NHDF, B) HeLa, C) MCF-10A, D) MCF-7. E) Clonogenic assay results of colony formation after treatment with 1 mM ascorbate, 15 µM MnTnHex, or both.
of ascorbate with a non-toxic amount of porphyrins to a cancerous cell line could render the porphyrins toxic.

5.3.3 Porphyrin Action Against Brain Cancer Cells

I explored the action of porphyrin, ascorbate, and dox against the brain cancer cell line, D-245 MG. D-245 MG is a human cell line that is used to create tumors in a mouse model of brain cancer, so we investigated the toxicity of porphyrins, ascorbate, and dox, alone and in combination, to this cell type. The porphyrins I tested were MnTE, MnTnBuOE, and FeTnBuOE.

IC50s of Porphyrin, Ascorbate, and Doxorubicin on D-245 MG

I determined the IC50s for three porphyrin compounds, ascorbate, and dox on D-245 MG cells. In all cases, we were interested in finding nontoxic concentrations of each of the compounds so that we could apply them together and observe their combined toxic effect.

Porphyrins For D-245 MG cells, the IC50s of MnTE (Figure 5.4A), MnTnBuOE, and FeTnBuOE were 4.1 mM, 1.7 mM, and 65 µM. These results support the previous observation in other cell types that Fe porphyrins and more lipophilic porphyrins are the more toxic.

Ascorbate The IC50 of ascorbate for D-245 MG cells was 4.0 mM (Figure 5.4B). Ascorbate concentrations between 0.3 and 3 mM enhanced cell viability before becoming quite toxic at 9 mM.

Doxorubicin The IC50 of dox for D-245 MG cells was 6.0 µM (Figure 5.4C). The lowest concentration of dox measured in this experiment was 30 nM, which killed
Figure 5.4: Selected MTT assay plots of A) MnTE, B) ascorbate, and C) dox administered to D-245 MG cells.
Viability Studies of Porphyrin, Ascorbate, and Doxorubicin

I also studied the combined effect of two independently less-toxic concentrations of MnTE and ascorbate or MnTE and dox on D-245 MG cells.

Porphyrin and Ascorbate  Co-treatment of two non-toxic concentrations of MnTE and MnTnBuOE, 3 and 15 µM, with a non-toxic concentration of ascorbate, 1 mM, showed that the combined effect of the two drugs is much greater than the action of
either one alone, significantly decreasing cell viability by between 40 and 120% (Figure 5.5) \((p < .05)\). FeTnBuOE had a similar result at 3 \(\mu\)M of a 40% reduction but not at 15 \(\mu\)M, for which the cell viabilities were all above 100%, and the differences were not significant at \(p < .05\).

**Porphyrin and Doxorubicin** While we observed a similar combined effect of MnTE with dox, it was is not as strong as the effect seen with ascorbate. The viabilities of the cells dosed with dox at 30 and 300 nM and two nontoxic concentrations of MnTE, 15 and 30 \(\mu\)M, were not significantly lower than that of dox alone, but the
difference is on the order of 5 to 10% instead of 40 to 120% (Figure 5.6).

5.3.4 Conclusions

Nontoxic dosages of porphyrin can effectively kill D-245 MG cells by the addition of a nontoxic concentration of ascorbate. The toxic effect of dox on D-245 MG is not be enhanced or suppressed by the addition of nontoxic concentrations of MnTE.

5.4 Encapsulation of Porphyrin, Ascorbate, and Doxorubicin

To test the flexibility of our microfluidic platform, we encapsulated both hydrophilic and hydrophobic molecules in PLGA microparticles. Using a DE, I separately encapsulated porphyrins, ascorbate, and the salt form of dox. Using a single emulsion (SE), I encapsulated the base form of dox. I characterized the microparticles through SEM, a release study, and their encapsulation efficiencies and loading levels.

5.4.1 Porphyrin

I encapsulated MnTE, a relatively hydrophilic porphyrin, using a microfluidic DE protocol. First, I stabilized the primary droplets, then tested two outer phase pHs, and finally characterized the microparticles.

Development of Microparticles

I screened surfactants and made primary droplets that are stable into the secondary chip without using protein. I then tested two outer phase pHs to see if there was an effect on the microparticle characteristics.

