Using the surfaces of droplets formed using droplet-based microfluidic technologies to study biomolecular interactions

by

Alex R. McDonald

B.Sc., Vancouver Island University, 2018

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We acknowledge and respect the lək^{w}əŋən peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

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ABSTRACT

This thesis explores droplet-based microfluidic technologies to fabricate bespoke emulsions, focusing on lipid- and protein-based interactions on the surface of the droplets. I introduce microfluidic technologies for droplet formation and factors that influence droplet shape and size. Different approaches to forming single and double emulsions using droplet-based microfluidic technologies are discussed along with considerations such as surface chemistry, droplet stability, and applications. First, I use double emulsions to produce biomimetic vesicles (liposomes) and explain why dewetting is a key step in liposome fabrication. I fabricated a lipid-based surface on aqueous droplets as a bottom-up cell membrane model using a novel combination of naturally-derived lipids in the aqueous phase and a simple plug-and-play microcapillary platform. These asymmetric liposomes remain stable in an asymmetric conformation for over 24 h and are within the size range of actual eukaryotic cells. I show that this cell membrane model is more biomimetic than other current models based on the lipid composition and conditions it is fabricated in. Second, I create a protein-based surface on oil droplets to explore the roles of proteins on droplet stability in beer. This was the first time a hop-oil-in-beer emulsion was made on a microfluidic device to explore the role that proteins have in long-term emulsion stability, which serves as the first step in understanding the unknown stabilization mechanism that keeps beer shelf-stable. By digesting gluten, a protein commonly found in beer during fermentation, with a gluten-specific enzyme, I show that hop-oil emulsion stability is influenced by the concentration of gluten present in solution. This thesis highlights the potential of droplet-based microfluidic technologies to create custom surfaces on emulsions and characterize their properties in two distinct applications: academic and industry.

Contents

List of Abbreviations

GUV	giant unilamellar vesicle
PDMS	polydimethylsiloxane
PC	phosphatidylcholine
SM	sphingomyelin
PE	phosphatidylethanolamine
PS	phosphatidylserine
PTFE	polytetrafluoroethylene
PVA	polyvinylalcohol
HEPES	4-(2-hydroxyethyl)-1-piper azineethane-sulfonic acid
DI	de-ionized
ID	inner diameter
OD	outer diameter
BC	Brewers Clarex
ABV	alcohol by volume

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I always remind my students when I'm teaching that Chemistry is a team sport. I owe everything in this thesis to so many people, I don't know where to start. Alas, here is my attempt:

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Myself, because there's nothing quite like starting a Masters later in life than most people, while in the middle of a pandemic, then losing your grandma and your dog, getting diagnosed with a rare bone disease after thinking you were in the clear six knee surgeries later, realizing your neurochemicals were all out of whack, barely being able to afford to live, and getting some semblance of a thesis together anyways. I feel privileged to have gotten this far.

I'll see you on the dark side of the moon

Pink Floyd

DEDICATION

For my Grandma, Betty Anne Bulawka, and for my first dog and best boy, Jack. We said goodbye to both of them before this thesis was finished, but it would not be complete without their love.

Special thanks to Gracie, PhDog and unyielding supporter of academics everywhere.

Chapter 1

Introduction

This thesis details how droplet-based microfluidic technologies can fabricate custom emulsions, emphasizing the outer layer of droplets as a surface for lipid- and protein-based interactions. Applications for these emulsions can be found in academia and industry, with this research characterizing an example of each.

1.1 Microfluidic technologies for droplet formation

Using microfluidic technologies for chemical research can be traced back to 1940[1], and the use of microfluidic devices has been widely integrated into techniques used for both academic and industrial applications since then[2]. Microfluidic systems work on a micron scale (tens to hundreds of micrometres), and this conveys certain advantages over conventionally scaled systems. These advantages include a large surface area to volume ratio, promoting better thermal uniformity and faster heat transfer[2]. This allows researchers to use smaller volumes of reagents, use less energy during heat transfer and work within a smaller footprint, saving valuable space and economic resources[2]. With those advantages in mind, microfluidic devices have been used for applications such as chemical synthesis, food technology, drug discovery, and more[3].

The devices are highly customizable depending on the application. Diverse types of devices include hydrogels, lab- and organ-on-chip, devices integrated with electronics or 3D culture technology, single cell analysis devices, and droplet-based microfluidics[3], which will be the type of microfluidic technology focused on in this thesis.

1.1.1 Droplet-based microfluidic devices

Droplet-based microfluidic devices consist of channels containing two immiscible phases, one phase continuously flowing and the other dispersed into droplets[3;4] (Figure 1.1). There are active and passive methods to produce droplets. Active methods include the use of electrowetting on dielectric and electrophoresis[4]. Passive methods, which will be the focus of this thesis, use the geometry of the device and the properties of the phases to create droplets when the dispersed phase meets the continuous phase[4]. Some geometric options for passive droplet-based microfluidic devices include T-junction, flow-focusing, and co-flowing, as illustrated in Figure 1.1. The main factors influencing the shape and size of the droplets produced include the flow rate, the interfacial tension of the phases, and the dimensions of the device channels[4]. Droplet-based microfluidics enables the high-throughput production of droplets so researchers can customize the size, structure, and interaction between phases in a stable micro-environment[4].

Microfluidic devices may be fabricated using silicon, glass, quartz, polycarbonate, polymethylmethacrylate, and polydimethylsiloxane (PDMS), which is the most used material for device production as it is chemically stable, provides functional optical and mechanical characteristics, and is highly customizable for creating microstructures with soft lithography procedures[5]. As the device geometries differ with the application, so do the fluid phases used in a droplet-based microfluidic system. The immiscible phases most used are water (or an aqueous solution) and oil, forming an

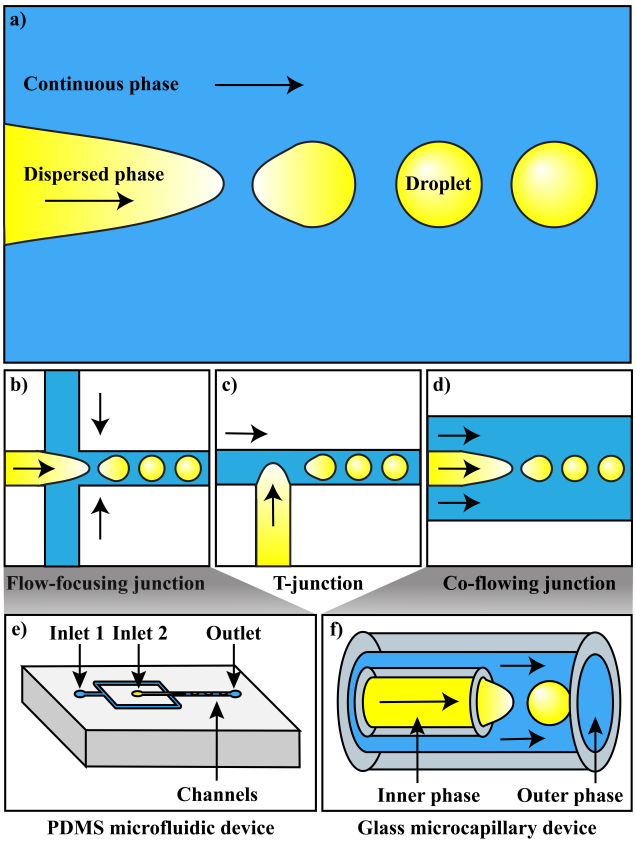

Figure 1.1: Droplet-based microfluidic device concept, device geometry options, and device types. a) Two immiscible fluid phases, a continuously flowing phase (blue) and a dispersed droplet phase (yellow). b-d) Device geometry options for how fluid phases meet are based on application, with b) flow-focusing junction, c) T-junction, and d) co-flowing junctions illustrated. e-f) Two main device types are fabricated from e) PDMS with microfluidic channels or f) glass with microcapillaries.

emulsion[6]. When only two phases are used, what is created is either an oil-in-water (O/W) or water-in-oil (W/O) emulsion, depending on which phase is continuous and which is dispersed, with the nomenclature detailing the composition of the dispersed phase first (see Fig. 1.1)[7]. These are known as single emulsions. Double or multiple emulsions may be formed using two or more phases to create droplets within droplets, which will be discussed in greater detail later in the introduction.

1.1.2 Single emulsions

Which phase is continuous and which is dispersed is controlled by the surface chemistry of the device, as one fluid will preferentially interact with the surface of the channels more than the other. These properties can be influenced by modifications to the channels as well as modifications to the interfacial tension of the fluid, by the addition of surfactants, which are amphiphilic molecules that reduce surface tension between the two phases[8–10]. The use of hydrophobic channels favours the formation of W/O emulsions, while a hydrophilic channel favours the formation of O/W emulsions[8]. PDMS is inherently hydrophobic, so W/O droplets are preferentially formed, while glass is more hydrophilic than PDMS and works better for O/W droplet formation. In addition, both materials may be modified via surface treatment to enable stable droplet formation. One such surface treatment method is used in Chapter 2.

Some products in the cosmetic, food, and pharmaceutical industries contain W/O emulsions; however, much of the academic microfluidic literature deals with W/O systems in colloid (solid in a liqud) structures due to their stability. While both steric and electrostatic forces stabilize O/W emulsions, W/O emulsions rely only on steric forces for stability in liquid form as the continuous phase (oil) has low electrical conductivity[11;12]. Aqueous droplets are more likely to coalesce or flocculate than oil droplets, so when designing a W/O emulsion system, the hydrodynamic interactions, viscosity, and interfacial tension of all phases and surfactants added must be carefully designed[11], as an emulsion is considered to be stable long-term if the droplets stay a size and shape that is consistent over time[12]. Stability over time is equally essential in O/W emulsions, and O/W fluid emulsion industrial products are common. They are found in the petroleum and food industries, along with biological research where they are used as self-contained rudimentary micro-reactors modified to mimic biological systems at the same size scale as the cells and tissues being studied[13;14]. For

both O/W and W/O emulsions, designing bespoke systems to produce monodisperse, reproducible droplets rapidly is best done using microfluidic technologies.

1.1.3 Double emulsions

Moving beyond single emulsions, the field of multiple emulsions (droplet systems containing at least three immiscible fluids) has widely expanded across both academic and industrial applications due to the precise control over droplet composition and overall system conditions that droplet-based microfluidic systems provide[15]. Multiple emulsions contain several layers of fluids in each droplet, and this thesis will focus entirely on all layers consisting of liquids, still denoted as aqueous or oil phases. The two predominant multiple emulsions in the literature are oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) emulsions. Other iterations are possible but will not be focused on in this thesis. O/W/O and W/O/W are also a specific subclass of multiple emulsion called a double emulsion, where two fluids are separated by a third liquid which is not miscible in the other two (Figure 1.2). Adding complexity

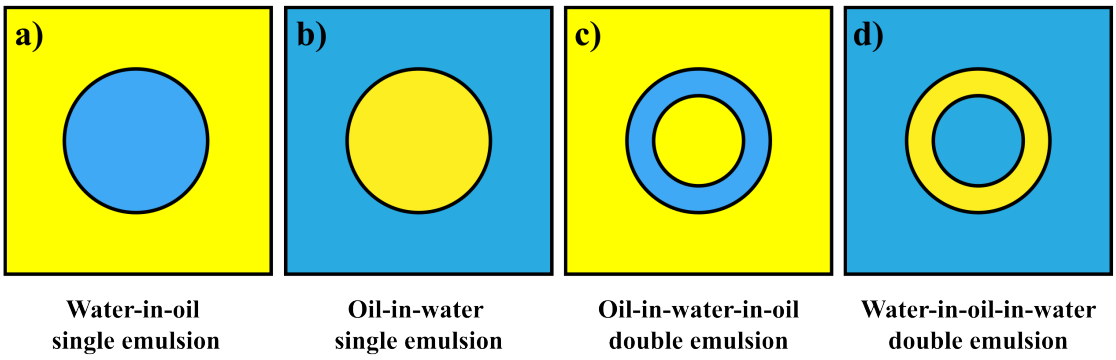

Figure 1.2: Emulsion nomenclature. Emulsion nomenclature where blue is water (W) and yellow is oil (O). a) W/O single emulsion. b) O/W single emulsion. c) O/W/O double emulsion. d) W/O/W double emulsion.

to droplets in a microfluidic system by creating multiple emulsions means we can answer more complex questions in fields such as synthetic biology[16;17], food science[18;19],

and medicinal research[20;21] by using the multiple droplet interfaces present in the system. Multiple emulsions made using droplet-based microfluidics can create a versatile range of structures, including porous, solid, and multi-phase particles and functional vesicles[6]. Double emulsions are fabricated mainly by two approaches, a one- and two-step emulsification[2;22;23]. Like O/W and W/O emulsions, the composition of the double emulsion plays a pivotal role in the stability of the droplets, so the properties and concentrations of the oil and aqueous phases, along with any surfactants added, must be well understood to create a stable, reproducible double emulsion.

The optimization of the geometry of the device and the composition of the individual phases is used to create stable microfluidic emulsions, allowing us to build more complexity into the system through many avenues. This thesis will focus specifically on the surfaces of the droplets created as viable surfaces for modification and customization. In Chapter 2, a novel technique is used to fabricate a W/O/W double emulsion where both aqueous phases are individually customized, and the oil phase is designed to be removed, creating an asymmetric vesicle. The aqueous droplets contain a biomimetic distribution of lipids, and when combined in an asymmetric conformation, they closely replicate the natural phospholipid bilayer in living eukaryotic cells. In Chapter 3, an O/W emulsion is created and protein-based properties are explored in relation to the surface of the oil droplets.

1.2 Formation of lipid-based surfaces on droplets using microfluidic technologies

Droplet-based microfluidic technologies allow us to create a biologically relevant compartment in a controlled environment. If the droplet is vesicle-like in composition, then the outer layer of the droplet acts as a membrane separating the inside and

outside of the structure from the rest of the environment. Specifically, the first steps are focused on stability and permeability when building a simple and robust model membrane. Before we create an entire model membrane, though, the interactions between the constituents of that membrane should be well characterized. A bottom-up approach to understanding life begins with assembling the functional components that make up the basic cell[22]. If we simplify cell architecture to its most basic components, we see a droplet with an aqueous internal compartment separated from the extracellular environment by a membrane made of amphipathic molecules[24].

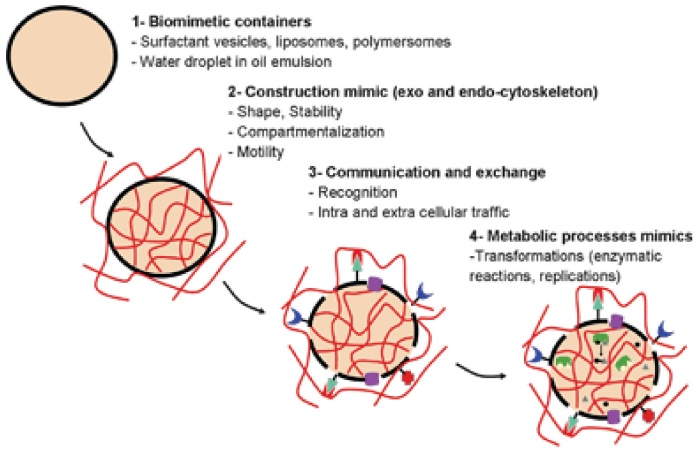

Figure 1.3: Schematic illustrating a bottom-up strategy for constructing simplified cell systems. A bottom-up approach is based on progressively implementing different components into biomimetic compartments 1) that reproduce the cell architecture 2) undergo recognition and exchange events 3) or some basic metabolic processes such as enzymatic reactions or 4) DNA replication. Reproduced from Brizard and van Esch[25] with permission from the Royal Society of Chemistry.

