UNIVERSITY OF CALIFORNIA,
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# An Investigation of Double Emulsion Droplet Composition, Generation, and Stability Using Microfluidics

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

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**ABSTRACT OF THE THESIS**

An Investigation of Double Emulsion Droplet Composition, Generation, and Stability Using Microfluidics

By

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Master of Science in Biomedical Engineering

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Professor Abraham P. Lee

Synthetic biology is the study and engineering of organisms with novel, unique functions. Specifically, bottom-up synthetic biology involves assembling complex cells or structures from simpler units. One such component of this field involves the production of artificial cells. By employing a bottom-up approach, researchers can investigate individual properties of the cell such as lipid membrane characteristics, specific biological pathways, and encapsulation phenomena.

Microfluidic methods enable the uniform generation of artificial cell precursors, otherwise known as Double Emulsions Droplets (DEDs). Although DEDs are versatile in both composition and application, they can be limited by their instability and susceptibility to rupture. This is observed by a reduction in DED concentration over time. Here we present a study for the optimization of the external aqueous phase resulting in the most stable and highest concentration of DEDs over a period of two weeks. By post-processing the DEDs in squeezing channels, the oil layer is thinned resulting in less depletion of the sample.

# Chapter 1: Introduction

## 1.1 Problem statement

There are more cells in the human body than galaxies in the known universe. Even by conservative estimates, the difference is on the order of hundreds of billions[1]. Furthermore, cells interact with one another via junctions and respond to physical and chemical signals which ultimately affects their function. Understanding and manipulating these functions has been a primary goal of synthetic biology for decades. The sheer number of cell types, their inherent complexity and interactions makes this no trivial goal.

Double emulsions have the potential to serve as a template for studying the interactions of various cell types. Their ability to encapsulate both hydrophilic and lipophilic cargo makes DEDs a suitable candidate for creating artificial cells[4,16,17]. However they pose limitations such as the tendency to coalesce and rupture, which reduces their storage times and usability for downstream applications[2,14]. Thus, optimization of DED composition and methods to prolong their storage are required.

## 1.2 Proposed Solution

The study and creation of artificial cells is an undertaking that has captivated engineers and biologists worldwide. The prospect of creating synthetic cells that can interact and even replace biological ones may seem in the realm of science fiction, but recent research suggests this goal may not be so impractical. What constitutes an artificial cell is not strict in definition, but they are generally understood as being able to perform one or more functions of biological cells[3,12]. For the study of complex biological pathways, artificial cells can provide a framework for determining how individual components contribute to a certain response. For instance, by functionalizing an artificial cell with one or more antigens, the role of each antigen in specific immune responses can be further elucidated.

Microfluidics provides a low-cost method of generating monodisperse artificial cell precursors, or Double Emulsions Droplets (DEDs). Typically, these DEDs come in one of two forms: liposomes or polymersomes. The difference between these two particles resides in the middle layer - the interface between the inside of the DED and the outside. As the names suggest, liposomes are composed of a lipid-based middle layer while polymersomes contain block copolymers at the interface. Liposomes, however, are more representative of biological cells due to their higher membrane fluidity and higher permeability at the cost of being less stable[4]. Here we present a microfluidic method for generating lipid based DEDs that are stably stored on the order of weeks. Because oil shell thickness has been correlated with DED instability, it is desirable to thin the oil layer immediately after droplet generation[9,14]. This is accomplished by flowing the DED sample through squeezing channels resulting in shearing and a smaller oil droplet being released from the shell.

### 1.3 Scope of Report

This thesis describes the design, methods, results, and outlook on generating double emulsion water-in-oil-in-water (w/o/w) droplets as the first step in synthesizing an artificial cell. A brief review of generation methods as well as a comparison between different double emulsion compositions is provided. Quantification of DED stability was determined by image analysis of the collected sample over a two-week period. Post-processing of the DED sample through squeezing channels was also performed in order to thin the oil shell.

### 1.4 Summary of Conclusion

The results of this work demonstrate the approach taken to optimize the external phase composition of double emulsion droplets. It was found that 9% Pluronic F68 surfactant with 10% glycerol resulted in the highest concentration of DEDs. By post-processing the sample in squeezing channels, oil shell thinning and reduction in sample depletion from 55.6% to 31.3% was achieved.

## Chapter 2: Background

### 2.1 Double Emulsion Droplets (DEDs)

Synthesizing and mimicking cellular functions is a task that has captivated researchers worldwide. Artificial cells have many potential applications from drug encapsulation and delivery to cell interaction studies. DEDs are often used as a framework for constructing artificial cells due to their ability to encapsulate both hydrophilic and lipophilic components. This is possible due to their structure and composition, notably the presence of an oil shell that partitions a smaller aqueous droplet from the external aqueous fluid[4,10-14].

For double emulsions to become more representative of an artificial cell, they must first undergo a process called dewetting. This involves the removal of oil from the middle phase, which is governed primarily by interfacial tension. Control of this dewetting process is crucial in ensuring sample integrity because while DEDs are generally stable when stored at room temperature, the lipid bilayer that remains after dewetting, is not[5]. Thus, it is desirable to generate DEDs that retain their oil shell rather than shedding it immediately.

Dewetting is not a binary process, which means DEDs can experience no, partial, or complete dewetting. Depending on the degree of dewetting, the DEDs take on unique forms. The complete engulfing scenario refers to a DED, partial engulfing refers to a single compartment multisome (SCM), and non-engulfing refers to a giant unilamellar vesicle (GUV). To quantify the tendency of the DED to take on a particular form, a metric called the spreading coefficient is used, which is a linear combination of the interfacial tension between each phase[5]. A positive spreading coefficient indicates that the phase will wet or spread on the interface while the opposite is true for a negative spreading coefficient. Thus, by tuning the composition of each fluid or phase, it is possible to generate DEDs with a preference to one of the three equilibrium forms.