Primary Droplet Stabilization In our microfluidic system, the primary droplet in a DE is more difficult to stabilize than the secondary droplet. While developing this two-chip system, we used protein as the payload in the inner phase, which as an amphiphilic molecule does not require a separate surfactant to make very stable
primary droplets (Chapters 3 and 4). While translating our system to encapsulate porphyrins, we tested different ways to stabilize the primary droplets. At first, Michael Keane and I co-encapsulated porphyrin with bovine serum albumin (BSA), which is commonly used as an inert stabilizer. Then I screened several molecular weights and concentrations of PVA to find one that stabilized the porphyrin-in-DMC primary droplets.

BSA in the Inner Phase In our first attempt to encapsulate porphyrin, we stabilized the primary droplets with BSA.

The inner phase was 1 mg/mL MnTnBuOE in 7.5% BSA, the middle phase was 2% PLGA (50:50 lactide-to-glycolide, 38-54 kDa) in DMC, and the outer phase was 3% PVA. DE droplets were generated with flow rates of 1 µL/min for the inner phase, 10 µL/min for the middle phase, and 20 µL/min for the outer phase and the droplets were collected in 10 mL 3% PVA (Figure 5.7A). We evaporated the organic phase using a rotary evaporator set at 100 mBar and 100 rpm over a room temperature water bath, washed the microparticles, and lyophilized overnight (Figure 5.7B).
Encapsulation with *PVA in the Inner Phase* I screened five PVAs of different molecular weights at 1, 2.5, 5 and 10% to find which PVA molecular weight and concentration best stabilized the primary droplets. The higher concentrations of PVA often interacted with the PLGA to coat the PDMS channel walls and clog the channels. Some of the PVA molecular weights were ineffective at stabilizing the droplets even at high concentrations. The lowest concentration surfactant that stabilized the primary droplets in both the primary and secondary chips without clogging the chips was 2.5% 9-10 kDa PVA (Figure 5.8 A and B).

*Outer Phase pH comparison* I made microfluidics-generated MnTE-loaded PLGA microparticles at two different outer phase pHs. I chose pH 8.6 to mirror the dox particles and pH 5.0 because lower pHs yielded less clumped particles in previous studies.

*Microparticle Characterization*

I characterized the pH 5.0 and pH 8.6 MnTE-loaded microfluidics-generated microparticles through a release study (Appendix A.2). At the conclusion of that study I digested of the particles in DMSO to release the remaining MnTE and calculated
Figure 5.9: A) Primary droplets of MnTE and PVA emulsified in PLGA in DMC and B) MnTE-loaded secondary droplets.

the encapsulation efficiency and loading level (Appendix A.4). I also imaged the pH 8.6 MnTE-loaded microparticles with SEM (Appendix A.1).

Method The formulations consisted of 19.6 mM MnTE with 2.5% 9-10 kDa PVA as the inner phase, 4% PLGA 50:50 38-54 kDa in DMC as the middle phase, and 3% PVA as the outer phase. The flow rates for the inner, middle, and outer phases were 1 µL/min, 20 µL/min, and 10 µL/min. I collected the droplets in 10 mL of the outer phase in a round-bottom flask and then evaporated the organic phase using a rotary evaporator at 100 mBar, washed the microparticles, and lyophilized overnight. I collected the pH 5.0 droplets over ice and the water bath under the rotary evaporator flask contained ice, while the pH 8.6 droplets were collected at evaporated at room temperature.

Release Study Timepoints were taken at 1, 6, 24, and 48 hours; after the 48 hour timepoint the microparticles were digested for two hours in DMSO (Appendix A.4). I measured the concentration of the releasates and digested microparticles using a UV-Vis spectrophotometer at 454 nm. The absorbance was converted to concentration using the extinction coefficient provided 179718 L mol⁻¹ cm⁻¹ (by Artak Tovmasyan).
Figure 5.10: Cumulative MnTE release from microfluidics-generated PLGA microparticles. Microparticles made with pH 5.0 outer phase are shown as blue diamonds; microparticles with pH 8.6 outer phase are shown as in green triangles.

The pH 5.0 microparticles released 89% of the porphyrin in the first hour and 1% remained after 48 hours. The pH 8.6 microparticles released 83% of the porphyrin in the first hour and 3% remained after 48 hours.