1.2.1 Synthetic unilamellar cell membranes

The amphipathic membrane that makes up the outer layer of all eukaryotic cells is commonly referred to as the phospholipid bilayer and consists of two layers of lipid molecules with their hydrophobic tails facing inwards and hydrophilic heads facing outwards. This forms a boundary between the interior and exterior of the cell,

providing the cell with stability (preventing merging or coalescing) and controlling the transport of materials into and out of the cell[24]. Further, the bilayer can be specifically functionalized to enable applications such as targeted molecular transport and delivery[26]. Liposomes act as biomimetic compartments that self-assemble from lipids to form a membrane structure closely related to a living cell membrane but easier to study in isolation in a simpler physical environment[27].

As discussed in Section 1.1 and seen in Figure 1.3, the first step in a bottom-up approach to creating an artificial cell is to create a biomimetic container using an emulsion. Then, a semi-permeable membrane can be formed, mimicking the shape and stability of a natural cell membrane. The use of a droplet-based microfluidic technology to create vesicles from lipids was first done by Tan *et al.*[28], and a wide variety of methods have been developed since then to fabricate lipid vesicles (known as liposomes)[22]. It is important to note that this thesis focuses more on the morphology aspect of mimicking a living cell membrane than functionality.

1.2.2 Liposome generation techniques

Traditional methods of forming liposomes involve either mechanical dispersion, solvent dispersion, or detergent removal[20]. Techniques include electroformation, film hydration, extrusion, and manual manipulation, and they generally lack the precise control over size, content, reproducibility, and high-throughput consistency that microfluidic techniques offer[20;22;29]. When forming liposomes, the type of lipid, surfactant, solvent, or oil used, along with the size, distribution, stability, usability, and encapsulation efficiency, must all be considered[27]. Figure 1.4 outlines the standard classification of sizes of liposomes and illustrates the different lamellarity options that may be fabricated. This thesis works in the giant unilamellar vesicles (GUV) range, above 1 μm in size with a single bilayer. GUVs resemble living eukaryotic

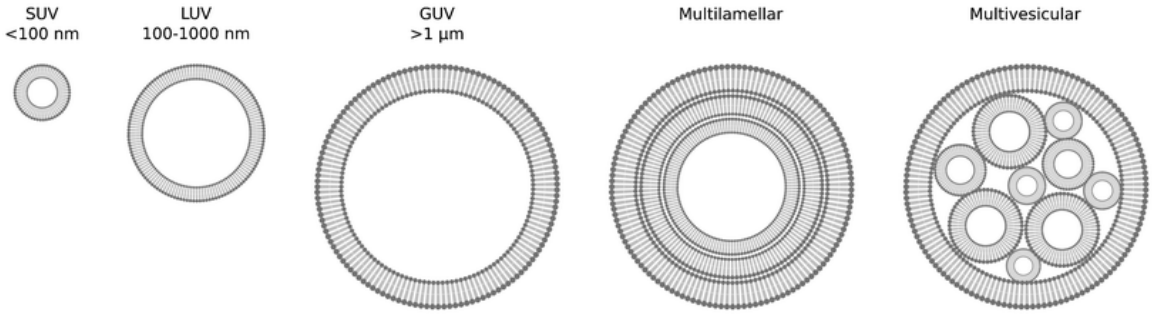

Figure 1.4: A common vesicle size and lamellarity classification system. Small unilamellar vesicles (SUV) are less than 100 nm in diameter; large unilamellar vesicles (LUV) are between 100 and 1000 nm; and giant unilamellar vesicles (GUV) are larger than 1 micron. Multilamellar vesicles have many membrane layers, and multivesicular vesicles encapsulate smaller vesicles. Reproduced from Swaay and de Mello[27] with permission from the Royal Society of Chemistry.

cells closely in size, structure, and function[27]. Has[30], and al Badri *et al.*[20] outline the current formulation techniques for liposome formulation in their reviews. Specifically using droplet-based microfluidic technology, single or multiple emulsions are formed with defined droplet size, structure, and composition, serving as a template for producing GUVs[30]. Methods include creating O/W emulsions in microfluidic devices and injecting the emulsion into a solvent to form closed vesicles[28], using lipid-coated W/O/W ice droplet hydration[31], and creating droplet emulsions containing lipids where device geometries promote self-assembly of liposomes[32].

1.2.3 Dewetting

One disadvantage of using droplet-based microfluidic technologies to form liposomes from double emulsions is that two immiscible phases must be used to form droplets, but the oil phase must then be removed from between the two aqueous phases, as illustrated in Figure 1.5. If oil remains between the lipid leaflets formed between the two aqueous phases, it can affect the physical and mechanical characteristics of the membrane, altering the biomimicry of the liposome[20]. There are limited proposed

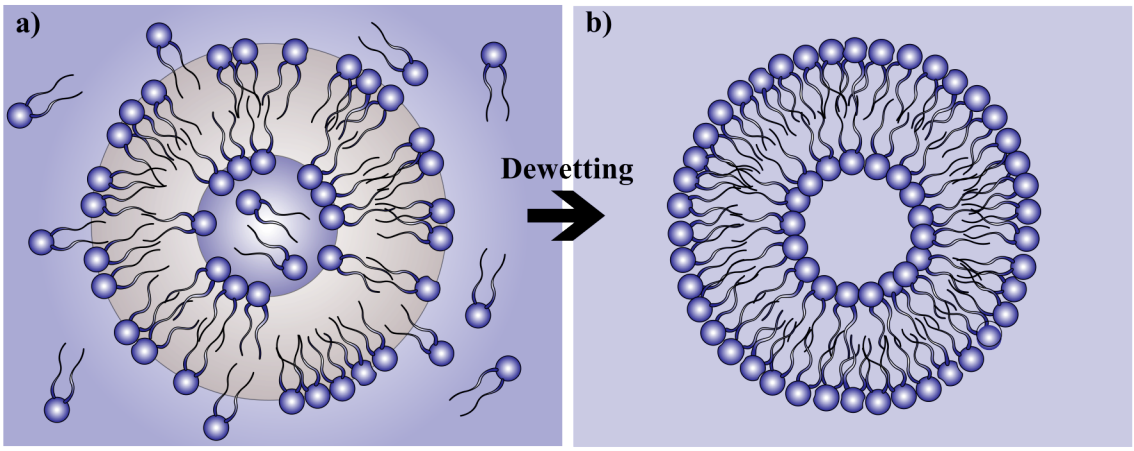

Figure 1.5: Liposome dewetting. a) Two aqueous phases (purple) with an oil phase (brown) in the middle. b) Removal of the oil phase results in a lipid bilayer forming.

strategies to separate residual oil from the phospholipid membrane during formation. Shum *et al.*[33] proposed a new approach to preserve phospholipid bilayer structure during oil removal based on a phenomenon called dewetting. Dewetting is a spontaneous phenomenon where a thin film undergoes a catastrophic rupture and rearranges itself in a more energetically favourable conformation[34]. Shum *et al.* used a mixture of volatile organic solvents designed to slowly evaporate into the air. As the solvents evaporated, the force between the phospholipids accumulating in the remaining solvents induced dewetting[33]. Since then, a few methods have utilized dewetting in W/O/W double emulsions to form liposomes[16;33;35–38]. These methods rely on lipids dissolved in the oil phase and employ synthetically-derived lipids, enabling a more reliable but less biomimetic method of forming liposomes, as synthetically-derived lipids are artificially produced through chemical methods rather than being naturally occurring. Chapter 2 will talk more about our novel method, which uses lipids in the aqueous phase and naturally-derived eukaryotic lipids.

1.3 Formation of protein-based surfaces on droplets using microfluidic technologies

While there has been much attention in the literature placed on lipid-based outer layers on droplets, lipids are not the only functional molecule on the surface of a droplet that is of interest. Along with lipids, proteins are one of the four major classes of biological macromolecules[39]. Droplets can be stabilized by protein layers that encapsulate, or coat, their surfaces, preventing coalescence or merging. Many proteins are surface active, and their multi-polar nature may serve to attract or repel other molecules or droplets in solution[40]. A protein layer is also a barrier between the droplet contents and the exterior environment, modifying the droplet morphology and functionality[41].

1.3.1 Impacts on emulsion droplet size and stability

Specifically for O/W emulsions, proteins coating the surface of an oil droplet help lower interfacial tension and slow droplet merging[42]. The interfacial membrane that is formed between oil droplets has been shown to play a role in stabilizing the droplets during long-term storage, which is a very useful quality for an industrial emulsion-based product to have[43], as O/W emulsions are commonly found in food and pharmaceutical applications. Proteins in O/W water emulsions may act as surfactants or be mixed with other surfactants. Much is still to be discovered about the composition of stabilizing interfacial layers and how their structures may affect the emulsion properties[44].

Good emulsion stability indicates that no change in size distribution or spatial arrangement of droplets is observed over the time-scale of the experiment[41]. Proteins may undergo conformational changes once adsorbed to an emulsion oil droplet, which

may also affect the desired droplet size and dispersion within the solution[45]. Other factors influencing how a protein will influence emulsion stability include protein type and concentration, aggregation, pH, ionic strength, and temperature[44]. As time scales and environmental factors affect emulsions differently, so do the conditions that disrupt them[41].

1.3.2 Emulsion digestion

The mechanisms behind how proteins promote emulsion stability are known and used industrially, but not all emulsions are designed to remain stable for long periods. When designing a custom emulsion, the mechanisms behind what and how specific emulsions are broken down or digested should also be understood. A protein-stabilized emulsion may become unstable due to physical or chemical processes such as modifications to spatial arrangement, oxidation, and enzyme action, along with many others[45;46]. Emulsion-based systems are carefully designed with structure, preparation, and utilization in mind, as are applications for the breakdown of those emulsions for controlled digestion and targeted release[47]. Proteases, enzymes that break down proteins, are utilized in a wide range of research and industrial processes, including pharmaceutical,[47] food[48], and biomedical sciences[46]. In an emulsion, they may be used to digest the droplet substance itself or the physical barrier that a protein-based outer layer provides, leading to emulsion instability[46;47].

1.3.3 Preparation and industrial applications

Preparation of protein-based outer layers on droplets requires similar steps on both a macro and micro scale. First, suitable proteins are selected to stabilize the emulsion. A protein solution is created in an aqueous solvent. The protein solution is mixed with another immiscible phase, such as oil, forming an emulsion using methods such

as those outlined in Section 1.1. The proteins in the solution stabilize the droplets, creating a surface layer on the droplet. Depending on the application of the emulsion, additional processing steps may be required, such as temperature or pH adjustment or the addition of other molecules to adjust the emulsion properties. While these methods can be done on a conventional macro-scale using large reaction volumes when developing a new experimental process, the micro-scale can be highly advantageous[49]. Precise control over fluid mixing and droplet size, minimal waste of valuable reagents, and high flow rates offer an efficient technique for understanding emulsion kinetics in industries including, but not limited to, biomaterials, petroleum, and food[14;49;50]. In Chapter 3, I explore the effects that a protein (gluten) has on emulsions of hop oil in beer and how understanding the mechanism behind the emulsion stabilizing process may impact the brewing industry.

1.4 Aims

I generate emulsions capable of mimicking biologically and industrially relevant conditions using droplet-based microfluidics. The methods used allow for precise control over size, composition, and the droplet environment. I focus on the outer layers of immiscible droplets in the solution.

The two aims that I address throughout this thesis are:

1. To use a simple microcapillary platform to generate a novel biomimetic artificial cell membrane using only natural lipids and biologically relevant conditions.

2. To explore the role of a protein as an emulsion stabilizer for the brewing industry, using a microfluidic device to create a novel hop-oil-in-beer emulsion.

First I fabricated an asymmetric artificial cell membrane using a plug-and-play

microcapillary platform to generate W/O/W double emulsions. The novel use of only natural lipids in the aqueous phase mimics the morphology of a living red blood cell. The goal was to generate a more biomimetic cell membrane model using an accessible microfluidic platform.

Secondly, I generated an O/W emulsion using a droplet-based microfluidic approach to replicate the macro conditions of a brewery on a micro-scale. I explored the role of gluten on the stability of hop oil droplets in beer, seeking to determine what role proteins may play in the long-term stability of beer. This was a collaboration with Phillips Brewing and Malting Co., and Raft Brew Labs.

1.5 Outline

My thesis contains the following chapters:

Chapter 1 contains a literature review that is required to understand the thesis. Specifically, it contains a review of microfluidic technologies for droplet formation and the formation of lipid- and protein-based surfaces on droplets.

Chapter 2 describes my work on developing an approach to model asymmetric cell membranes using a simple microcapillary platform. This work is currently being prepared as a manuscript.

Chapter 3 describes my investigation of the stability of hop oils in beer using a microfluidic approach to focus on the roles proteins have on droplet stability. This work is being resubmitted to Lab on a Chip based on the request of the Editor.

Chapter 4 summarizes the work done in my thesis and details its impact, along with a description of goals for future work.

Chapter 2

Biomimetic asymmetric liposomes to model passive drug transport across red blood cell membranes

I used a custom plug-and-play microfluidic platform designed by our group[51], and fabricated reproducible asymmetric liposomes using only naturally-derived lipids in the aqueous phase. I demonstrate the creation of double emulsions followed by a dewetting process, allowing for control over the properties of the resulting liposomes. I then characterized those asymmetric liposomes compared to symmetric ones produced via the same methods. This work provides insights into the fabrication of asymmetric liposomes and offers a versatile and accessible method for their production.

Kaitlyn Ramsay conceived the idea based on prior work she did with Sean Farley while in our group[51]. She performed initial data collection and analysis for Figure 2.1, and helped draw Figures 2.2, 2.3, and 2.6. I performed all other data collection and analysis and drew Figures 2.4 and 2.5. Kaitlyn wrote an early draft of the Introduction and Materials and Methods section that I adapted and changed as I

developed the method further. I wrote the first draft of the Abstract, Results and Discussion, and Conclusions sections. Katherine Elvira, Kaitlyn and I wrote the manuscript and aim to submit it this year. We also published an early version of this research in the 25th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS) Technical Digest in 2021 (See Appendix A). MicroTAS requires the submission of a 2-page abstract for admission to the conference. These abstracts are peer-reviewed by external reviewers and a technical review committee.

2.1 Introduction

Despite recent microfluidic advances in liposomal generation, most liposomes are made of symmetric lipid bilayers, using synthetically-derived lipids. This limits the biomimetics of the model membranes, as *in vivo* all biological cells have inner and outer leaflets which differ in lipid composition. Eukaryotic cell membranes are composed of primarily phosphatidylcholine (PC) and sphingomyelin (SM) in the outer layer and phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the inner layer[52]. Membrane asymmetry affects multiple bilayer properties including membrane potential, permeability, shape, surface charge and stability.[53] Loss of asymmetry has numerous physiological consequences.[53] For example, the externalization of PS, a lipid which is primarily contained in the inner red blood cell leaflet, to the outer leaflet has been linked to blood coagulation and erythrocyte adhesion.[53] Along with better mimicry of biological membranes, asymmetric liposomes have been shown to have the potential to entrap large molecules with less chance of drug leakage and greater protection of drugs being entrapped[20].