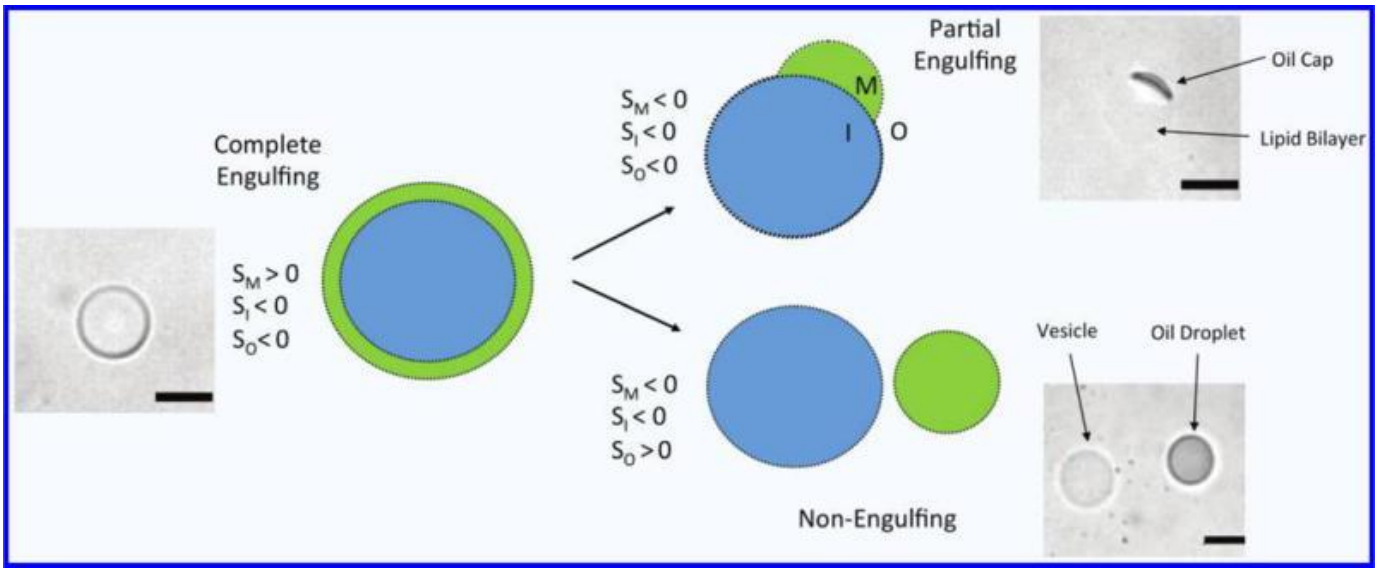


Figure 2.1 - Three possible configurations of Double emulsion droplet from Vallejo et. al paper[5]. Scale bar is 20µm.

## 2.2 Generation Methods

There are a variety of methods of generating DEDs and lipid-based vesicles, each with their own set of advantages and shortcomings. They all broadly fall into one of two categories: solvent free methods or solvent displacement methods. The main difference between these methods is the presence or absence of an organic solvent (e.g. Oleic acid). For solvent free methods, the final sample is free of any organic solution and contains lipid vesicles in an aqueous medium. In contrast, displacement methods result in a sample where lipid molecules are dissolved in an organic solvent and formation of vesicles requires dewetting, or solvent removal to occur[5,19].

The most common solvent free method is film hydration. In this process, a lipid solution placed on a surface forms a thin film after complete evaporation of the solvent. After applying an aqueous solution, the film swells, and vesicles begin to form. This method has the advantages of being low cost, relatively simple, and lacks organic solvent in the final sample. However, the vesicles produced have high polydispersity and can be both unilamellar and multilamellar[4]. Thus, post-processing such as sized-based filtration is necessary to obtain a more homogenous sample.

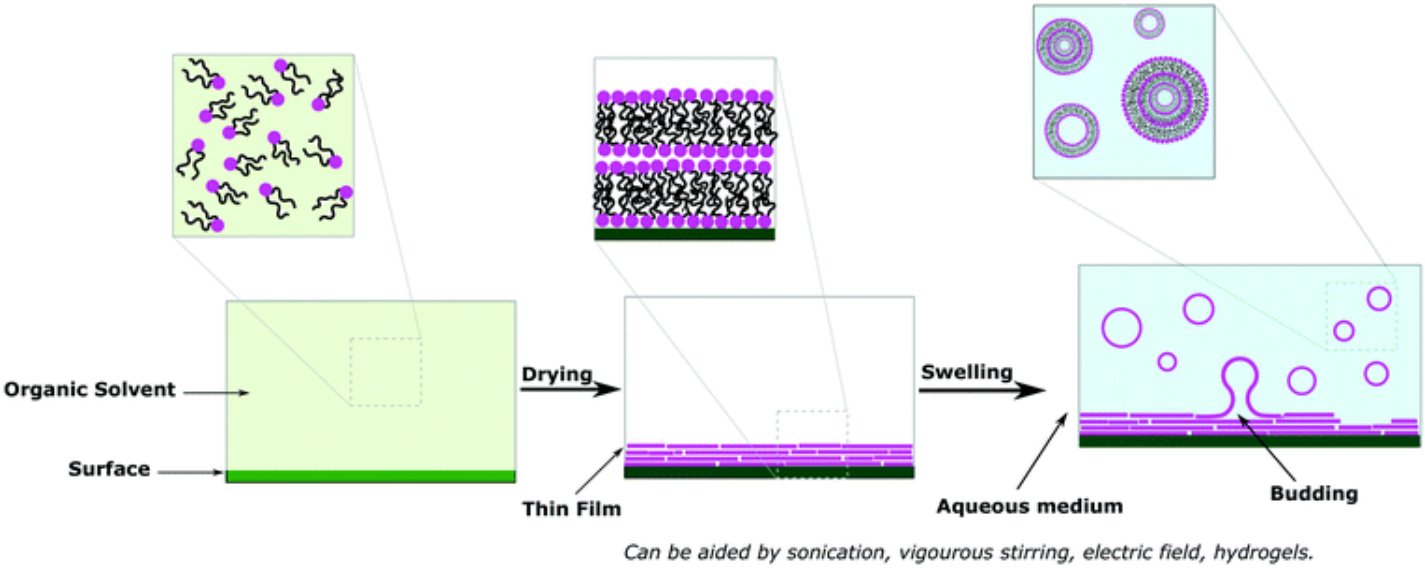


Figure 2.2 - Vesicle formation via thin film hydration[4]