*Encapsulation Efficiency and Loading Level* The pH 5.0 microparticles had an encapsulation efficiency of $5.6 \pm 0.3\%$ and a loading level of $6.76 \pm 0.13$ nmol/mg. The pH 8.6 microparticles had an encapsulation efficiency of $7.5 \pm 0.7\%$ and a loading level of $5.5 \pm 0.4$ nmol/mg. While both of these encapsulation efficiencies and loading levels seem low, they are sufficient to be a starting point for further optimization.
SEM The pH 8.6 MnTE-loaded microfluidics-generated microparticles were heterogeneous in shape and some had holes on their surfaces or appeared to be collapsed (Figure 5.11).

Conclusion

Both the pH 8.6 and pH 5.0 microparticles were quite similar in their attributes, indicating that in this range the pH did not have a great effect on the microparticles. The burst release from these microparticles is high, but the strategy of lowering the theoretical loading level may not be the best approach. The loading level is already such that the mass of microparticles required to produce a sufficient MnTE concentration in our *in vitro* experiments is rather high for the available volume and we may not be able to resuspend such a dense concentration of microparticles. The microparticles have rough surfaces, which may be improved with further optimization of the formulation, especially the components and concentrations of the outer phase and the drug concentration.
5.4.2 Ascorbate

I explored encapsulating the hydrophilic drug ascorbate in microfluidics-generated PLGA microparticles.

Development of Microparticles

I encapsulated ascorbate in PLGA microparticles using a DE method with my microfluidic system. The inner phase was 250 mg/mL ascorbate and flowed at 4 µL/min; the middle phase was 4% PLGA in DMC and flowed at 40 µL/min; the outer phase was 1% PVA pH 7.0 and flowed at 50 µL/min. I evaporated the organic phase using a rotary evaporator at 100 mBar, washed the microparticles, and lyophilized overnight.

Microparticle Characterization

The primary droplets slightly merged when entering the secondary chip, but were overall quite stable, even without a surfactant in the inner phase (Figure 5.12 A). However, a release study showed that 0.001% of the total ascorbate added to the system was released on day 1 and 0.000006% on day 2 (spectrophotometric measurements at 265 nm by Artak Tovmayson) (Section A.4). The microparticles were
spherical and slightly heterogeneous in size, but contained holes on the surfaces (Figure 5.12 B).

Conclusions

Since virtually no ascorbate was encapsulated in the PLGA microparticles and what was encapsulated was likely only surface-associated, we decided not to pursue encapsulating ascorbate in this manner.

5.4.3 Doxorubicin

Dox, in addition to ascorbate, can work synergistically with porphyrins to selectively target cancer cells while sparing healthy cells. Dox can be water-soluble or organic-soluble depending on the form of the drug. I made DE and SE particles loaded with dox and characterized them through a release study, encapsulation efficiency and loading level calculations, and SEM.

Doxorubicin in PLGA Particles

Many groups have previously encapsulated dox in PLGA particles using the emulsion/solvent evaporation method. Dox can be solubilized either in its salt form in an aqueous phase or in its base form in an organic phase. Aqueous-soluble dox requires a W/O/W DE [92, 93, 94, 95] while organic-soluble dox only requires an O/W SE [96, 97, 98, 99].

Tewes et al. directly compared dox encapsulation by the SE and DE methods [100]. The SE method had a 95% encapsulation efficiency while the DE method had a 67% encapsulation efficiency. However, within the first 24 hours of release, the DE formulation had released 6.5% of its payload while the SE formulation had only released 1.5%. As the study was not extended past 24 hours, it is not clear whether the overall release rate would be similar between the two, as the authors conclude, or whether the dox was bound more tightly in the SE formulation and ultimately
Figure 5.13: Dox-loaded droplets made using DE (top) and SE (bottom) droplets.

less overall would have been released. Contrary to Tewes et al., Khemani et al. also directly compared SE and DE dox encapsulations and found the higher encapsulation efficiency in the DE particles [101].

Development of Microparticles

Because there have been varying reports on the relative encapsulation efficiencies and release curves of dox encapsulated using the DE and SE methods, I decided to generate PLGA microparticles using our microfluidic system to encapsulate dox using both methods.

Doxorubicin-Loaded Double Emulsion Droplets I formed the DE dox particles with an inner phase of 1 mg/mL dox in 2.5% 9-10 kDa PVA flowed at 1 µL/min, a middle
phase of 4% PLGA 50:50 38-54 kDa flowed at 10 \( \mu L/min \), and an outer phase of 3% PVA pH 8.6 flowed at 7.5 \( \mu L/min \) (Figure 5.13 A and B).