Lipid asymmetry is a dynamic process and lipids are translocated across the cellular membrane to maintain an energetically favourable equilibrium[52]. Son and Lon-

don[54] discuss the importance of the acyl chain structure in lipids composing asymmetric membranes, noting that the rate of translocation (and therefore maintenance of the asymmetric conformation) was related to the asymmetry of the membrane. Therefore, to maintain asymmetry in biological membranes, choosing the right combination of acyl chains on lipids is important.

Perhaps due to their complexity and the extra steps needed to custom-make asymmetric lipid bilayers, there are few publications which use microfluidic technology to fabricate them. The most common approach is a layer-by-layer technique.[55–57] In this technique, a lipid monolayer is formed at the water-oil interface of droplets. The benefit is that this technique is relatively simple and reliable. Nevertheless, the layer-by-layer method involves the use of droplet capture posts which are difficult to run at high throughput while also controlling monodispersity[32]. Air is also easily captured in these regions and droplets can fall apart when higher flow rates are required[3]. These liposomes can also contain residual solvent within the lipid membranes which limits their applicability as synthetic cells or drug delivery vessels[58]. The pulsed-jet flow method involves the use of a pulsed jet flow against two parallel planar asymmetric lipid bilayers to form liposomes.[59;60] The advantage of this method is that it allows for the construction of more complex artificial cell models. The downside is that it requires highly precise alignment as well as complex engineering to generate a functional platform. The final approach makes use of a platform with a triangular post region and two flow-focusing regions.[32] The main steps involved in the liposome fabrication process were: 1) the formation of monodisperse water emulsions in an oil solution containing the inner leaflet lipids, 2) the replacement of the inner-leaflet-lipid solution with an outer-leaflet-lipid solution surrounding the water-oil emulsion, 3) the formation of W/O/W double emulsions, and 4) the extraction of the excess oil/outer-leaflet-lipid solution.[32] This approach is particularly advantageous when one considers

the versatility of polydimethylsiloxane (PDMS) microfluidic platforms. That said, the micro-fabrication techniques required to design these platforms are highly complex, requiring specialized training on precisely aligned and costly machinery that all researchers may not have access to.

Here we use a versatile microfluidic platform to generate simple, reproducible asymmetric liposomes from naturally-derived lipids, capable of red blood cell membrane-like biomimeticity. There were several challenges we needed to address. The first is that the precedence for the microfluidic formation of liposomes using double emulsions in the literature involved putting the lipids in the oil phase.[16;27;37;61] In order to generate asymmetric liposomes, the separate inner and outer lipids must be mixed into distinct inner and outer aqueous solutions, which has not previously been done. The surfactant and lipid portions of the aqueous phases were prepared separately, allowing for further customization. The second challenge involved the development of a facile, reproducible, and reliable microfluidic platform that supported the solvents used for liposome formation. We determined that a microcapillary platform which we recently developed[51] could be re-purposed for the generation of these liposomes. We use parameters that closely replicate a living system with regard to pH, temperature, and scale, without the cost and complexity of working within an actual biological system. For the first time, we fabricate asymmetric liposomes from naturally-derived lipids using a method that combines an accessible microcapillary platform and more biologically relevant conditions than previous methods. We show that our method is capable of fabricating asymmetric liposomes that mimic the lipid composition of the red blood cell membrane, which we aim to use as a biomimetic model membrane for passive drug transport.

2.2 Results and discussion

The simple, plug-and-play microcapillary microfluidic device we adapted for this work was first developed and described by our group as an alternative to PDMS and traditional microcapillary glass devices for forming multi-compartmental double emulsions[51]. It consists of two junction boxes cast from flexible polyurethane resin using 3D printed moulds (junction boxes 1 and 2 in Figure2.1a). The junction boxes hold three capillaries, the inner glass capillary, the middle polytetrafluoroethylene (PTFE) tubing, and the outer surface-treated glass capillary (Figure2.1b). The surface treatment enabled consistent, reproducible double emulsion formation by producing a hydrophilic surface. The junction boxes allow the insertion of PTFE tubing or glass capillaries through the top protrusion and act to link the capillaries together and form a seal to prevent leaks. They hence eliminate the need to align and create a seal around the capillaries used as we require in traditional microcapillary devices. The inner and outer aqueous phases contained custom naturally-derived lipid blends (pink and purple in Figure2.1c) and the middle oil phase was 50:50 v/v chloroform and hexanes (brown in Figure2.1c))[16]. Double emulsion formation was observed on- and off- chip, at an average rate of 1.4 $\pm$ 0.2 droplets per second (Figure 2.1d). Each component of the microcapillary device, the components of each phase, and the flow rates can all be easily customized based on the desired double emulsion to be formed. Not only is the device customizable, but it utilizes simple, off-the-shelf materials, enabling use by researchers that are not microfluidics specialists. The limiting factor of this device is the size of the glass syringes delivering the three phases. Therefore, the method could be scaled up by using larger syringes to fabricate double emulsions in higher quantities.

All liposomes in the literature to date use synthetically-derived lipids in the solvent (oil) phase. Starting with lipids in the aqueous phase theoretically allows for

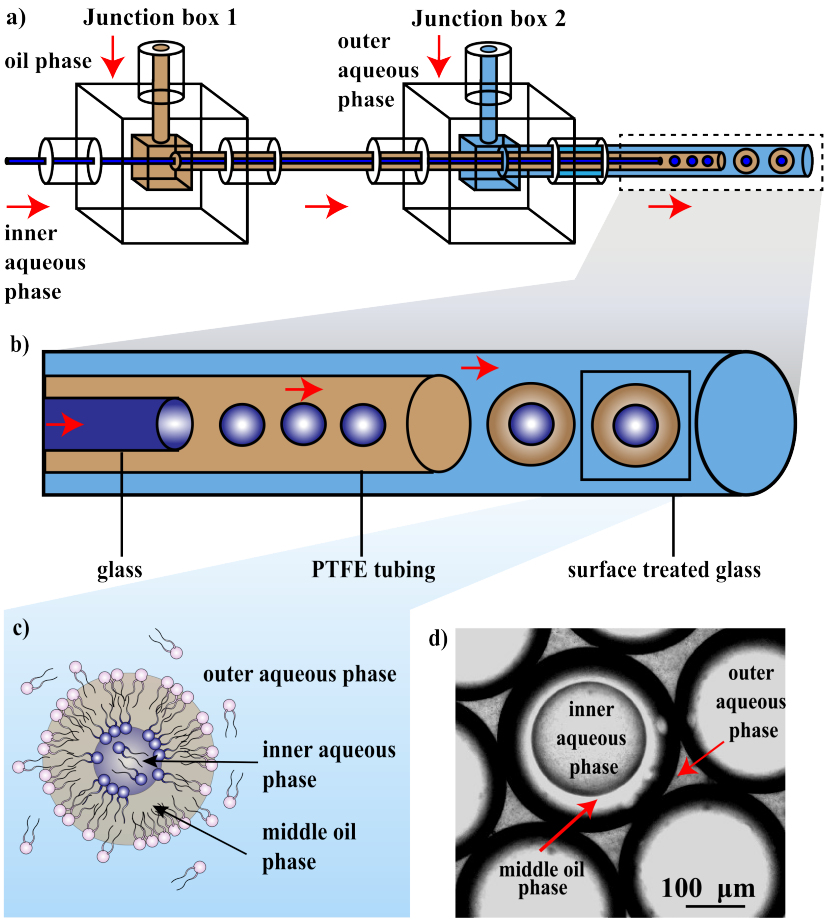

Figure 2.1: Liposome generation using our plug-and-play glass microcapillary device. a) Schematic of the plug-and-play glass microcapillary device used to generate liposomes. Junction boxes 1 and 2 are made from flexible polyurethane resin formed using custom 3D printed moulds. W/O emuyslions are formed at junction box 1 and W/O/W emulsions are formed at junction box 2. Purple indicates the inner aqueous phase, brown indicates the middle oil phase and light blue indicates the outer aqueous phase. b) Enlarged schematic of the microcapillaries and droplet formation. The inner capillary is a glass capillary, the middle capillary is PTFE tubing, and the outer capillary is a surface-treated glass capillary. c) Schematic showing the double emulsion composition. The inner phase was 50:50 v/v PE in HEPES buffer and 2% w/w PVA and 8% w/w PEG in Milli-Q water, the middle phase was 50:50 chloroform and hexanes and the outer phase was 50:50 v/v PC in HEPES buffer and 10% w/w PVA, and 0.5% w/w F-68 Pluronic surfactant in Milli-Q water (outer surfactant aqueous solution). d) Double emulsion formation observed off-chip prior to oil removal. Scale bar is 100 µm. Double emulsions are formed at an average rate of 1.4 ± 0.2 droplets per second. Image captured using brightfield microscopy.

self-assembly of the hydrophilic heads of the phospholipids at the aqueous phase and the hydrophobic tails towards the oil phase that causes bilayer formation upon the dewetting of the oil phase. That said, methodological changes had to be conducted for this to be possible. It has been shown in droplet interface bilayer (DIB) research that lipid extrusion is a necessary step in the formation of the bilayer between two lipid-filled aqueous droplets[62], because lipids are less soluble in aqueous solutions and extrusion allows them to be better solubilised[16]. Hence, our new methodological approach involves the preparation of the lipid and surfactant aqueous solutions separately. Lipids were hydrated in an aqueous 4-(2-hydroxyethyl)-1-piper azineethane-sulfonic acid (HEPES) buffer at pH 7.4, underwent five freeze-thaw cycles, and then were heated to 37 °C and extruded through a polycarbonate membrane. Lipid and surfactant aqueous solutions were mixed directly before the experiment began for a final lipid concentration of 5 mg/mL. By preparing the aqueous solutions separately based on the inner or outer phase lipid composition and extruding the lipids into a biologically relevant aqueous solution at 37 °C, we more accurately represent the environment that natural red blood cells exist in, allowing us to observe how our asymmetric liposomes behave in a biomimetic environment.

2.2.1 Asymmetric aqueous-phase liposome generation

Figure 2.2 shows a schematic of the fully formed asymmetric liposome after dewetting of the middle phase. In Figure2.2a, the two different lipids (PC is pink, PE is purple) are contained in two different aqueous solutions (outer is pink, inner is purple), separated by the middle oil phase (50/50 chloroform and hexanes is brown). The double emulsions are formed and then collected from the microcapillary device into HEPES buffer at 37 °C and pH 7.4. Double emulsions dewet within 15 minutes post-collection, forming an asymmetric liposome as illustrated in Figure2.2b. Deng *et*

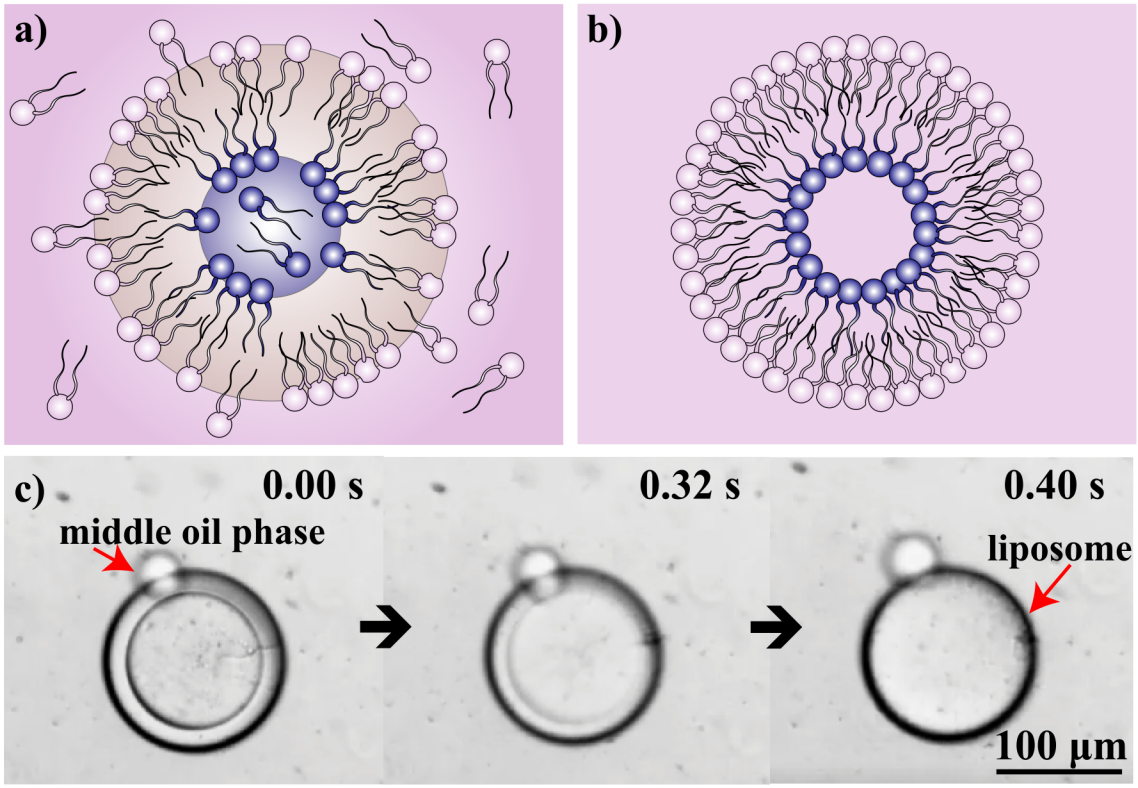

Figure 2.2: PC/PE asymmetric aqueous-phase liposome generation. a) Schematic of double emulsion formation with two different lipids (PC is pink, PE is purple) contained either in the inner (dark purple) or outer (light purple) aqueous phases. The middle oil phase (50/50 chloroform and hexanes) is shown in brown. b) Schematic of the asymmetric liposome formed after dewetting of the oil phase (a and b are not to scale). c) Bright-field microscopy time lapse images showing dewetting of the oil phase and formation of the asymmetric liposome. Double emulsions dewet within 15 min and liposomes were observed immediately after dewetting and up to 24 h after collection. Scale bar is 100 µm for all images.

al showed that the rate of dewetting was influenced by the amount of Pluronic F-68 present in the outer aqueous phase[16]. The formation of the bilayer happens rapidly once the oil phase (brown) has thinned enough to cause a spontaneous rearrangement of the PC/PE lipid molecules in the inner (PE) and outer (PE) phases into a more energetically favorable conformation (Figure2.2b,c). Liposomes were observed 2 hours after formation and up to 24 hours after collection.