Conversely, solvent displacement methods are more conducive for encapsulation of hydrophilic cargo at the cost of requiring solvent removal to generate lipid bilayers. In many cases, these organic solvents are toxic to cells, making the dewetting process essential before the sample can be used in experiments. Most solvent displacement methods are a variation of emulsion phase transfer. Single emulsion (water-in-oil) droplets are first made by agitating a small volume of water into an organic solvent containing dissolved lipids. This single emulsion is then poured through a medium containing both the organic solvent and an aqueous solution separated by an interface containing lipids. Due to the amphiphilic nature of lipids and the density difference between the organic and aqueous solutions, the single emulsions are able to transition into double emulsions (w/o/w) upon passing through the interface[4]. While emulsion phase transfer may also be appealing due to its relative simplicity and capability of encapsulating hydrophilic components, it has little control over vesicle diameter and lamellarity. Recently, microfluidics has emerged as a gold-standard method of generating DEDs, vesicles, and ultimately artificial cells. Specifically, microfluidics enables greater control over droplet diameter, unilamellarity, and the composition of each fluid phase.

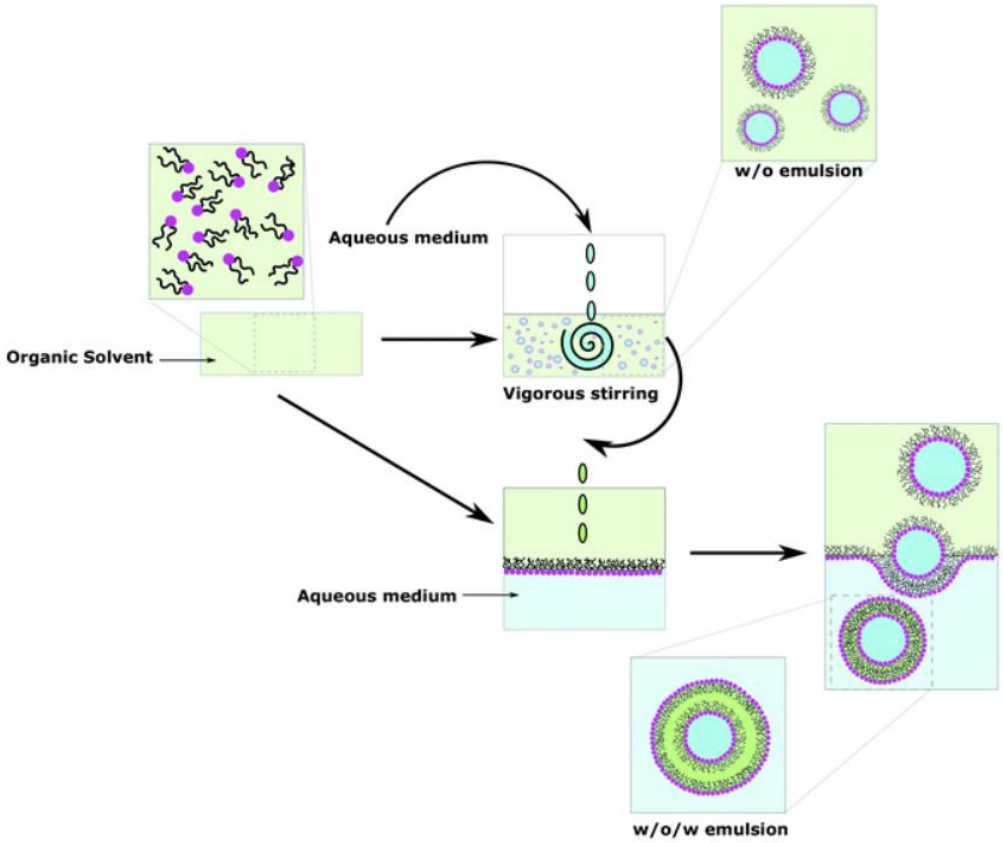


Figure 2.3 - Formation of Double emulsions using phase transfer[4]

## 2.3 Microfluidics

The advent of microfluidics has enabled greater control over many droplet parameters such as size and oil shell thickness. The variety of microfabrication techniques in this field allows for numerous channel geometries and features - each capable of generating DEDs. Like the methods mentioned previously, each microfluidic approach has its benefits and drawbacks. Selection of the "best" design would thus depend on the desired features of the double emulsions.

One of the earlier designs for DED generation consisted of a double-T junction. In this design, a single emulsion first forms at the first junction then becomes a double emulsion at the second junction. The droplets are generated via shear force and their size is governed primarily by the flow rates of each fluid. In fact, many designs still rely on a two-step emulsification process. For instance, the double-cross shaped design uses this process, but offers greater control over droplet size due to shear being applied in from two directions as opposed to one[6]. However, both designs are limited by the fact that they result in DEDs with a thick oil shell, which can shorten the shelf life of the sample and make the dewetting process more difficult[9,14].