**Doxorubicin-Loaded Single Emulsion Droplets**  To solublize dox-HCl in the DMC phase for the SE dox particles, I stirred 0.9 mg dox-HCl in 4.5 mL DMC with 100 \( \mu L \) triethylamine (TEA) overnight. The outer phase was 3% PVA pH 8.6. The flow rate for the inner phase was 5 \( \mu L/min \) and the flow rate for the outer phase was 7.5 \( \mu L/min \) (Figure 5.13C).

**Microparticle Characterization**

I characterized the dox-loaded PLGA microparticles made by both the DE and SE methods through a release study, encapsulation efficiency and loading level calculations, and SEM.

**Release Study**  The release curves between the DE and SE microparticles were very similar over the first 48 hours, with the DE particles releasing slightly more drug overall (Figure 5.14). Timepoints were taken at 1, 6, 24, and 48 hours and after the 48 hour timepoint the microparticles were digested for two hours in DMSO. The concentration of the releasates and digested particles was measure using a UV-Vis spectrophotometer at 480 nm. Concentrations were calculated from the linear portion of a standard curve. Of the total amount of dox encapsulated in the DE microparticles, 33% was released in the first hour and 9% remained after 48 hours. Of the total amount of dox encapsulated in the SE microparticles, 27% was released in the first hour and 8% remained after 48 hours.

**Encapsulation Efficiency and Loading Level**  The encapsulation efficiency was 65\( \pm 9\% \) for the DE microparticles and 46\( \pm 7\% \) for the SE microparticles. The loading level
Figure 5.14: Cumulative release of dox from PLGA microparticles made from DE (blue diamonds) and SE (red squares) droplets.

was 7.8±1.1 μg/mg for the DE microparticles and 7.2±1.9 μg/mg for the SE microparticles.

SEM The DE dox microparticles were spherical and round with only slight variation in the diameters and a fraction of the particles have a small hole in the surface (Figure 5.15A).

There were two populations of SE dox microparticles present in the SEM. Some of the particles were reasonably spherical and smooth, and some appeared wrinkled or collapsed (Figure 5.15B).
5.4.4 Conclusion

Both DE and SE dox-loaded PLGA microparticles have release curves and encapsulation efficiencies that can be applied to \textit{in vitro} testing. This is an acceptable starting point for future optimization of the protocols. The SEM revealed an unusual morphology for the SE microparticle surface that we intend to investigate, possibly due to the presence of TEA.

5.5 Future Work

The future work of this project includes optimization of the microparticle formulations, \textit{in vitro} testing, and testing in a mouse tumor model.

5.5.1 Further In Vitro Study of Porphyrin and Doxorubicin Combined Action

The hypothesis that porphyrin protects health tissue from dox damage while maintaining dox toxicity in cancerous tissue has only been partially substantiated. Further testing is necessary of the application of naked MnTE and dox to healthy brain and cardiac cells.
5.5.2 Microparticle Formulation Optimization

Both the MnTE-loaded and the dox-loaded microparticles would benefit from further optimization, in particular reducing the burst release from the porphyrin-loaded microparticles. While the release curve of the dox-loaded microparticles is well-suited for 48-hour cell culture experiments, we would like to slow the release for possible in vivo applications.

We can attempt to encapsulate other porphyrins now that we have demonstrated that our platform and formulation can encapsulate one porphyrin. It may even be possible to encapsulate the more lipophilic porphyrins such as MnTnBuOE in an organic phase so that we can make only SE droplets.

5.5.3 In Vitro Tests of Drug Release

We propose to test the MnTE- and dox-loaded microparticles in vitro as we have done with the previous cell experiments (Section 5.3.3) in healthy and cancerous brain, breast, and heart cells. Instead of adding naked drug to the cells, we can simply resuspend the microparticles in media and dose the cells with the mass of particles that encapsulates the desired amount of drug for that group. Since we deliver the drugs in separate microparticles, we can include control groups of porphyrin-loaded microparticles delivered with naked dox and vice versa. The slowly released drugs should expose the cells to ROS over a longer period of time than the bolus delivery of naked drugs as they redox cycle and therefore be more effective in killing the cancer cells. We want to first test D-245 MG cells, but it is crucial to test the microparticle combination on both cancerous and normal human cell lines such as McF-7 and MCF-10A since porphyrin should enhance the dox toxicity in the former while reducing it in the latter.
5.6 Conclusions