2.2.2 Asymmetric membrane characterisation

After developing the microfluidic platform and observing PC/PE aqueous-phase asymmetric liposome formation, we confirmed the asymmetry of the lipid bilayer. Based on the fluorescence methods put forth by Lu *et al*[32], we added 1-palmitoyl-2-6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl-sn-glycero-3-PC (NBD-PC, green) to the outer PC lipid solution, and Texas Red 1,2-dihexadecanoyl-sn-glycero-3-PE, triethylammonium Salt (TR-PE, red) to the inner PE lipid solution and fabricated liposomes using the same method as above. Confocal microscopy was used to capture fluorescent images of both fluorophores present in the liposome bilayers (Figure 2.3). The presence of both green and red fluorescence surrounding the liposome qualitatively confirmed the presence of both lipids in the bilayer membrane. Over multiple replicates ($n > 3$) the inner layer (TR-PE, red) and outer layer (NBD-PC, green) of the liposome were visualized simultaneously and separately at the respective fluorophore emission wavelengths and consistently qualitatively confirmed the presence of both lipids in the bilayer. TR-PE fluorescent intensity (at 542 nm) inside the bilayer was compared to the aqueous solution outside the liposome, confirming that all TR-PE tagged lipids in the inner aqueous solution were found on the outer radius of the inside compartment in a bilayer conformation (Figure 2.3d,e). Similarly, the green NBD-PC outer layer shows the same amount of fluorescent background intensity (at 458 nm) inside and outside of the bilayer, with a higher concentration of green NBD-PC lipids found along the outside radius of the liposome. This confirms that PC/PE asymmetric aqueous-phase liposomes maintained confirmed qualitative asymmetry for over 24 h.

Then we made aqueous-phase symmetric liposomes using the same experimental method, with both inner and outer aqueous lipid phase solutions containing PC. Figure 2.4 shows the diameter and % relative frequency of both asymmetric PC/PE

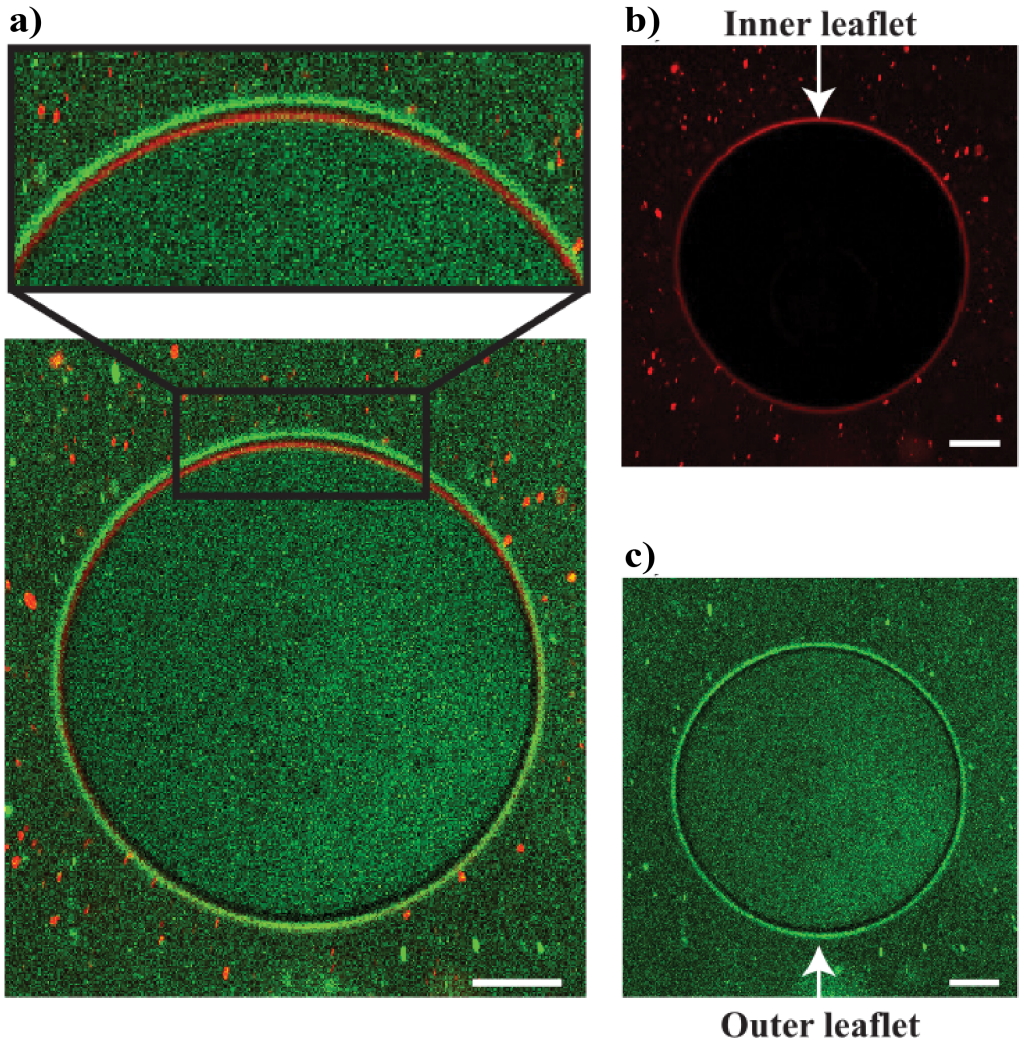

Figure 2.3: Qualitative measurement of PC/PE aqueous-phase bilayer asymmetry. Left) Merged confocal microscopy image of asymmetric bilayer showing outer green NBD-PC fluorescence and inner red TR-PE fluorescence layers. Right) Image of asymmetric liposomes separated by fluorophore (top, red is TR-PE, bottom, green, is NBD-PC). Scale bar is 50 µm for all images.

(2.4a) and symmetric PC/PC (2.4b) aqueous-phase liposomes. The size range was from 30 to 250 µm for asymmetric liposomes with an average diameter of 71 ± 39 µm. Symmetric liposomes were measured in a size range from 20 to 200 µm with an average diameter of 53 ± 31 µm. The size range of both symmetric and asymmetric liposomes were within ranges considered biologically relevant, as most eukaryotic cells are within the size range of 1-100 µm[63]. Our liposome size range is also comparable to other current microfluidic techniques for forming liposomes. Kamiya *et al.* formed two types of asymmetric liposomes using synthetic lipids and a pulsed-jet flow method, one with diameters between 100-200 µm and the other with diameters between 3-20 µm[59]. Lu *et al.* and Deng *et al.* both employ droplet-based microfluidic technology to fabricate double emulsions, forming symmetric liposomes with diameters between 57-69 µm and 99-115 µm[16] and asymmetric liposomes with diameters between 55-63 µm[32], though both methods use only synthetically-derived lipids which likely contributed to a narrower size distribution.

Table 2.1: T-test (Two-sample) data showing the difference between the average diameters of PC/PE asymmetric liposomes and PC/PC symmetric liposomes formed using our plug-and-play microcapillary platform.

Liposomes	Mean	t Stat	p-value*
Asymmetric	71.5	4.6	3.6E-06
Symmetric	52.6		

α = 0.05

The mean diameter of asymmetric and symmetric liposomes was calculated to be significantly different ($p = > 0.05$) (Table 2.1). We expected to see these differences in diameter between asymmetric and symmetric liposomes[64]. The symmetric PC/PC liposomes are packed more closely together due to the headgroup structure and acyl chain saturation of PC lipids, which provides a stable, ordered bilayer structure *et al*[64]. It is known that PE has a strong reluctance to interdigitate compared to PC[65] and that the rigidity of an asymmetric bilayer is higher than in a symmetric bilayer[66].

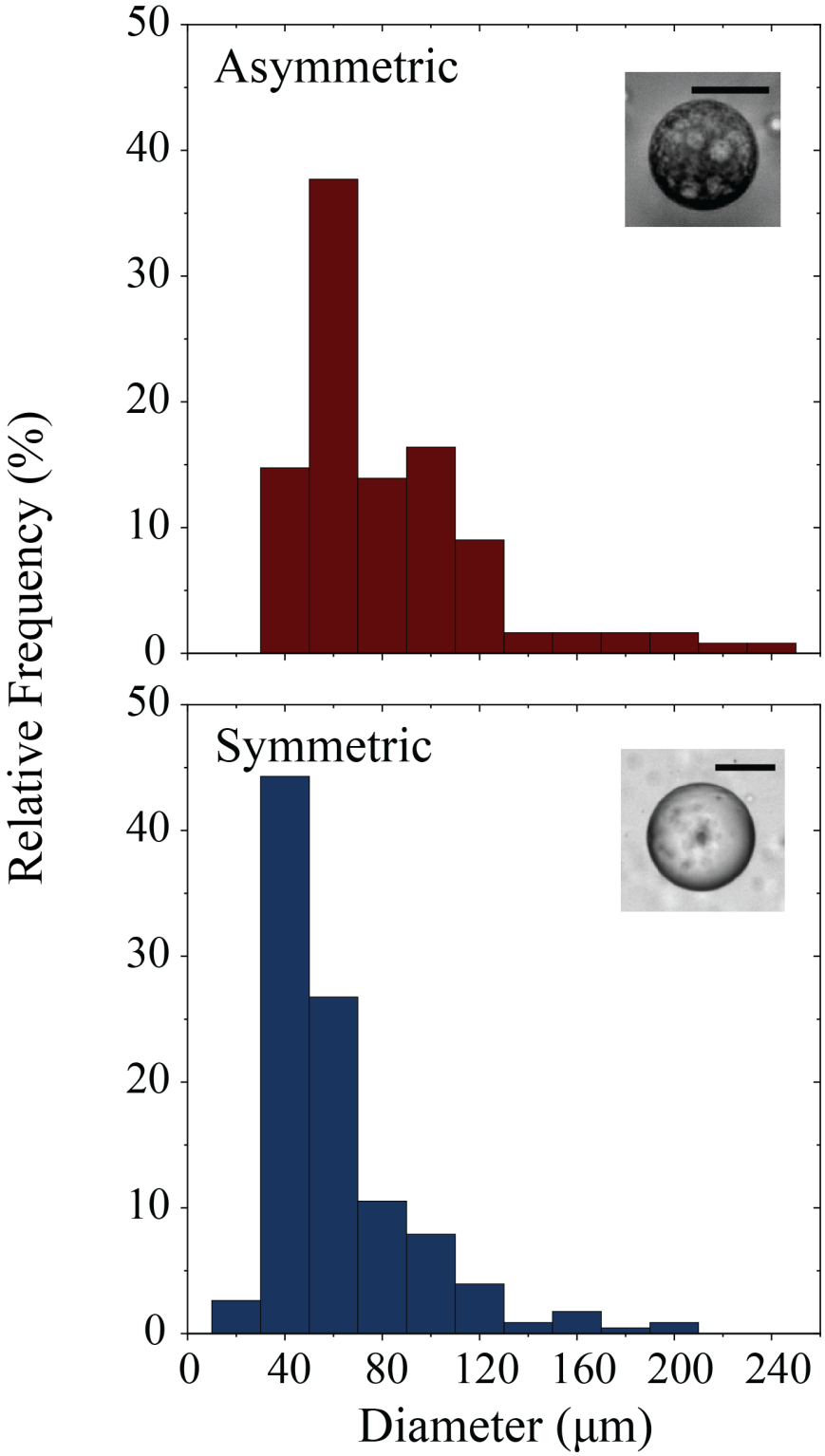

Figure 2.4: Liposome diameter relative frequencies. Diameter (µm) and % relative frequency of asymmetric PC/PE (top, red bars) and symmetric PC/PC (bottom, blue bars) liposomes post-dewetting. Asymmetric liposomes had an average diameter of 71 ± 39 µm (n = 122) and symmetric liposomes had an average diameter of 53 ± 31 µm (n = 228). Gathered over 3 replicates. Scale bars represent 100 µm. Images of liposomes captured with brightfield microscopy

Working in this size range allows for easy analysis by light and confocal microscopy to confirm size and asymmetrical properties. Farley *et al*[51] and Lu *et al*[32] also proved that controlling flow rates allowed for custom double emulsion size, hence allowing us to fabricate asymmetric aqueous-phase liposomes in a biologically relevant 1-20µm range for future work.

Table 2.2: T-test (Two-sample) data showing the difference between the average diameters of PC/PE asymmetric liposomes 2 h and 24 h after collection.

Time	Mean	t Stat	p-value*
2 h	71.5	8.2	1.7E-14
24 h	38.5		

α = 0.05

Asymmetric PC/PE liposomes were collected off-chip, and dewetted spontaneously within 15 min, at which time the majority of the oil was removed. They were then visualized on a depression slide in HEPES buffer using an inverted microscope. Images were taken at approximately 2 h after collection and an average diameter of 71 ± 39 µm was measured (Figure 2.5a). They were stored in closed vials at 4 °C overnight and visualized again after approximately 24 h. The n was similar (n = 122 and n = 169, respectively) but overall a statistically significant reduction in diameter was observed, with an average diameter of 38 ± 25 µm measured (Figure 2.5b, Table 3.1). The literature shows that lipids with high transition temperatures (above body temperature) are more stable than those with lower transition temperatures and when used to form a bilayer they experience a decrease in the rate of leakage[67]. As naturally-derived lipids have a lower phase transition temperature, liposomes fabricated with naturally-derived lipids may exhibit lower overall stability over time than synthetic lipids[68]. We know that different lipid formulations produce different retention profiles and therefore different *in vivo* properties[67]. The next step towards fabricating a biomimetic asymmetric red blood cell membrane model was the addition of PS and SM to the lipid mixtures. SM has been shown to stabilize the bi-

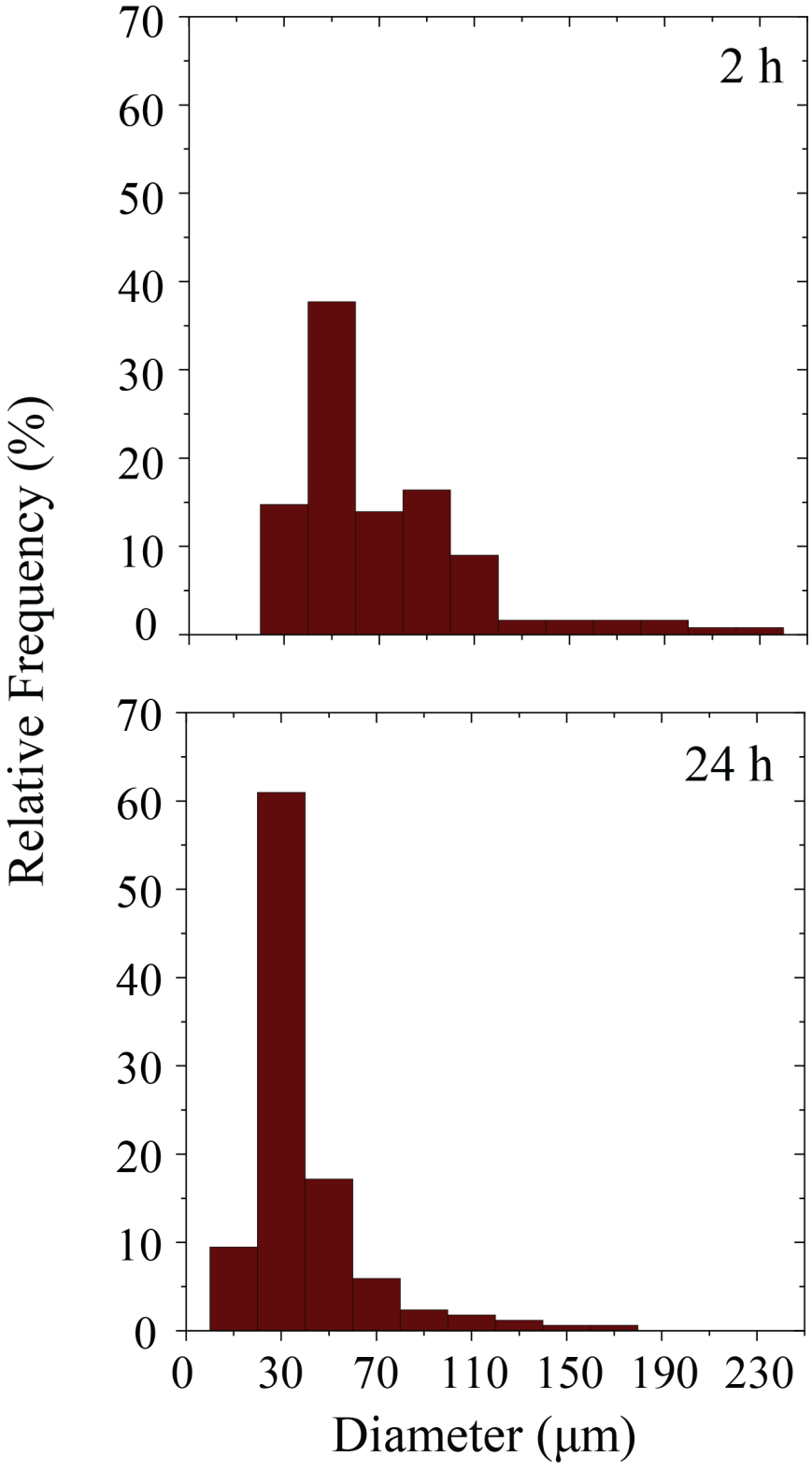

Figure 2.5: Asymmetric PC/PE aqueous-phase liposome stability over time Comparison of diameters of asymmetric PC/PE aqueous-phase liposomes a) 2 h after collection (n = 122) and b) 24 h after collection (n = 169) showing an average reduction in diameter of 60%, from 71 ± 39 µm to 38 ± 25 µm.

layer more effectively than PC (which adopts a bilayer conformation independently), maintaining bilayer structure with and without the presence of cholesterol[69].