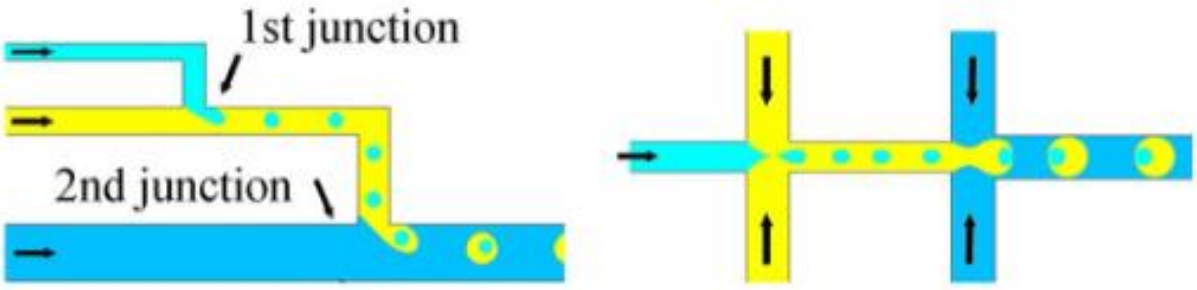


Figure 2.4 - Microfluidic methods of generating double emulsion droplets; double-T junction (left), double-cross shaped (right)[6]

The use of glass capillaries as flow focusing microchannels is another commonly used method of generating double emulsions. In this method, three microcapillaries are aligned coaxially, with the innermost providing the internal aqueous solution and the outermost providing the external aqueous solution. Diameter of the DEDs is mainly dependent on the size of the opening at the end of the middle injection tube[10,13,14]. While this method can generate double emulsions with thin oil shells at high throughput, it is limited by the difficulty of fabricating the device[6]. To elongate and thin the glass capillaries, they are pulled from each end over a flame. This process is prone to breaking the capillaries, especially if the desired double emulsion size is much smaller than the initial diameter of the glass.

To address these limitations, we utilize a microfluidic device with a single flow focusing junction to generate double emulsions with a thin oil shell.

# Chapter 3: Research Methods

### 3.1 Device Fabrication and Setup

All microfluidic devices were fabricated by first pouring a 10:1 mixture of polydimethylsiloxane (PDMS) and curing agent on silicon wafers patterned with SU-8 photoresist forming positive channel features. This mixture was cured overnight at 65°C to allow the mixture to harden. The PDMS was then peeled from the silicon wafer and inlet and outlet holes were made using 1mm biopsy punches. Devices were then cleaned of dust and bonded to a glass slide using a Harrick Plasma chamber.

Because PDMS is hydrophobic, a surface treatment was applied in order to ensure that the external phase channel became hydrophilic. This is necessary because the external phase consists of mostly water, so the surface treatment enables the solution to wet the channel at the droplet generation region[5]. Directly after bonding, a vacuum was applied at the outlet and 0.4% poly vinyl alcohol (PVA) was flowed through the external phase channel for one minute

followed by 0.1% PVA for 4 minutes to wash away any residual crystals. This was done for each of the ten sets of channels per chip.

DEDs were generated by first attaching microtubes containing the external, middle (oil), and internal phases. Each microtube contained two lengths of tygon tubing, one of which was connected to the device and the other attached to a pressure regulator system operated by a LabView controller. This allowed for independent adjustment of the inlet pressure for each phase. Double emulsions were then collected via tubing that ran from the outlet into an Eppendorf tube.

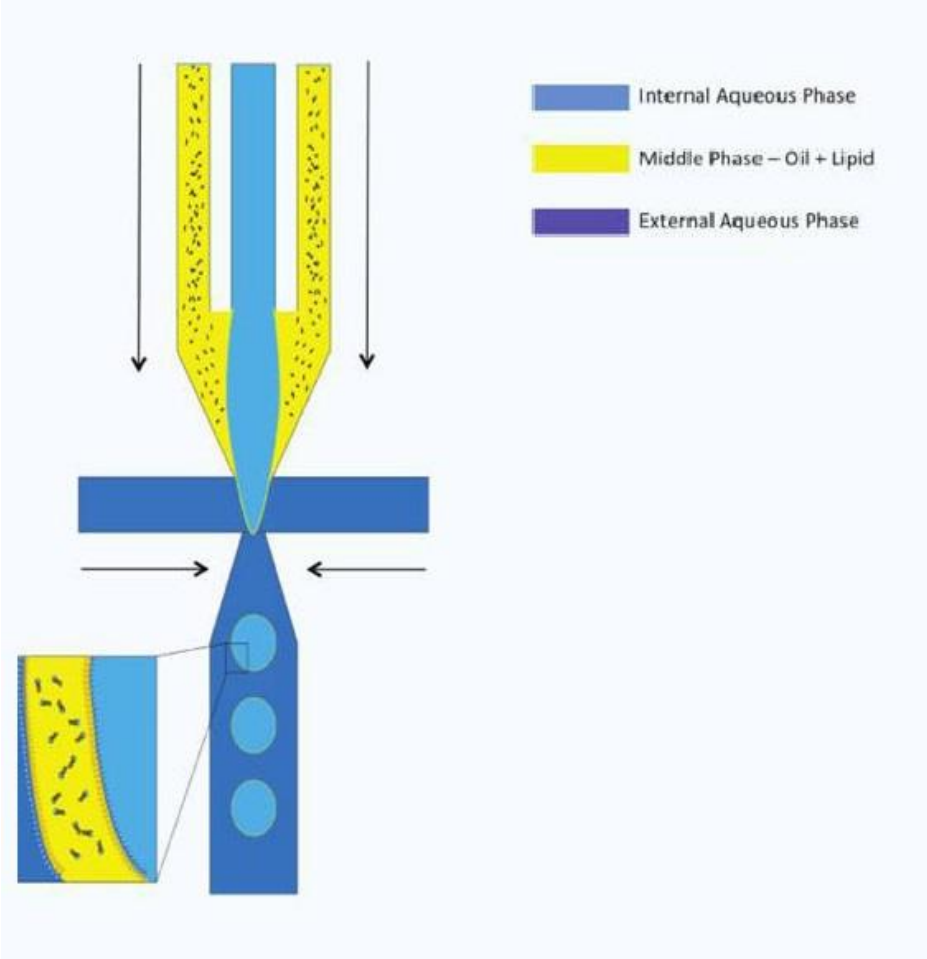


Figure 3.1 - Schematic of DED generation device from Vallejo et al. paper[5]

### 3.2 Reagent composition

The internal aqueous phase consisted of 1% Pluronic F68 and 250mM sucrose. A stock solution of 10% F68 was purchased from Sigma Aldrich and diluted in deionized water to the desired concentration. The middle oil phase was prepared from DOPC and DPPC lipids (Avanti

Polar Lipids) dissolved in oleic acid at a concentration of 10mg/mL. This lipid solution was placed in a sonication bath for 1h to aid with the breakup of lipid clumps. The final oil phase composition was a 3:1 ratio of DOPC to DPPC with 5mg/mL of cholesterol. The external phase contained either 1%, 6%, or 9% F68 with 10% glycerol. For all experiments, internal and middle phases remained the same while three different external phase compositions were tested to determine which resulted in the least depletion of DEDs during storage at room temperature.