In this chapter, I demonstrated the flexibility of our microfluidic platform to encapsulate a hydrophilic drug (porphyrin) and a hydrophobic drug (dox). I met the challenge of forming DE droplets that were stable enough to encapsulate small molecule drugs. I accomplished this for two types of porphyrins, ascorbate, and dox. While the phases and surfactants we used were not effective in encapsulating ascorbate, I successfully encapsulated porphyrin in DE microparticles and dox in DE and SE microparticles. *In vitro* cell experiments that I have performed in cancerous and normal human cells have laid the groundwork for future validation of the efficacy of our drug-loaded microparticles in killing cancer cells while preserving normal cells.
I have developed a PDMS-based two-chip microfluidic device that generates double emulsion droplets in two independent single emulsion droplet-makers. I used this device to make poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with several different hydrophilic drugs and one hydrophobic drug and tested the microparticles for potential clinical use.

In Specific Aim 1, I moved the microfluidic platform from generating single emulsion droplets to double emulsion droplets to particles loaded with protein. In my initial comparison of microfluidics-generated and bulk-generated microparticles, I found that the microfluidics-generated microparticles were much more uniform in size and shape and had a more sustained drug release profile, but had much lower encapsulation efficiency. Finally, I tested and implemented several ideas that increased the microparticle production from the device; I altered the microfluidic device design and manufacturing protocols to make for more smooth manufacturing of the double emulsion droplets.

In Specific Aim 2, I manufactured peanut protein-loaded microparticles using bulk and microfluidic methods and our collaborators in the Burks lab tested them
in *in vitro* and *in vivo* models of peanut allergy. Through the course of our collaboration, I moved from making the particles using a bulk method to a microfluidic method. I raised the encapsulation efficiency and loading level and lowered the burst release of peanut protein from the microparticles as we tailored them to the mouse model. We found that treating peanut-allergic mice with encapsulated peanut protein increased the safety of a subcutaneous immunotherapy protocol but did not improve the efficacy of the protocol over delivery of naked peanut protein.

In Specific Aim 3, I validated the flexibility of our microfluidic platform by encapsulating a water-soluble small-molecule drug, a porphyrin, and a water-soluble or oil-soluble drug, doxorubicin. Through a series of cell experiments, I showed that porphyrin, ascorbate, and doxorubicin are selectively toxic to cancerous cells.

The future directions of this project are to continue to improve the microfluidic platform and to further our applications of the microparticles. We have started experimenting with on-chip solvent evaporation to eliminate the last bulk step in the particle manufacturing protocol. With another collaborator, we have begun making drug-loaded double emulsion PLGA droplets and microparticles in a glass capillary microfluidic device. For our peanut allergy application, we would like to make and test *in vivo* microparticles that are loaded with both peanut protein and an adjuvant and also microparticles loaded with only single peanut protein. For our porphyrin delivery application, we would like to further optimize the formulations and test the efficacy of the drug-loaded microparticles *in vitro* on cancerous and non-cancerous cell types.
Appendix A

Characterization Methods

A.1 Scanning Electron Microscopy and Diameter Measurements

I used a FEI XL30 FE-SEM to generate SEM images at 1.5 to 3 kV. I measured the microparticle diameters from these SEM images using ImageJ.

A.2 Release Study

I compiled release curves of each of the encapsulated drug compounds through a release study.

In some cases, I resuspended an entire batch of microparticles in 1 mL PBS without measuring the mass, but knowing the mass of drug that went into that batch of particles based on the generation time. In all other cases, I weighed an entire batch of particles and then divided the batch into accurately weighed replicates and resuspended them in 1 mL PBS. In either case, the mass of particles that went into each mL of PBS was between 2 and 30 mg.

At the desired timepoints of 24-hour intervals for all release studies and 15 and 60 minute timepoints for the burst release studies I centrifuged the samples at 1 krpm
for 5 minutes. I pipetted off the supernatant for measurement and replaced it with 1 mL of fresh PBS, pipetting to resuspend.

I assessed the protein concentrations of the release sample supernatants of the peanut protein microparticles using a microBCA assay (Thermo Scientific 23235). The porphyrin, ascorbate, and dox concentrations were assessed spectrophotometrically by Artak Tovmasyan or myself.