2.2.3 Asymmetric liposome generation to model red blood cells

Building on confirmation that our method creates asymmetric liposomes where asymmetry is maintained for 24 h using a combination of two phospholipids (PC outer leaflet and PE inner leaflet), we added two more phospholipids (PS and SM) and changed the PC and PE concentrations of the inner and outer lipid solutions in a distribution proposed to mimic the lipid composition of the cell membrane of red blood cells *et al* (Figure 2.6a, right) for a total of four naturally-derived lipids distributed differently depending on position in the inner or outer layer[70]. This lipid distribution was determined by observing the differences in lytic and non-lytic actions of phospholipases on an intact red blood cell membrane[?] To replicate biological conditions in our study, we used only naturally-derived eukaryotic lipids, which have a heterogeneous composition of polar head groups and fatty acid tails, further adding to the asymmetric nature of the bilayer while still maintaining reproducibility and functionality[68]. Naturally derived lipids contain more unsaturated fatty acids than specifically-formulated synthetic lipids, leading to a lower phase transition temperature. This promotes more structural flexibility but decreases overall stability over time, leading to the preference towards liposomes fabricated with synthetic lipids traditionally[68]. First, we fabricated and characterized simple liposomes using only PC in the outer phase and PE in the inner phase. Next, we added further complexity by adding SM to the outer phase and PS to the inner phase in the distribution first proposed by Verkleij *et al*[70].

We kept all other methodologies consistent, including the use of HEPES buffer

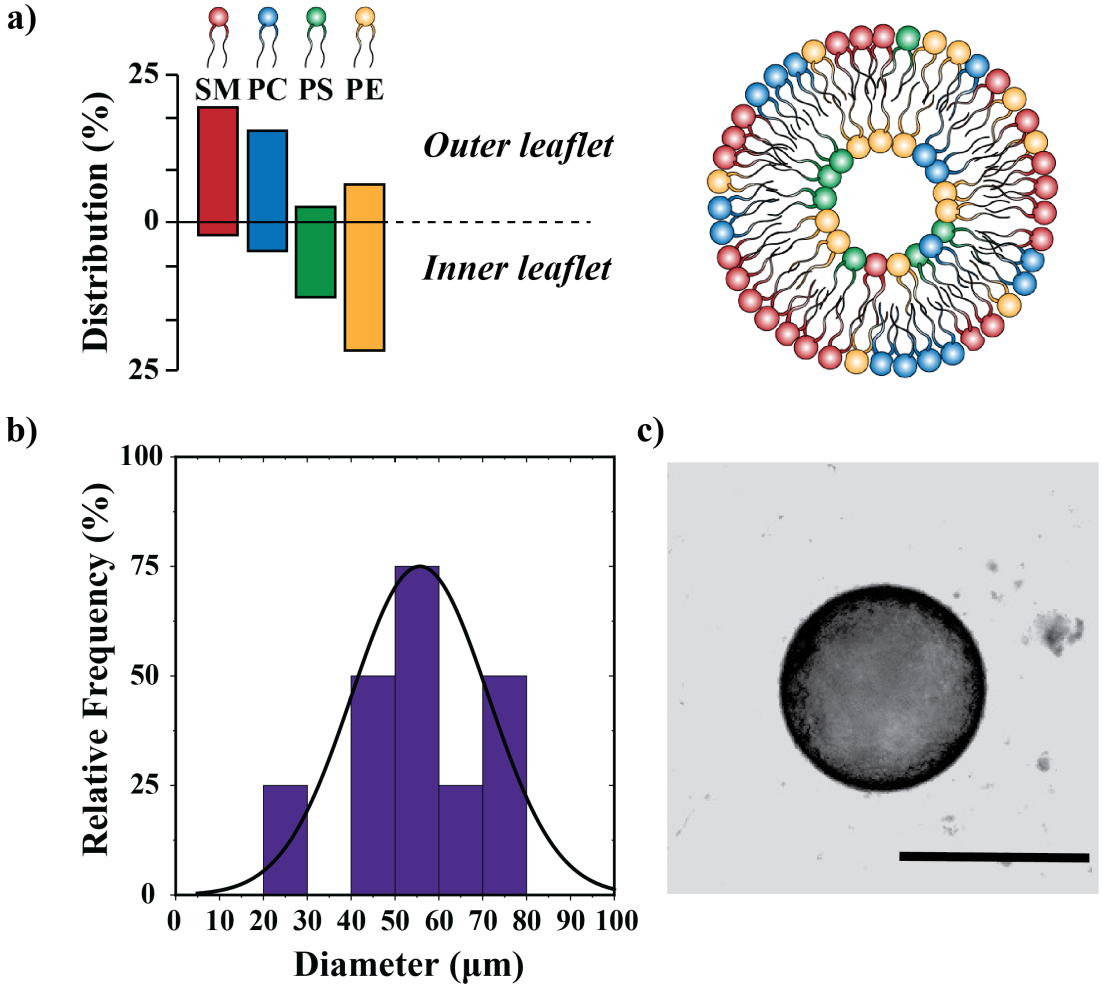

Figure 2.6: Red blood cell asymmetric phospholipid membrane composition. a) Proposed distribution of phospholipids in human red blood cell membranes [70] b) Preliminary red blood cell diameter measurements (n = 9, average = 56 ± 15µm.) c) Red blood cell liposome brightfield microscopy image. Scale bar is 50 µm.

at pH 7.4 and a temperature of 37 °C. Figure 2.6b shows the relative frequency (%) distribution of liposome diameters measured, with an average of 56 ± 15 µm (n = 9), which is in the same size range as the PC/PE asymmetric and PC/PC symmetric liposomes in Figure 2.4. Qualitatively, the red blood cell membrane liposomes also appeared visually similar to the PC/PE asymmetric liposomes (Figure 2.4a, Figure 2.6c). SM is known to be distributed mainly in the outer leaflet of the red blood cell membrane, playing a stabilizing role in lipid bilayer configurations[69]. PS is the second most abundant phospholipid in the inner leaflet and serves as a signal in cell-cell interactions when present on the outer leaflet surface instead of the inner leaflet surface[71].

2.2.4 Future work

The next steps are to validate the composition of both the inner and outer leaflets for asymmetry. Post-validation, this model is intended to be used to mimic passive diffusion in and out of the red blood cell membrane *in vitro*, starting with quinine, a fluorescent molecule and known treatment for malaria. As we know, lipid bilayer chemical structure has an impact on stability and permeability[64]. We anticipate that this model would more closely replicate actual passive diffusion in and out of the red blood cell membrane compared to the current standard of artificial cell membranes used for malaria research without the time consuming and costly steps of creating red blood cell "ghosts" to do the same drug testing trials[72].

2.3 Materials and methods

2.3.1 Materials

All reagents were used as received unless otherwise stated. Poly(vinyl alcohol) (PVA, MW = 13 000–23 000 g/mol, 87–88% hydrolyzed), 1-palmitoyl-2-6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl-sn-glycero-3-phosphocholine (NBD-PC), Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-PE) sphingomyelin (egg, ≥95%), L-phosphatidylserine (brain, porcine), L-α-phosphatidylcholine (egg), and phosphatidylethanolamine (egg) were purchased from Sigma Aldrich. Pluronic F-68 was purchased from Thermo Fisher Scientific. PTFE tubing (1/16 inch outer diameter, OD, 750 μm inner diameter, ID) was purchased from Chromatographic Specialties Inc. 1 mL glass gas-tight syringes (model 1001 TLL, PTFE Luer Lock) were purchased from Hamilton. Polyethylene glycol (PEG, MW = 6000 g/mol) was purchased from VWR. 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane was purchased from Gelest. Polyurethane resin (Vytaflex 30) and release spray were purchased from Smooth-on. Glass capillaries were purchased from Vitrocom. 100 nm polycarbonate filters and Avanti Mini Extruder were purchased from Avanti Lipids.

2.3.2 Surface treatment of glass microcapillaries

The outer glass capillaries were surface treated to create a more hydrophilic surface than untreated glass naturally has. First, the outer capillaries were cleaned with soapy deionized (DI) water, DI water, isopropanol, ethanol and acetone. The capillaries were then dried with a filtered air gun and placed in a glass petri dish on a hot plate at 95°C for 30 min. The capillaries were then plasma treated at 100 W with air plasma for 1 min. Capillaries were chemically modified immediately after plasma treatment. By submerging them fully in 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane

for approximately 15 min, we generate a highly hydrophilic surface. Afterwards, the excess silane was dabbed off with Kim wipes and capillaries were dried using a filtered air gun. They were used within 48 h of surface treatment to maximize treatment efficiency.

2.3.3 Lipid preparation

All lipids were suspended in chloroform at a starting concentration of 25 mg/mL.For PC/PE asymmetric liposomes, 400 μL of PC was used for the outer aqueous phase and 400μL of PE was used for the inner aqueous phase. 4 μL of fluorescently-tagged PC and PE were added to the outer and inner aqueous phases respectively to visualize the bilayer asymmetry.For PC/PC symmetric liposomes, 400 μL of PC was used for the inner and outer aqueous phases. For the red blood cell membrane liposomes, the outer layer consisted of 160 μL PC, 180 μL SM, and 60 μL PE. The inner aqueous phase consisted of 60 μL PC, 40 μL SM, 200 μL PE, and 100 μL PS. Lipids were added to a 10 mL glass round-bottom flask and excess chloroform was removed via evaporation using a steady stream of filtered air. The flask was then placed in a glass desiccator under vacuum for 1 h. Next, 1 mL of aqueous HEPES buffer at pH 7.4 was added to the flask and the solution was vortexed for 30 s to re-suspend the lipids. The lipid solution was then subjected to 5 freeze-thaw cycles in liquid nitrogen and warm water, respectively. Then, the lipid solution was heated to 37 °C using a water bath and extruded through a polycarbonate membrane to create unilamellar liposomes.

Surfactant aqueous solutions were prepared separately and added to the lipid solutions post-extrusion. For the inner aqueous phase, 2 wt% PVA and 8 wt% PEG were added to DI water. For the outer aqueous phase, 10 wt% PVA and 0.5 wt% Pluronic F-68 were added to DI water. The solutions were placed in glass vials with a magnetic stir bar and mixed for 30 min at 95 °C to fully dissolve the surfactants. These

solutions were added to their respective lipid solutions at a 1:1 ratio, resulting in a final concentration of 5 mg/mL inner and outer lipid solutions with added surfactants.

2.3.4 Assembly of the modular microcapillary platform

The microcapillary device was assembled as described previously[51]. Only a low-cost 3D printer, standard glass capillaries and PTFE tubing, and a syringe pump were necessary to assemble and use the device. The device was then assembled using a 0.20 mm ID glass microcapillary for the inner phase (heat-ligated to 0.25 mm ID PTFE tubing), 0.75 mm ID PTFE tubing for the middle phase, and a 1.5 mm ID glass microcapillary, surface treated as described above, for the outer phase.

2.3.5 Liposome formation and visualization

The three solutions (outer aqueous, middle oil, and inner aqueous) were kept at 37 °C and inserted into 1 mL gastight glass syringes. The middle oil phase was a 50:50 v/v mixture of chloroform and hexanes. Syringe tips were inserted into the top protrusions of the junction boxes of the microcapillary device as illustrated in Figure 2.1a. Using neMESYS low pressure syringe pumps (Cetoni), the outer phase flow rate was set to 200 μL/min, the middle phase flow rate to 100 μL/min, and the inner phase flow rate to 50 μL/min. Droplet formation using the microcapillary device was visualized using a stereomicroscope (SMZ800N, Nikon). Double emulsions were carefully collected in HEPES buffer at 37 °C, taking special care to not shear the double emulsions prior to dewetting of the middle phase by jostling the emulsions or letting them fall through air instead of flowing directly into the buffer. The dewetting process was visualized using a depression slide on a Ti-U inverted research microscope (Nikon). Post-dewetting, residual oil droplets were removed by exploiting the density differences between the liposomes and residual oil using a sucrose solution as outlined

in Deng *et al*[16], by adding the as-formed samples to a 100 mM sucrose solution so the oil droplets (lower density) floated above the sucrose solution and the liposomes (higher density) settled below. Images and videos were captured using a Phantom high-speed camera (VEO 710L).

Liposomes were observed on a Nikon Ti-U2 inverted research microscope using a 40X/0.60 objective. For the time series, emulsions were sealed in a vial and stored at 4 °C. Images were taken again after 24 h. Brightfield microscopy images were processed using ImageJ (version 1.52a) and confocal microscopy images were processed using Zen Blue (Zeiss).

2.4 Conclusions

We show a simple platform for creating asymmetric liposomes using naturally-derived lipids in the aqueous phase for the first time. We show that the device created by our group[51] is capable of forming double emulsions that dewet to form asymmetric liposomes in a more biomimetic environment than methods currently used. We characterize the liposomes and asymmetry of the bilayers formed and use a custom blend of lipids to mimic the composition of phospholipids in a red blood cell membrane. Our platform offers a versatile and accessible solution for creating double emulsions and producing biomimetic cell bilayers. This could be of benefit in drugs development research, where having accurate, biomimetic models can help researchers develop a more robust understanding of a drugs behaviour in the body *in vitro* before moving to *in vivo* trials.