### 3.3 Squeezing Channels

Previous studies have used mechanoporation techniques, namely squeezing, to deliver cargo to cell cytoplasm[7,8]. It was hypothesized that passing the DEDs through a constriction could result in oil layer thinning and transient poration of the lipid layer. Three gap widths (10µm, 12µm, 15µm) were fabricated using standard soft lithography methods. Each constriction region also stretched for a length ranging from 50µm to 200µm, resulting in 12 unique squeezing channel dimensions per device. DEDs were collected and flowed through squeezing channels to determine which channel geometry resulted in the most thinning of the oil shell.

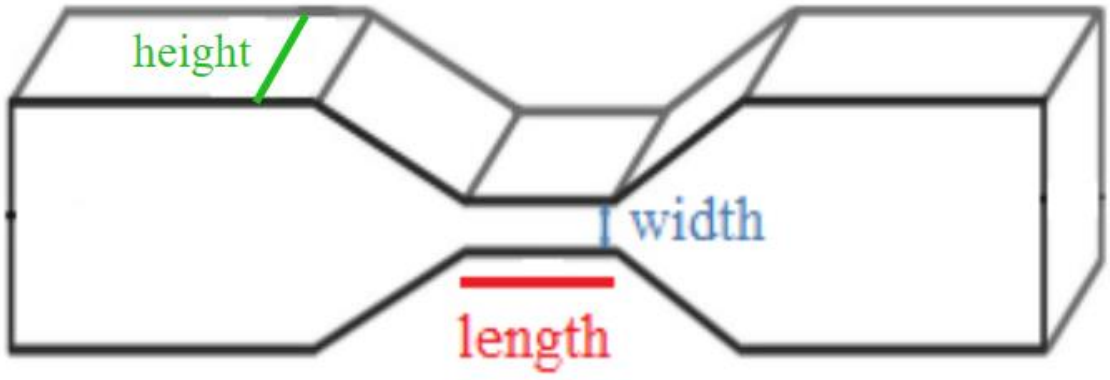


Figure 3.2 - Squeezing Channel Design[9] with Width (10µm, 12µm, or 15µm), Length (50µm, 100µm, 150µm, or 200µm), and Height (20µm)

## Chapter 4: Results and Discussion

### 4.1 External Phase Optimization

DED generation was observed real-time under an inverted microscope with the aid of a high-speed camera. Thickness of the oil phase was first determined qualitatively based on droplet breakoff at the orifice and droplet adherence just before the outlet. Varying the surfactant (Pluronic F68) concentration in the external phase had visible impact on droplet oiliness and resulted in differing levels of DED depletion during storage. As seen in Figure 4.1, droplet

generation characteristics vary significantly between 1% and 9% F68 in the external phase. It was observed that 1% F68 in the external phase resulted in DEDs adhering to one another downstream of the orifice. Increasing the F68 concentration resulted in discrete formation of DEDs with thinner oil shells.