A.3 Microparticle Digestion and Protein Extraction Assay

For the protein-loaded microparticles, I assessed the total protein content using a digestion and extraction assay adapted from [54]. This assay works on either dry or wet particles. For dry particles, I began with either an entire batch of particles or with an accurately weighed portion of a batch of particles. Wet particles were assessed at the end of a release study after removing the last timepoint’s supernatant.

I added 200 µL of DMSO to the particles and incubated for 1 hour at room temperature, occasionally hand-shaking. I then added 1 mL of 0.05 M NaOH 5% SDS solution, pipetted to mix with the DMSO, and incubated for 1 hr at room temperature with occasional hand-shaking. Finally, I centrifuged the samples and performed a micro BCA assay (Thermo Scientific 23235) on the supernatants. I made the microBCA standards with a 5:1 ratio of 0.05 M NaOH to 5% SDS:DMSO.

A.4 Microparticle Digestion and DMSO-Soluble Compound Assay

Porphyrin, ascorbate, and doxorubicin are all soluble in DMSO. I digested the PLGA particles in 700 µL DMSO and I or Artak Tovmasyan measured the concentration of the payload in the DMSO spectrophotometrically at 454 nm for porphyrin, 265 nm for ascorbate, and 480 nm for doxorubicin. We used a small amount of PLGA dissolved in DMSO as the blank.
Appendix B

Hydrophilic PDMS for O/W Emulsions

With Michael Keane, I developed a new protocol for creating hydrophilic PDMS chips to use as our secondary chips in the two-chip system.

B.1 Prior Methods

Until summer 2013, I had used two coating protocols to turn the channel walls of PDMS chips hydrophilic. The first method was to coat the channel walls with a sol-gel and then deposit acrylic acid [53]. This method had the advantage of the ability to pattern the wettability. We could selectively flow the acrylic acid solution over only the flow-focusing device that was for making O/W or W/O/W emulsions. The second method was to flow PVA into chips and let it adsorb to the channel walls, repeat, and finally heat-immobilize the polymer on the surface [55] (Section 3.5). I did not use the PVA coating method for patterned wettability.

Each of these methods was time-consuming to treat the chips after the initial fabrication and bonding process. Both methods produced coatings of varying quality, with chips frequently becoming irreversibly clogged during the process or upon use.
during droplet-making.

B.2 Literature Claims

Yao et al. investigated how to turn PDMS hydrophilic by doping it with the surfactant poly(dimethylsiloxane-ethylene oxide) (PDMS-b-PEO) (Polysciences 09780-100, molecular weight 600). They cured PDMS with PDMS-b-PEO at a range of concentrations between 1 and 1.9%. (I will refer to the cured PDMS doped with PDMS-b-PEO as PEO-PDMS.) They observed a decrease in the contact angle between a drop of water and PEO-PDMS over time and that lower contact angles correlated with higher concentrations of PDMS-b-PEO. They also reported the stabilized contact angles (after 200 seconds) of several concentrations of PDMS-b-PEO in PDMS. 0% PDMS-b-PEO yielded a 102° contact angle. The lowest contact angle measured was approximately 24° for 1.9% PDMS-b-PEO in PDMS [57].

Roman explains that under air block copolymers like PDMS-b-PEO expose their hydrophobic moiety, the PDMS segment. However, when exposed to water, the copolymer will undergo block migration to expose the PEO moiety, thus changing the surface to become more hydrophilic [56].

Bonding PEO-PDMS chips to glass coverslips by plasma ashing could not be accomplished by Yao et al. if the PDMS-b-PEO concentration was 2% or higher [57].

Finally, Yao et al. measured the contact angle of liquid entering microfluidic channels by capillary force. The higher the concentration of PDMS-b-PEO, the faster the liquid moved through the channel. A minimum concentration of 0.8% achieved the high speed capillary movement [57].
B.3 Hypothesis

We expected that we would be able to use PEO-PDMS as hydrophilic chips based on the contact angle measurements from [57]. From the information about block migration when exposed to water, we were hopeful that PEO-PDMS would help us automatically pattern the wettability of compound double emulsion chip (one hydrophobic droplet-maker and one hydrophilic droplet-maker). However, as Yao et al. had not made droplets in their microfluidic chips, we wanted to replicate their results before trying to generate O/W droplets.