Chapter 3

Microbrewidics: A microfluidic platform to investigate what stabilises hop oil emulsions in beer

Here we use a microfluidic platform to explore the role of proteins on hop oil emulsion stability in beer. The terpenes linalool and α-pinene, which are major components of the hops employed in brewing, were used to form emulsions with a Kolsch-style ale on a microfluidic device (oil-in-beer emulsions). Oil-in-beer emulsions were also created with additional gluten (as a protein present in beer) added to the beer aqueous phase to test how this protein affects emulsion stability. Then Brewers Clarex, an enzyme commonly used in brewing to degrade proteins in beer, was used to digest the oil-in-beer emulsions. We show that hop oil droplet stability in an aqueous beer solution containing gluten was impacted by the addition of Brewers Clarex. This suggests that the protein in finished beer may affect the stability of the hop oil emulsions present, which would have a direct impact on the shelf life and sensory quality of the beer. Additionally, the two hop oils tested differed in structure and droplet stability with

protein and enzyme treatment, suggesting that this effect may also be dependent on the type of hop oil present.

This work was a collaboration with Phillips Brewing and Malting Co. and Raft Brew Labs. It was also done before, during, and after the COVID-19 pandemic lockdowns, with undergraduate and graduate students contributing to method development and experimental work. Euan Thomson, Ph.D., of Phillips and Raft Brew Labs and Professor Katherine Elvira conceived the experiments. Kaitlyn Ramsay, Ph.D., developed the microfluidic device, the surface treatment protocol, and created Figure 3.2. Nicole York, Danielle Hanke, Jaling Kersen, and Caitland Stagg performed preliminary experiments including protein solubilization and enzyme condition optimization. Alexandra Schauman processed all ImageJ data. Euan wrote the first draft of half of the Introduction. Danielle wrote the first draft of half of the Materials and Methods. Jaime Korner, Ph.D., and I did all the experimental work. I analyzed all the data, created all other figures, and wrote the first draft of the Abstract, Results and Discussion, and Conclusion sections, and the other halves of the Introduction and Materials and Methods sections. Professor Elvira and I wrote and revised the manuscript, which we are resubmitting to Lab on a Chip on request from the Editor. We also published an early version of this research in the 26th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS) Technical Digest in 2022 (See Appendix A). MicroTAS requires the submission of a 2-page abstract for admission to the conference. These abstracts are peer-reviewed by external reviewers and a technical review committee.

3.1 Introduction

While the brewing industry is well known for experimentation in creating balanced, flavourful, and nuanced beverages, the mechanisms by which brewers achieve these remain, in some cases, unknown. Beer is a complex mixture of saccharides, esters, alcohols, organic acids, metals, proteins, tannins, and many other classes of compounds contributed by raw materials including malted barley, hops and water, as well as by-products of yeast fermentation[73]. A detailed understanding of how these ingredients and their by-products affect flavour allows researchers and brewers alike to have more control over both the sensory and visual characteristics of their beers, with the "Holy Grail" being the ability to maintain the flavour characteristics typical of a fresh beer over many months of shelf life.

A recent trend in beer brewing is the emergence of "hazy" pale ales, characterised by their intense hop aromatics, low bitterness, and high turbidity, which causes their distinctive cloudy appearance. This turbidity, or "haze", is caused by complexes of excess proteins, starches, and tannins originating from the raw ingredients[73]. Haze is thought to be responsible for some of the emulsification of aromatic terpenes from hops in hazy beer styles[74], though the mechanism is not known. Given the greater stability of aromatic terpenes in hazy beer styles, a likely mechanism that merits further exploration is microencapsulation, where a water-soluble molecule coats a hydrophobic molecule, encapsulating the enclosed material and allowing it to remain separate from the surrounding aqueous solution. As a result, the guest molecule gains protection from destabilizing agents such as ultraviolet radiation, metals, and oxidation[75], all known to contribute to early staling and flavour deterioration[76].

In cases where haze is not desired, brewers apply stabilization techniques and preventative measures to address visual and sensory consistency in the product. Techniques to ensure colloidal stability include cold conditioning to encourage particulate

settling, filtration regimes, and the addition of enzymes or precipitants that target proteins and/or polyphenols responsible for the majority of beer haze[73]. Two such commercial additives that specifically target gluten protein and peptides are BrewTan and Brewers Clarex. BrewTan is a commercial tannic acid that forms an insoluble complex with haze-active proteins via hydrogen bonding. The complex then precipitates out of the solution. However, the solubility of the complexes can depend on the degree of polymerization, so smaller tannin-peptide complexes are formed but may remain soluble and pass into the finished beer[77]. Tannins also have the potential to alter the sensory profile of the finished product, especially if overdosed[77;78]. Brewers Clarex is a commercially available enzyme extracted from *Aspergillus niger* that contains a proline-specific endoprotease[79]. It is commonly used in the brewing industry to combat a phenomenon known as "chill haze" by degrading proline-rich proteins such as gluten[79;80], since "chill haze" is a result of the undesired aggregation of proteins produced during fermentation[80]. Brewers Clarex is capable of cleaving peptides and entire proteins efficiently, leaving behind smaller peptides that are less hydrophobic and less capable of forming large protein-polyphenol complexes that contribute to beer haze[79], as shown in Figure 3.1. Furthermore, it has been shown to significantly reduce gluten mass and can target both large and small peptides with no impact on the sensory profile of the beer, which is why it was chosen for this study[77].

Microencapsulation has been widely studied in food science for the preservation of reactive or volatile compounds[81]. Aromatic terpenes from hops are large contributors to the taste and smell of beer. However, many of them exhibit high volatility and undergo structural changes during the brewing process, it is hard to predict the final sensory profiles they will create[82]. Types of compounds that naturally occur in brewing grains include dextrins and proteins, which are both known to exhibit microencapsulation[81;83], though they have yet to be explored for their ability to emulsify

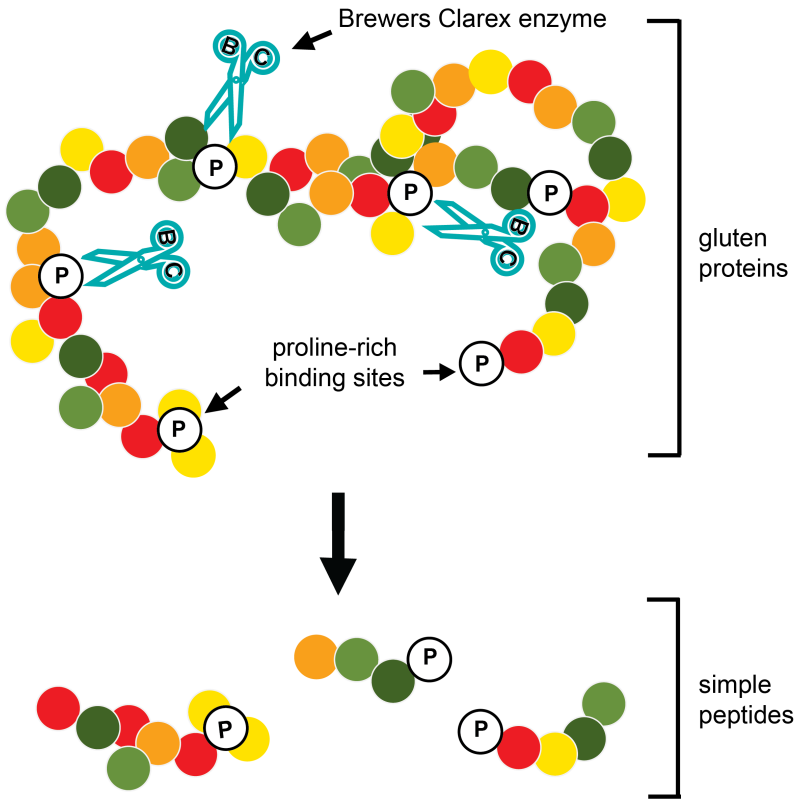

Figure 3.1: Mechanism of action of the Brewers Clarex enzyme. Brewers Clarex (blue scissors labelled BC) targets the carboxyl end of the amino acid proline (white circle denoted "P") on the gluten protein (multi-colored chain), breaking down the gluten proteins into smaller peptides which are unable to form beer haze.

and stabilise volatile hydrophobic substances in the context of brewing.

In fact, there is minimal prior work in the brewing field regarding microencapsulation, and research is mostly focused on the characterization of haze-active proteins, starches, and tannins in beer[84]. Some studies have investigated microencapsulated brewing yeast (*Saccharomyces cerevisiae*) for the primary alcohol fermentation of sparkling wine[85] and beer[86;87]. Almonacid et al. found that during fermentation, the encapsulated and unencapsulated samples differed in both volatile chemical and sensory properties[87], however their study was focused only on yeast metabolites and the microencapsulation had no sensory effect on the finished beer. Benucci et al. used a similar method to microencapsulate yeast and carried out a more in-depth analysis

of the volatile compounds produced and the sensory properties of the beer, finding that the product fermented by encapsulated yeast yielded a significantly different terpene and sensory profile than the unencapsulated yeast[86]. Notably, neither of those yeast encapsulation studies showed any significant changes to the physical-chemical parameters (pH, alcohol content, bitterness, colour) of the finished beers fermented using encapsulated yeast, a desirable outcome when managing consistency in finished products. There is currently no research focused on the mechanism by which hop oil emulsions are stabilised in finished beer and whether haze-active molecules play a role in the emulsion stabilization of hop oils in beer.

A useful approach for studying the properties of an emulsion is to observe the stability of oil-in-water (or water-in-oil) mixtures over time while disrupting the suspected emulsifying agents. Miniaturizing these emulsions using microfluidic technologies holds great promise in food science[18], as has been seen in other academic and industrial fields for the unveiling of key emulsion constituents[2]. Microfluidic platforms allow control over the formation of individual droplets in an immiscible stream, whose volume can be controlled by regulating the relative flow rates of the two fluids, and hence volume can be ignored as an experimental variable. In addition, each component of the emulsion (oil phase, aqueous phase, emulsifier) can be varied for analysis.

Here we have developed a microfluidic method of investigating whether the stabilizing agent of terpenes from hop oils in beer has a proteinaceous component. Our hypothesis was tested by examining the effects of Brewers Clarex on emulsion stability. Emulsions were created using beer as the aqueous phase and two hop terpenes (as the main components of hop oils) as the oil phase. Choosing two individual hop terpene oils enabled us to narrow down whether structural differences between each oil had an impact on stability. The components of beer that potentially confer emulsify-

ing properties were explored by manipulating concentrations of key components such as haze-active proteins and enzymatic digestion. The oil-in-beer emulsions created using our microfluidic platform were digested with different concentrations of Brewers Clarex, and the average number of droplets and average droplet volumes were monitored over time to assess the stability of the emulsions. Control experiments were performed to ensure that emulsion stability could be attributed solely to digestion with Brewers Clarex. These assays allowed us to investigate the role of proteins in the stabilisation of hop oils in beer, which is a longstanding question in the brewing industry.

3.2 Results and discussion

Our microfluidic platform was designed to create oil-in-beer emulsions (Figure 3.2a). We used a flow-focusing junction with a pinched inlet and outlet to create the oil-in-beer droplets, and we collected them off-chip for digestion and analysis over the course of 7 days (Figure 3.2b). Figure 3.2c shows the composition of the oil (terpene) and aqueous (beer) phases. The oil phase consisted of either linalool or α-pinene, as two relevant terpenes present in finished beer[88]. The aqueous phase consisted of degassed beer to which was added all possible combinations of a protein (gluten) at different concentrations (none, low, and high) and/or an enzyme (Brewers Clarex) in different formats (none, active, and deactivated). We chose to use a "clear" (transparent with no suspended particulate) Kolsch style of beer with low hop content to allow control over the hop oil content in the emulsions we created on our microfluidic platform. To mimic a hazy beer, gluten was added to the beer before insertion into the microfluidic platform. It was then tested whether this protein contributed to stabilizing hop oils in beer. The oil-in-beer emulsions produced on-chip with different concentrations

of gluten were then treated off-chip with the enzyme Brewers Clarex to determine whether digestion of the protein occurred. The oil-in-beer emulsions were analyzed for droplet volume and frequency post-digestion once they had spent 20 minutes in a water bath at 50 °C, at this temperature that the enzyme has 100% activity[89]. Emulsions were then sealed and stored in the refrigerator at 4 °C until they were observed again after 1 day and after 7 days. Average oil droplet volume was used as a proxy for emulsion stability, since a stable average droplet volume over time indicates relative stability of the droplet population.

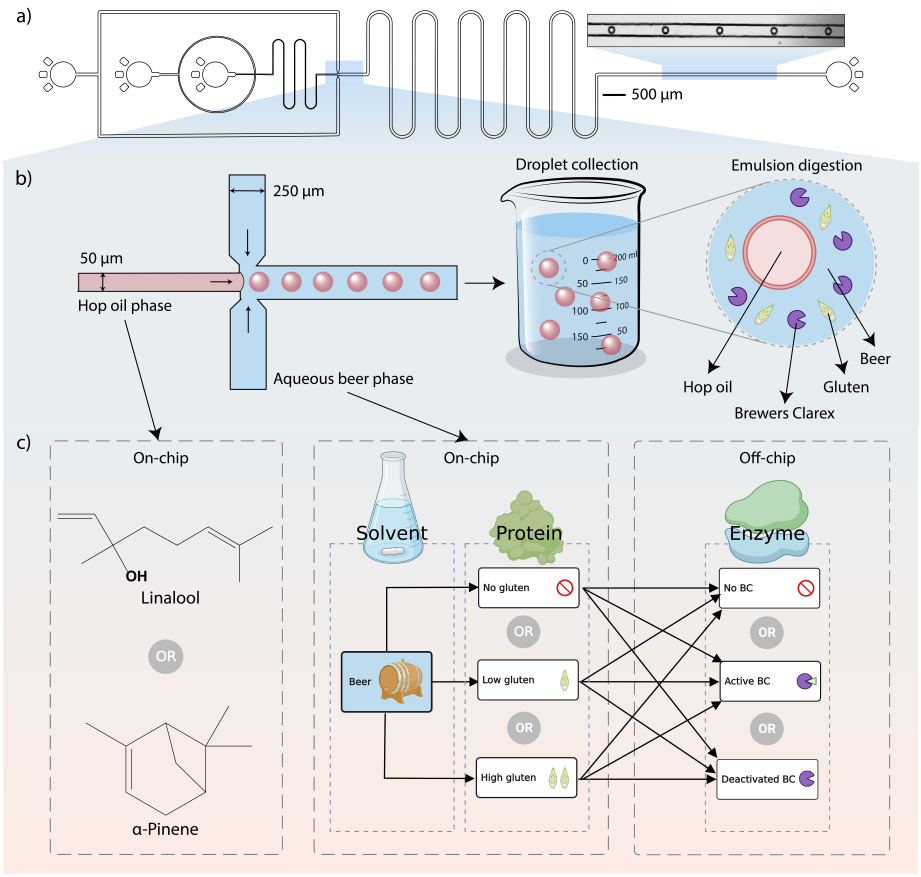

Figure 3.2: Microfluidic platform and assay design. a) Design of the microfluidic platform used for the formation of oil-in-beer emulsions. Emulsions were created in the flow focusing junction highlighted by a blue box. The other flow focusing junction was not used in these assays. A brightfield image of the oil-in-beer emulsions created is shown near the outlet of the microfluidic device. b) Dimensions and design of the flow focusing junction for the creation of hop oil droplets (red phase) in beer (blue phase), which sometimes also contain a protein (gluten). Emulsions were collected off-chip in a glass vial to allow for digestion with Brewers Clarex and analysis. The inset shows a graphical representation of the oil-in-beer emulsion after the addition of Brewers Clarex, where the red circle denotes the oil droplet, the green leaf represents gluten, and the purple sector represents Brewers Clarex. c) Graphical representation of the assay, showing the composition of the hop oil and aqueous beer phases inserted into the microfluidic platform. The hop oil phase consisted of a terpene, either linalool or α-pinene. The aqueous phase is predominantly beer, to which a protein is added at two different concentrations (low or high gluten) on-chip. An enzyme is added in two different formats (active or deactivated Brewers Clarex) off-chip in all possible combinations. Control experiments where no protein or enzyme was added were also performed. Droplet frequency and volume were recorded directly after enzyme treatment (Day 0), after 1 day and after 7 days. Image partially created with BioRender.com.