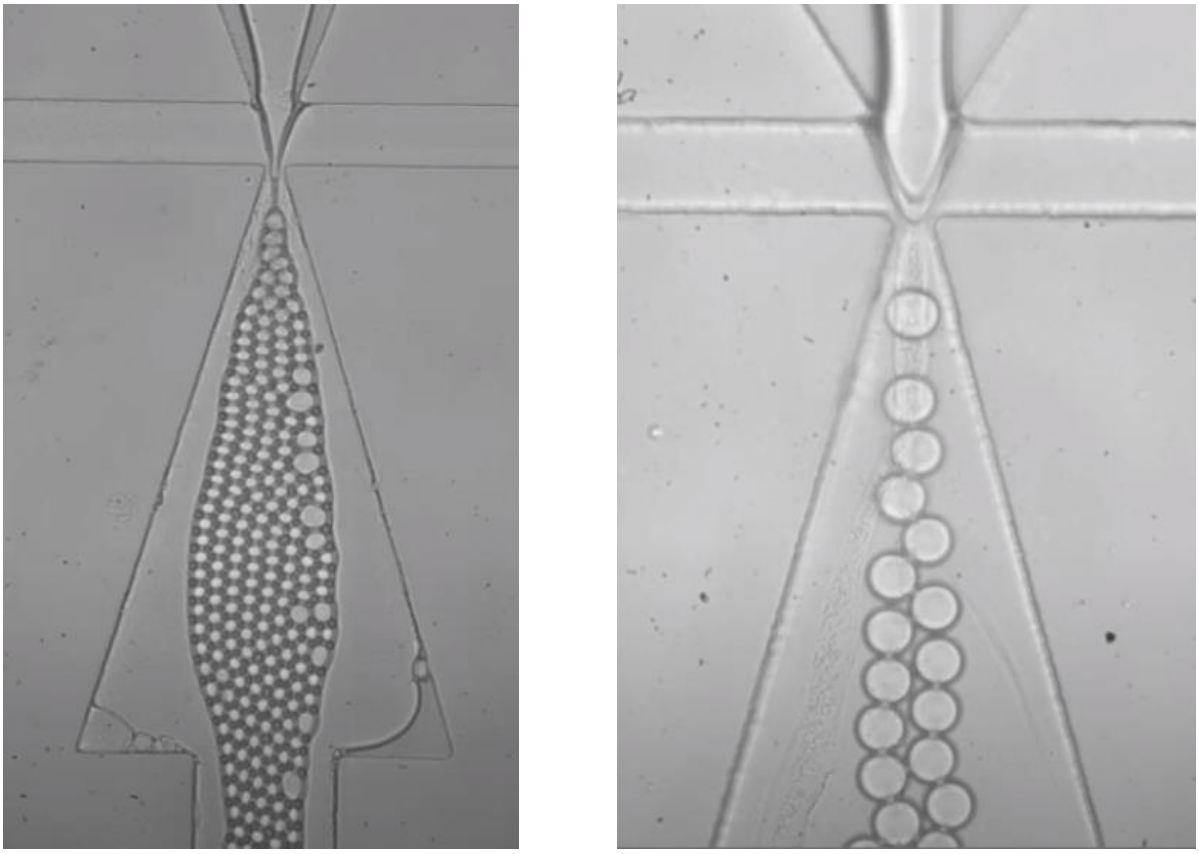

Figure 4.1 - Brightfield image of DED generation using external phase with 1% F68 (left) and 9% F68 (right)

Minimizing sample depletion is crucial for future applications of the DEDs. Thus, collected DEDs were counted in a hemocytometer slide (Incyto) at Day 0, Day 7, and Day 14 to determine the optimal composition of the external phase. In some samples, immediate dewetting occurred causing the DEDs to transition into an SCM morphology. Because this is undesirable, only in-tact double emulsions that retained their oil shell were counted. DED counts over a two-week period are shown in Figure 4.2.

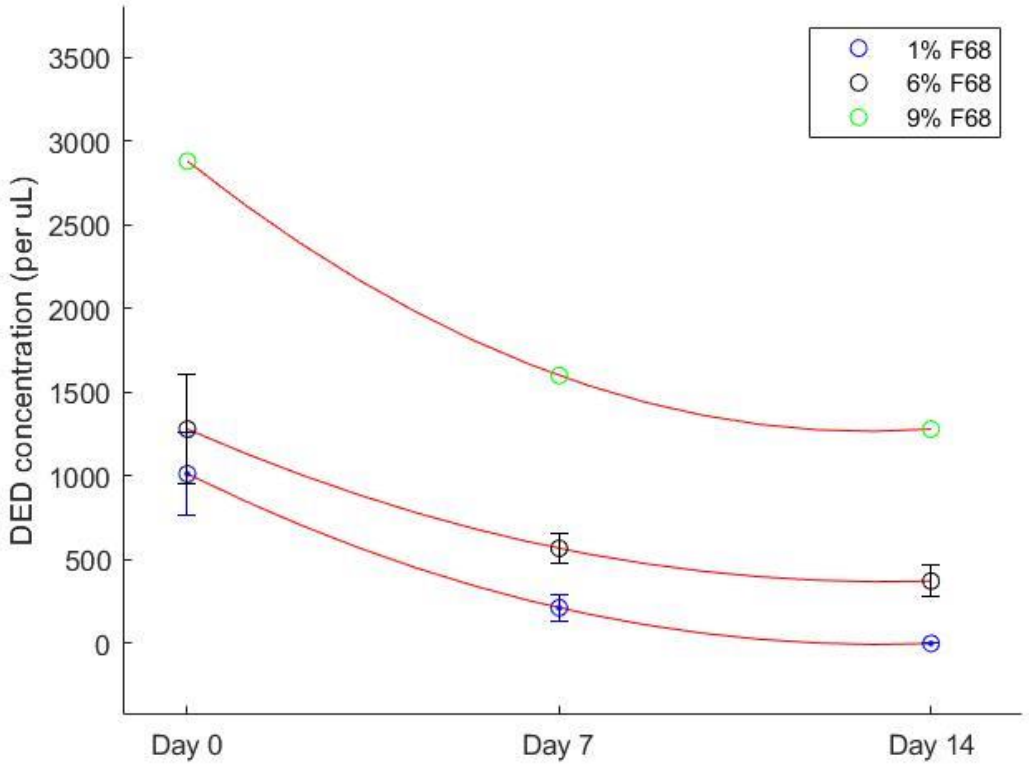


Figure 4.2 - DED concentration over two weeks using external phase composition containing 1% F68 (n=3), 6% F68 (n=3), and 9% F68 (n=1)

Curves of best fit were applied to the data using a second-degree polynomial regression. By taking the derivative of the curves at Day 0 and Day 7, the decay rate of the sample was approximated as shown in Table 4.1. All decay rates are in terms of DED loss per day in 1µL of the sample.

Table 4.1 – DED decay rate at Day 0 and Day 7

| | 1% F68 | 6% F68 | 9% F68 |
|---|---|---|---|
| Decay rate – Day 0 | -156 | -139 | -250 |
| Decay rate – Day 7 | -72 | -61 | -114 |

## 4.2 Squeezing Channel Experiments

Double emulsions were flowed through squeezing channels of various dimensions to optimize the conditions for oil shell thinning. All DEDs had a diameter ranging between 25µm and 35µm, so gap width for the squeezing channel were designed to be approximately half of droplet diameter (10µm to 15µm). As mentioned previously, a total of twelve designs were

tested using three different widths and four different lengths. It was observed that upon passing through the squeezing region, the DEDs became elongated. After exiting the squeezing region, the DEDs remained in-tact with a smaller oil droplet being pinched off. Thus, the purpose of this experiment was to determine the channel dimensions that resulted in the greatest volume of oil being removed from the double emulsion.