B.4 Curing

The fabrication of PEO-PDMS chips added only one sub-step to the PDMS curing process. While combining the PDMS base and curing agent at a 9:1 ratio, we simply added the desired amount of PEO-PDMS. We then mixed, degassed, and cured as with typical PDMS (Section 3.5).

B.5 Contact Angle Measurements

Before attempting to make droplets with the chips, we first repeated the contact angle experiment from [57]. Michael Keane doped PDMS with PEO-PDMS at five concentrations between 1 and 1.95%. He measured the contact angle of water with the surface of the PEO-PDMS and a PDMS control (Figure B.1). Michael observed a decrease in the contact angle over time as the surface of the PEO-PDMS was exposed to the polymer, while the contact angle of the PDMS remained almost unchanged. He also observed lower contact angles with higher concentrations of PEO-PDMS added. However, the magnitudes of the contact angles he measured for the PEO-PDMS were generally higher than what was reported in [57].
Figure B.1: Water contact angles measured on PDMS and PEO-PDMS. The earliest measurement is represented by blue circles, the middle measurement is represented by red plusses, and the latest measurement is represented by green Xs. Data collected by Michael Keane.

B.6 Device Fabrication

Through experimentation, we found that it was necessary to swell and deswell the PEO-PDMS chips so that they would firmly bond to glass coverslips. After curing the PEO-PDMS, cutting the chips from one another, and punching the holes for the tubing, we commenced a swelling/deswelling protocol. We put the chips into hexane overnight, then acetone overnight, then water overnight, and finally let them air-dry.
overnight.

Michael and I tested two PEO-PDMS bonding protocols. The first was to spin uncured PEO-PDMS onto coverslips, press the cured PEO-PDMS chips onto the coverslips, and then baked for 30 s at 110°C. However, this method produced inconsistent results. The second method was to use the same bonding protocol we did with regular PDMS chips: plasma ashing for 30 s at 40 W, then baking for 1 hr at 80°C. This method was consistent and we went forward with it.

B.7 Droplets

Despite our expectations based on Yao et al.’s report, we were unable to make O/W droplets using PEO-PDMS chips made as outlined above. We tried incubating with water in the channels, the mechanism suggested by [56], even overnight, but were unable to turn the channels hydrophilic enough to sustain droplets. However, by oxygen plasma ashing the chips just before use, the chips were able to produce droplets. The chips could be used for 4 to 8 hours, but would have to be re-ashed before use the following day. We found that 1% PDMS-b-PEO in PDMS was sufficient to sustain the hydrophilic surface. We empirically found that a sufficient oxygen plasma ashing protocol was 50 W for 60 s. Undoped PDMS chips that we exposed to oxygen plasma were not able to form O/W droplets.

Because we had to expose the entire chip to oxygen plasma, it did not seem that contact with water alone caused block migration. We were not able to achieve automatically patterned wettability on integrated chips and continued to use the two-chip system with the second chip made with PEO-PDMS.

The PEO-PDMS chips do not have the issues that plagued the coated chips. Their channel walls are just as smooth as plan PDMS chips so they are not prone to clogging. It is also nearly always possible to reuse a chip on multiple days, whereas trying to clean a coated chip often left it irreversibly clogged. For these reasons and
the reduced fabrication time, we switched to using PEO-PDMS for our secondary chips that formed O/W droplets.
Appendix C

Ara h Proteins

The protein extracted from peanut is composed of 13 proteins, named Ara h 1 to Ara h 13. Their homologs, molecular weights, isoelectric points, and IgE reactivities are in Table C.1.
Table C.1: The homology, molecular weight, isoelectric point, and IgE reactivity of the thirteen Ara h proteins.

<table>
<thead>
<tr>
<th>Ara h Number</th>
<th>Homology</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>IgE Reactivity (%)</th>
<th>Reference</th>
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<tr>
<td>1</td>
<td>cupin (vicilin-type)</td>
<td>63.5</td>
<td>4.55</td>
<td>88</td>
<td>[65, 102, 103]</td>
</tr>
<tr>
<td>2</td>
<td>conglutin</td>
<td>17</td>
<td>5.2</td>
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<td>[65, 102]</td>
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<tr>
<td>3</td>
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<td>4.5</td>
<td>45</td>
<td>[65, 103]</td>
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<tr>
<td>3.02 (renamed from 4)</td>
<td>cupin</td>
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<td>44</td>
<td>13</td>
<td>[102]</td>
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<tr>
<td>5</td>
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<td>43</td>
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