Our initial assays were designed to determine whether gluten affects the stability

Table 3.1: One-way ANOVA data comparing the average droplet volume change between day 0 and day 7 for each gluten concentration (no gluten, low, or high gluten)

Sample	F value	F statistic	p-value*
No gluten	0.43	3	0.65
Low gluten	124	3	<0.05
High gluten	16.8	3	<0.05

α = 0.05

of oil-in-beer emulsions over time. Figure 3.3 shows how the average droplet volume changes over time for oil-in-beer emulsions of α-pinene when subjected to three different gluten concentrations (no gluten, low and high concentrations of gluten). No Brewers Clarex was added to these assays. The average droplet volume was calculated by taking the mean of all droplet volumes recorded for each different treatment and monitoring over time. At the first measurement (Day 0), the emulsion with no gluten added (black data) had the highest average droplet volume, with the addition of gluten at either concentration lowering this initial average volume. After 1 day, this trend remained stable. After 7 days, the low gluten (blue data) and high gluten (purple data) average droplet volumes had both changed, with the ANOVA between Day 0 and Day 7 showing a statistically significant difference in average droplet volumes ($p < 0.05$) for both gluten concentrations (See Table 3.1). For the emulsion with a low concentration of added gluten, the average droplet volume stayed smallest in size overall over 7 days, but changed more significantly than the group with no gluten added ($p = 0.65$). The high gluten group had the largest change in average droplet volume, with size increasing dramatically from the start to the end of the assay.

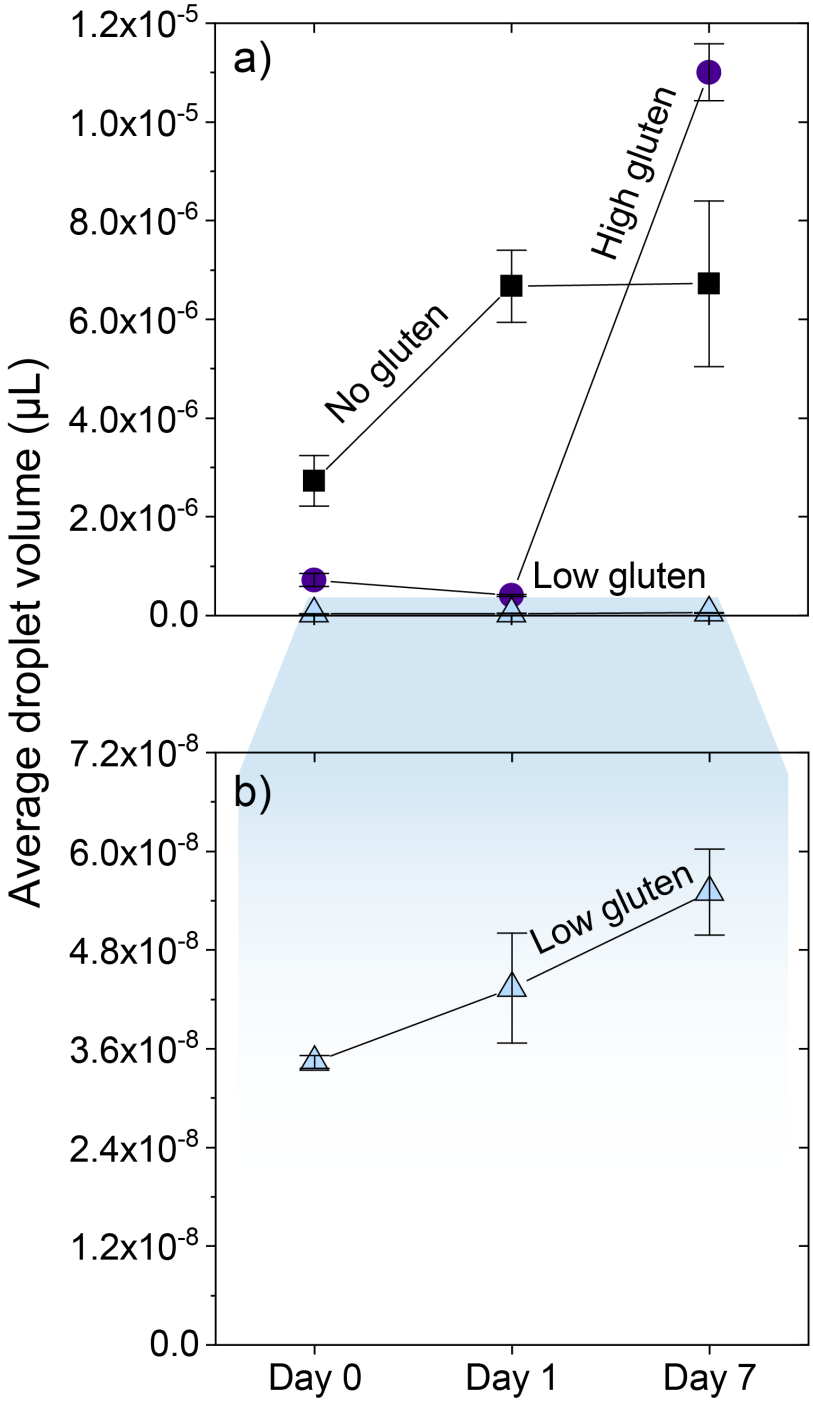

Figure 3.3: Average droplet volume over time for α-pinene oil-in-beer emulsions treated with different gluten concentrations. Oil-in-beer emulsions of α-pinene in beer treated with either no gluten (black data), low (blue data), or high (purple data) gluten concentrations were monitored over seven days to assess droplet volume, a metric for how stable an emulsion is over time. b) Same data for the low gluten assay as shown above using a smaller volume scale to enhance visualization of volume change over time. In both graphs, lines are added between data points of the same assay to aid visualisation.

The changes in average droplet volume over time for oil-in-beer emulsions of α-pinene with no gluten, or low, and high gluten concentrations indicate that the presence of gluten may contribute to emulsion stability over time and that this effect may also be sensitive to the amount of gluten present. The emulsion not treated with gluten (black data) showed no statistically significant change in average droplet volume over time (see Table 3.1), suggesting that the emulsion remained stable over 7 days. While the average droplet volume for the assay treated with a low gluten concentration (Figure 3.3b) did change significantly over time, the overall size range of the average droplet volume remained smaller than the other two treatment groups. Contrarily, although both the low and high gluten treatments changed significantly in average droplet volume over 7 days, the high gluten treatment assay (purple data) changed more significantly and showed the highest change in overall size, indicating that a high concentration of gluten present may destabilise the emulsion more than a low concentration of gluten. This fits with anecdotal industry knowledge that beer styles with higher terpene content (such as pale ales) can be subject to more particulate coagulation, which can be a result of interactions between proteins and polyphenols[90].

Adding gluten to oil-in-beer emulsions clearly affects emulsion stability. We then added Brewers Clarex, as an enzyme that digests gluten to further test our hypothesis that gluten stabilises hop oil in beer. Figure 3.4 shows oil-in-beer emulsions of α-pinene, or linalool treated with the same gluten treatments as in Figure 3.3 (no gluten, and low and high concentrations of gluten), with the addition of four Brewers Clarex enzyme treatments: no Brewers Clarex, deactivated Brewers Clarex, low Brewers Clarex, and high Brewers Clarex. Brewers Clarex was added to the emulsions off-chip. Figures 3.4a and 3.4c show the individual droplet volume of linalool or α-pinene emulsions when gluten and/or enzyme are added, and Figures 3.4b and 3.4d show

the relative droplet frequency of the linalool or α-pinene emulsions under the same conditions. Deactivated Brewers Clarex was added as second control to determine whether other components of Brewers Clarex may affect emulsion stability.

When no gluten was added to the linalool emulsions, the mean (green stars on black data in Figure 3.4a) individual droplet volume stayed relatively similar upon treatment with deactivated, low, or high concentrations of Brewers Clarex. However, the addition of deactivated Brewers Clarex caused a dramatic increase in relative droplet frequency (black data in Figure 3.4b), which may suggest that a non-enzymatic component of Brewers Clarex has a stabilizing effect on the linalool emulsions. When either concentration of Brewers Clarex was added, droplet frequency was similar to when no Brewers Clarex was added, so the deactivated enzyme or another component in the Brewers Clarex may influence the emulsion stability. When higher concentrations of Brewers Clarex are added, we see a larger spread of individual droplet volumes (Figure 3.4a, black data) than for low concentrations of Brewers Clarex. This could mean that the increased gluten digestion by Brewers Clarex is causing more droplet instability and, hence merging.

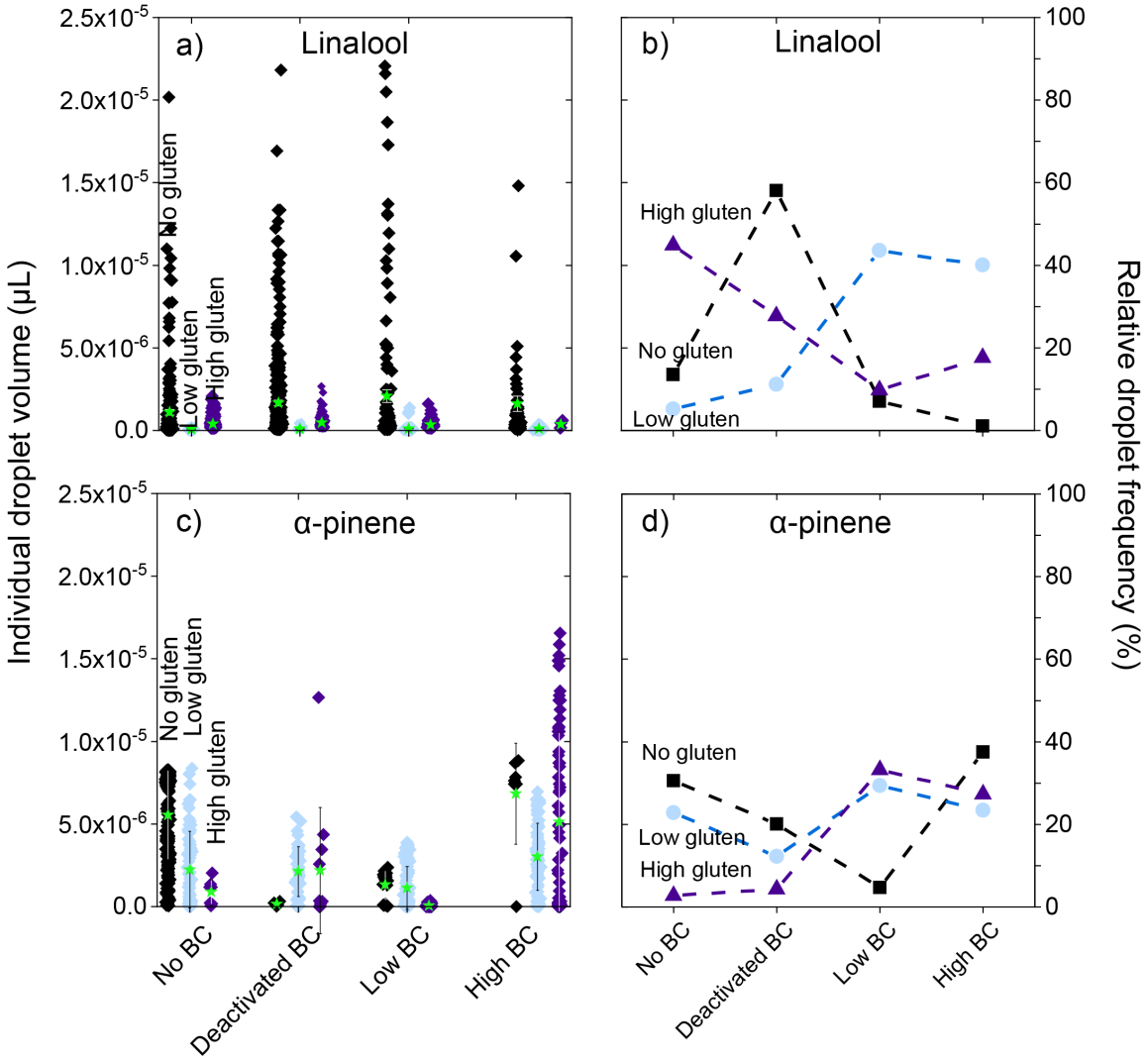

Figure 3.4: Changes in individual droplet volume and relative droplet frequency for oil-in-beer emulsion upon the addition of Brewers Clarex. Droplet volume when a) linalool and c) α-pinene emulsions are treated with different Brewers Clarex enzyme concentrations (no Brewers Clarex, deactivated Brewers Clarex, low concentration of Brewers Clarex and high concentration of Brewers Clarex). For each Brewers Clarex treatment, the emulsion also contains no additional gluten (black data), or low (blue data) or high (purple data) gluten concentrations. This illustrates the changes in individual droplet volume upon the addition of gluten and/or enzyme for both types of oil-in-beer emulsions. Green stars show the mean droplet volume for each treatment. Error bars represent standard deviation. Relative droplet frequency for each enzyme treatment illustrating the relationship between droplet frequency and the total volume of droplets for both b) linalool and d) α-pinene emulsions with no additional gluten (black data), or low (blue data) or high (purple data) gluten concentrations.

In the α-pinene assays (Figures 3.4c and 3.4d) we see the effect of the type of

hop oil used on emulsion stability. Monoterpene alcohols such as linalool with a hydroxy group are more water-soluble than hydrocarbons such as α-pinene[91], which could explain the spread in droplet sizes. In this case, when deactivated or low concentrations of Brewers Clarex are added, we see increased stabilisation of the emulsion, which again suggests that there are multiple stabilisation mechanisms in effect. This may be due to the active enzyme in Brewers Clarex in conjunction with a non-enzymatic component, since the deactivated Brewers Clarex treatment did not appear to affect the relative droplet frequency of the α-pinene emulsions in Figure 3.4d, or the spread in droplet volume in Figure 3.4c as it did in the linalool emulsions.