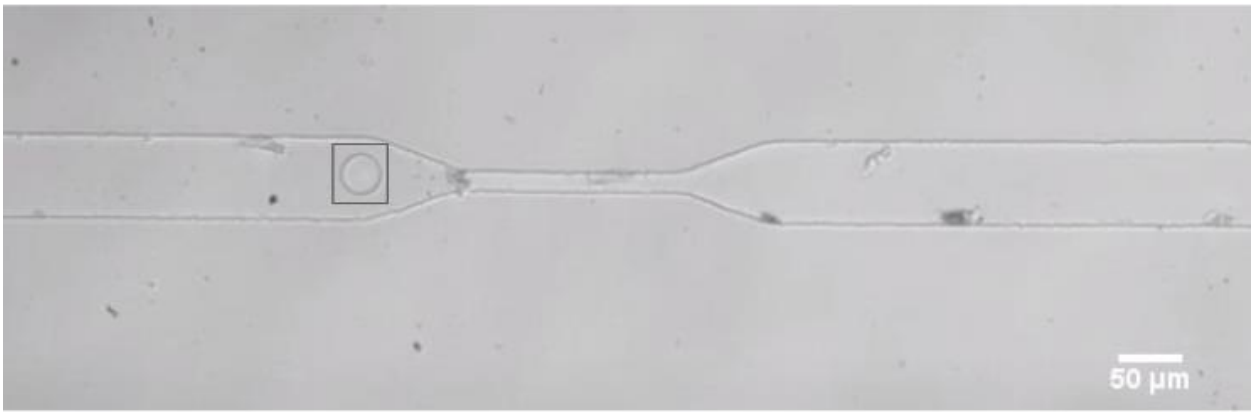

Figure 4.3 - DED prior to entering squeezing region

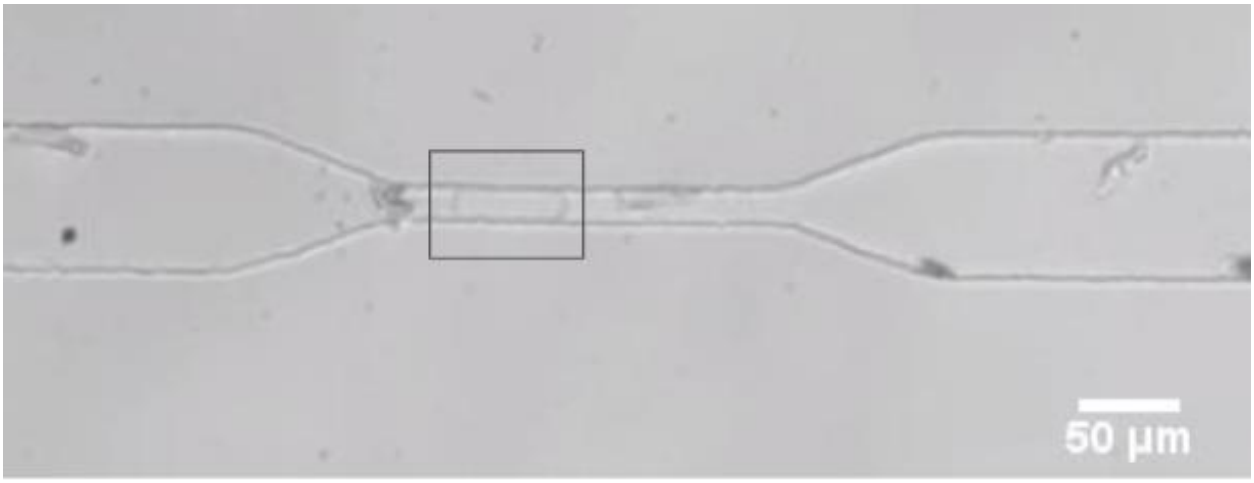

Figure 4.4 - Elongated DED during squeezing region

Figure 4.5 - DED with thinned oil shell (black) and released oil droplet (blue)

Initial results indicated that the 12µm and 15µm width channels were not able to cause any oil removal from the DEDs. The 10µm channel, however, did result in oil shell thinning. Furthermore, the diameter of the oil droplet released from the shell was found to be greater with increasing constriction length as shown in Figure 4.6. ImageJ was used to make all measurements of droplet diameter.

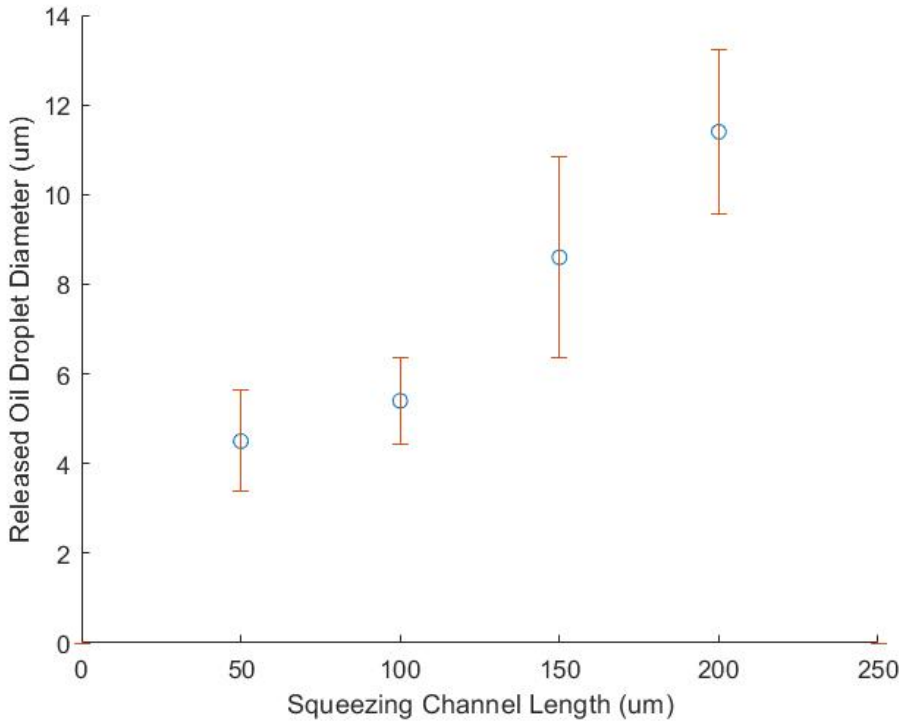

Figure 4.6 - Diameter of oil droplet released from double emulsion shell after squeezing (n=3)

### 4.3 Shelf Life Study

After observing oil shell thinning, it was hypothesized that passing the DEDs through the squeezing channels prior to collection would result in less depletion of the sample over time. This experiment was inspired by research done by Tanyeri et al. who found that the resistance that prevents the inner aqueous phase from coalescing with the external phase is inversely proportional to the oil shell thickness[9]. In other words, DEDs with a thinner oil shell are less likely to rupture.

DEDs were generated using 9% F68 and 10% glycerol in the external phase. Half of the sample was stored in a separate Eppendorf tube while the other half was pumped through the squeezing channel. After collection, both DED samples were stored at room temperature conditions. 10µL of each sample was removed via pipette for counting in a hemocytometer slide. Comparison of DED counts over a two-week period is shown in Figure 4.7. Counts of both samples at Day 7 and Day 14 indicate that DEDs that were processed in squeezing channels prior to collection experienced less depletion. As before, the decay rates were computed using a second-degree polynomial regression yielding a reduction in decay rate from -217 to -80 at Day 0 and a reduction from -103 to -57 at Day 7.

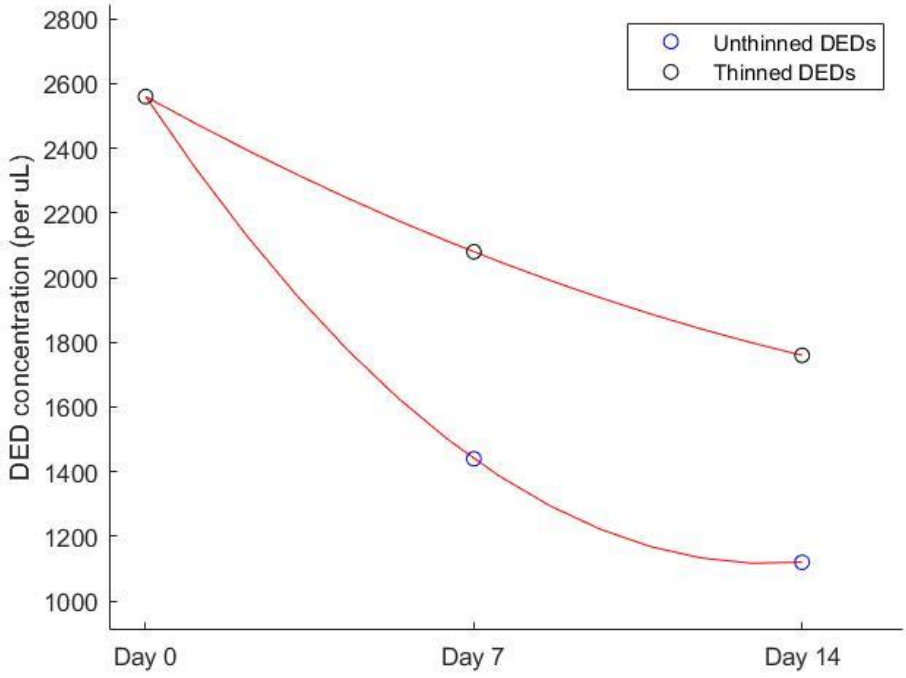


Figure 4.7 - DED Concentration with Thinned and Unthinned oil shells over 2 weeks (n=1)

# Chapter 5: Conclusion

## 5.1 Discussion of Results

As a result of testing external phase compositions containing either 1%, 6%, or 9% F68, it was found that 9% F68 with 10% glycerol resulted in the highest concentration of DEDs being generated. This indicates that surfactant concentration plays a key role in DED stability by preventing coalescence. Non-ionic surfactants such as F68 have been shown to assemble at the oil-aqueous interface thereby reducing both the surface energy and the likelihood of DED merging[15,18].

By then flowing the DED sample through squeezing channels a reduction of the oil shell volume was demonstrated as seen by the released oil droplet. Comparison of DED concentrations over a two-week period indicated that oil shell thinning resulted in less depletion of the sample and thus a longer shelf life as shown in Table 5.1

Table 5.1 - Percentage change in DED concentration over 7 and 14 days

| | 1% F68 | 6% F68 | 9% F68 | 9% F68 + squeezing |
|---|---|---|---|---|
| Day 7 | 79.0% | 54.2% | 44.4% | 23.1% |
| Day 14 | 100.0% | 70.9% | 55.6% | 31.3% |

It was also observed that the squeezing channels with the longest length (200μm) resulted in the most volume removed from the oil shell. This is due to the formation of an oil "tail" in the constriction region. In other words, while the DED is being squeezed, a portion of the oil shell begins to separate from and lag behind the internal aqueous phase. In squeezing channels of longer length, this separation is greater, and it becomes energetically more favorable for the oil tail to pinch off rather than retract back into the DED shell[9].

## 5.2 Limitation of the Study

While measurable improvements were made in DED generation and storage, the formation of a functionalized lipid bilayer as in a biological cell was not demonstrated. Longer squeezing channel lengths could have been tested to determine if oil shell thinning only occurs within a certain range. Despite demonstrating a reduction in DED loss from 55.6% to 31.3%, a longer shelf life study was unable to be performed due to time constraints.

## 5.3 Future Directions

The generation and dewetting of double emulsions comprise the first two steps in the formation of an artificial cell. While this study focused on the optimization of these processes,

the next direction would involve functionalization of the lipid bilayer after complete dewetting. For instance, the effect of simultaneous pumping of DEDs and biological components (eg. fluorescently labelled proteins) through squeezing channels could be investigated. Transmembrane protein integration into the lipid bilayer could lead to numerous cell interaction studies, potentially elucidating certain pathways or cellular functions.

Researchers worldwide are starting to realize the potential of microfluidics in the formation of double emulsions and GUVs. Although much remains in the development of biologically relevant artificial cells, this work represents a promising first step towards such a goal.

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