Individual droplet volumes for both linalool and α-pinene emulsions in Figures 3.4a and 3.4c did not change drastically with the addition of a low concentration of gluten (blue data). However, the relative frequency of droplets in both hop oil emulsions increased with the addition of both low and high Brewers Clarex doses (blue data in Figures 3.4b and 3.4d), indicating that the active enzyme may have influenced the frequency of droplets in the emulsions when a low concentration of gluten was present. When a high concentration of gluten (purple data) was added to the oil-in-beer emulsions, we saw contrary influences between the two terpenes, with a decrease in droplet frequency for linalool emulsions treated with low and high Brewers Clarex doses (purple data in Figure 3.4b), and an increase in droplet frequency for α-pinene emulsions treated with low and high Brewers Clarex doses (purple data in Figure 3.4d). The mean droplet volume (green stars) of linalool emulsions with a high gluten concentration did not change when treated with deactivated, high, or low Brewers Clarex doses (purple Figure 3.4a), while α-pinene emulsions showed dramatic changes in both mean volume and range of droplet volume sizes when a high dose of Brewers Clarex was used (purple Figure 3.4c). This suggests that gluten may influence the emulsion stability of certain hop oil emulsions such as α-pinene, but not

Table 3.2: Total number of droplets (n) included in each data set in Figure 3.4. Each oil-in-beer emulsion of linalool or α-pinene had a protein added on-chip and an enzyme added off-chip in all possible combinations outlined in Figure 3.2

Linalool

Protein	Enzyme	n total
No Gluten	No BC	216
	Deactivated BC	427
	Low BC	158
	High BC	98
Low Gluten	No BC	24
	Deactivated BC	53
	Low BC	191
	High BC	207
High Gluten	No BC	286
	Deactivated BC	236
	Low BC	5
	High BC	129

α-pinene

Protein	Enzyme	n total
No Gluten	No BC	131
	Deactivated BC	86
	Low BC	161
	High BC	20
Low Gluten	No BC	84
	Deactivated BC	45
	Low BC	108
	High BC	86
High Gluten	No BC	7
	Deactivated BC	11
	Low BC	85
	High BC	70

others such as linalool. As this is a preliminary study, we are observing associations between different treatment groups, however the experiments should be repeated before narrowing down the causation of these changes in trends. It remains to be determined whether this trends would hold across hydrocarbon and monoterpene alcohol groups.

Overall, oil-in-beer emulsions of α-pinene or linalool with a low concentration of gluten present (blue data) treated with a low concentration of Brewers Clarex showed

the smallest individual droplet volume and highest relative droplet frequency, indicating that in "hazy" style beers where there are more haze-active proteins present than in "clear" style beers, a low concentration of Brewers Clarex may serve to stabilise hop oil emulsions. The deactivated Brewers Clarex treatment groups had individual droplet volumes and relative droplet frequencies different than the no Brewers Clarex treatment groups, suggesting other components of Brewers Clarex had an impact on emulsion stability. Finally, different structures of the hop oils in the emulsion may influence the emulsion stability, which could have ramifications for brewers choosing which hops to include in "hazy" style beer recipes when aiming for a shelf-stable brew.

3.3 Materials and methods

Table 3.3: Oil-in-beer emulsion treatments of samples used in Figure 3.4. In each case 100 µL of beer and either α-pinene or linalool oil-in-beer emulsions were collected and treated off-chip with the solutions described under "Off-chip treatment". In the case of the "No Brewers Clarex" sample, an extra 100 µL of beer and either α-pinene or linalool oil-in-beer emulsions were added to ensure volume consistency. Likewise, in the case of the "Low Brewers Clarex" sample, an extra 50 µL of beer and either α-pinene or linalool oil-in-beer emulsions were added to ensure volume consistency

	Off-chip treatment	Final treatment concentration
No Brewers Clarex	100 µL of oil-in-beer emulsions	Pure oil-in-beer emulsions
Deactivated Brewers Clarex	100 µL of deactivated Brewers Clarex	50% (v/v) deactivated Brewers Clarex
High Brewers Clarex	100 µL of Brewers Clarex	50% (v/v) Brewers Clarex
Low Brewers Clarex	50 µL of Brewers Clarex and 50 µL of oil-in-beer emulsions	25% (v/v) Brewers Clarex

3.3.1 Materials

All reagents were used as received. Gluten from wheat, PVA (99%), chlorotrimethylsilane, sodium hydroxide, glacial acetic acid, linalool and α-pinene were purchased from Millipore Sigma. Acetate masks were printed at 10 µm resolution by CAD/Art Services. SU-83050 and developer were purchased from MicroChem. Silicon wafers (100 mm diameter) were purchased from Silicon Materials. Polydimethylsiloxane (PDMS, Dow Sylgard 184) base and curing agent (Sylgard 184 Silicone Elastomer Curing Agent) were purchased from Ellsworth Adhesives. Polytetrafluorethylene (PTFE) tubing (outer diameter 1/16 inch and inner diameter 0.75 mm) was purchased from Chromatographic Specialties. Brewer's Clarex was a free sample from DSM Food Specialties. The beer (78 Kolsch, 5.0% ABV) was donated by Phillips Brewing and Malting Co.

3.3.2 Microfluidic device fabrication

Microfluidic platforms were designed using AutoCAD (Autodesk Student 2017 LTD) and printed onto acetate masks to create positive photomasks. The SU-8 layer was spin-coated onto the wafer and the wafer was soft baked for 5 min at 25 °C, 2 min at 65 °C and 30 min at 95 °C. Following the soft bake, the wafer was exposed to UV light for 11 s at 19.96 mW cm^2 using an OAI Model 800 mask aligner through the acetate photomask. The wafer was then heated at 95 °C for 15 min and cooled for 30 min. Unexposed SU-8 was removed using developer. Adhesion of the features was enhanced through exposure to UV light for 90 s at 19.96 mW cm^2 and a final hard bake at 200 °C for 30 min. The wafer was placed in a vacuum desiccator with 50 µL of vaporised chlorotrimethylsilane for 45 min to silanise the wafer and prevent sticking. PDMS base and curing agent in a 10:1 ratio were mixed together thoroughly and poured over the silanised wafer, then placed in a desiccator under vacuum for 45

min to remove air bubbles. The PDMS-covered wafer was then placed in the oven at 65 °C to cure for a minimum of three hours to overnight. Once cured, the PDMS was carefully separated from the wafer mould and cut into individual devices using a scalpel. Access holes were punched using biopsy punches with a 1/16-inch outer diameter. PDMS was also spin coated onto glass microscope slides at 1200 rpm for 25 s and cured as above to create the bottom of the devices. Devices and PDMS-covered glass slides were washed thoroughly with soapy deionised water, isopropanol, and ethanol. The clean devices were placed in reverse osmosis water for 5 minutes, dried thoroughly with a filtered air gun, and then dried at 90 °C for 30 min on a hotplate. After the devices were dried, they were plasma bonded (Diener Electronic, Zepto ONE, 1 min, 100 W, 0.64 mbar) to the PDMS-covered glass slides.

3.3.3 Surface treatment of the microfluidic devices

The channels of the microfluidic devices were treated with a polyvinyl alcohol (PVA) solution as described previously[92] to ensure the production of oil-in-water emulsions. In brief, the PVA solution was made by dissolving 1.00 g of PVA in 100 mL of MilliQ water. The solution was stirred at room temperature for 45 min, then at 100 °C for 45 min, and then at 65 °C overnight. Immediately after plasma bonding the devices to the PDMS-covered slides, PVA solution was manually flowed through the channels using two gas-tight 1 mL glass syringes (Hamilton). The surface-treated devices were then placed in the oven at 65 °C for approximately 48 h before use.

3.3.4 Preparation of the beer aqueous phase

The beer used was a 375 mL canned Kolsch-style ale supplied by Phillips Brewing and Malting Co. A new can was used for each assay. Prior to insertion into the microfluidic device, the beer was degassed to ensure reproducible fluid dynamics on-

chip. Beer was poured into a glass test tube and sonicated with a Fischer Analog Vortex sonicator. Once a foam layer formed it was removed from the liquid layer and the sonication step was repeated until there was no carbonation present in the beer.

3.3.5 Preparation of gluten solutions

As shown in Figure 3.2c, the aqueous phase on the microfluidic platform consisted of degassed beer and gluten at one of three different concentrations (no gluten, low gluten, high gluten). For the addition of gluten, first a 5% w/w gluten stock solution was made using gluten from wheat dissolved in 0.05% w/v NaOH solution and stirred at 55 °C for 30 min. It was allowed to cool, and 10% v/v glacial acetic acid was added. Serial dilutions were performed to reach a $2.5\text{x}10^{-5}$% (w/w) final gluten concentration and $2.5\text{x}10^{-1}$% (w/w) final gluten concentration for the low and high gluten assays, respectively. These solutions were added to the degassed beer prior to forming emulsions. When no gluten was added to the beer phase, the same amount of solvents used to make the gluten solution (but without the gluten) were added to the degassed beer prior to emulsion formation.

3.3.6 Creation of oil-in-beer emulsions

The design of the microfluidic device is seen in Figure 3.2a. Hamilton Gastight glass syringes (1 mL) were filled with hop oil (inner oil phase) and degassed beer (outer aqueous phase) to make oil-in-beer emulsions. Syringes were connected to the microfluidic device using PTFE tubing. The flow rate was controlled using neMESYS low pressure syringe pumps (Cetoni) with an initial flow rate of 15 µL/min for both phases, which was then adjusted to reach the highest throughput possible and droplet formation at equilibrium. Once equilibrated, the emulsions travelled through the device to the outlet where PTFE tubing carried the emulsions to a glass collection

vial.

3.3.7 Brewers Clarex treatment of oil-in-beer emulsions

Oil-in-beer emulsions were collected in a glass vial until approximately 2 mL of emulsion was accumulated. Brewers Clarex was used as received for the high Brewers Clarex treatment and diluted to 50% v/v with reverse osmosis water for the low Brewers Clarex treatment. To prepare deactivated Brewers Clarex, it was heat-deactivated in a water bath at 70 °C for 15 min and then cooled to room temperature before use. Oil-in-beer emulsions were treated with these Brewers Clarex solutions as described in Table 3.3. Vials were then sealed and incubated in a water bath at 50 °C for 20 min prior to observation.

3.3.8 Droplet analysis

Droplet formation on the microfluidic devices was observed on a Nikon Eclipse Ti-U2 Inverted Research Microscope. Videos of emulsion formation and images of the on-chip emulsions were collected at 4x magnification using a Phantom high-speed camera (VEO 710L). After 20 min of emulsion treatment as described in Table 3.3, emulsions were observed on microscope using a 40X/0.60 objective. Images were taken in the 4 quadrants of an observation slide made using Fisherbrand glass microscope slides and slide covers. For time series experiments, emulsions were sealed in a vial and stored in a 4 °C refrigerator. Images were taken again after 1 day and after 7 days. Images were processed using ImageJ (version 1.52a). Results showed a normal distribution and outliers were eliminated using the InterQuartile Range (IQR) Rule (if value $>$ or $<$ 1.5xIQR).

3.4 Conclusions

We have shown that using a microfluidic platform to digest oil-in-beer emulsions in the presence and absence of gluten and Brewers Clarex is a rapid and effective way to assess the stability of the emulsions under multiple conditions that reflect the full-scale conditions found in the brewing industry. Oil-in-beer emulsions were affected by the addition of gluten to the emulsion, along with Brewers Clarex, a proline-specific endoprotease known to efficiently digest gluten in beer. Both α-pinene and linalool emulsions with a low concentration of gluten present showed higher stability in droplet volume and frequency when treated with a low concentration of Brewers Clarex, though it is not yet clear if the active enzyme or another component of Brewers Clarex were responsible for the overall emulsion stability.

As this is the first time a microfluidic platform has been used to explore the dynamics of hop oils in beer, this early work inspires many further questions. Future work in understanding how proteins affect hop oil emulsion stability could be done using the new methods we have developed, with other common molecules found in beer originating in brewing grains. We suggest starch, as a large-molecule polysaccharide commonly found in conjunction with gluten and enzymatically-processed by amyloglucosidase during brewing. We also suggest a more robust panel of hop oils, including a selection of both hydrocarbon- and monoterpene-structured hop oils to elucidate whether the structure of the individual hop molecules affects how stable they are when emulsified in beer. This could also lead to future work involving microfluidic analysis alongside an analysis of the individual molecular components of the oil-in-beer emulsion as it ages, to assess if structural changes in individual molecules contribute to overall emulsion stability.

This work and future studies based on it may lead to interesting conclusions on the macro scale of industrial brewing. If certain hop oils in conjunction with adjusted

proteinaceous components of beer are more stable over time while not sacrificing the sensory qualities of the final product, brewers could adjust recipes to create beers that better withstand the aging process. Work has already been done on individual hop oils and their respective shelf stabilities[93]. Combining that work with the proteinaceous and aqueous components used on the macro scale in the brewing industry by using a microfluidic platform could reveal what this means for the hazy beer brewing process for recipes yet to be developed, and drank by brewers, scientists, and market consumers alike.

Chapter 4

Conclusions and Future Work

4.1 Conclusions

This thesis describes the use of droplet-based microfluidic technologies to explore the behaviour of different biomolecular components on the surface of droplets in emulsions. Two applications were characterized, one with a lipid-based droplet surface and one with a protein-based droplet surface. The lipid-based work highlighted an accessible method for fabricating asymmetric liposomes as a simple, biomimetic cell membrane model. The protein-based work detailed a novel method for creating hop-oil emulsions in beer to explore how proteins affect droplet stability over time. There are two main conclusions I reached in this thesis:

1. Fabricating asymmetric liposomes using naturally-derived lipids in an aqueous solution can be done using a simple microcapillary device built from materials that are accessible to non-specialized researchers. By using a device that is far simpler to fabricate than traditional PDMSpolydimethylsiloxane or glass microcapillary devices, I presented a method for creating double emulsions capable of dewetting and forming asymmetric liposomes. Forming the asymmetric lipo-

somes using only naturally-derived lipids in the aqueous phase allowed for lipid self-assembly in an environment that is more biomimetic than most methods currently used, which put lipids in the oil phase. I characterized the asymmetric liposomes to confirm bilayer asymmetry, size, and stability, which are necessary to measure for future potential applications of this model. I offer a starting point for future biomimetic model membrane research with asymmetric liposomes capable of mimicking the composition of phospholipids found in a red blood cell membrane.

2. We developed a droplet-based microfluidic device to explore hop oil dynamics in beer on a micro-scale. A microfluidic device provided me with a rapid and effective method for forming hop-oil-in-beer emulsions, which could then be treated with gluten and a gluten-specific protease to understand if gluten was playing a role in hop-oil droplet stability. I recorded changes in emulsion stability over time based on the dosage level of gluten and Brewers Clarex (the enzyme) and found that the individual hop oil terpene structure may also affect emulsion stability. In a collaboration with Phillips Brewing, a local craft brewery, this is the first time a microfluidic system has been utilized to provide brewers with quality control information about hop oil emulsion stability. Further research is needed to understand the specific mechanism (as it is likely multi-faceted) responsible for overall emulsion stability in beer over time. I established a method for future researchers to conduct a comprehensive panel of hop oil experiments to further understand how individual terpene structure and protein stabilization may be related. By analyzing how individual components affect overall emulsion stability, the mechanisms discovered may have impacts on a macro scale in the brewing industry.

Both applications use the surfaces of microfluidic droplets in a novel way that

illuminates the need for future exploration into their functions in both academic and industrial research. The first method I developed allows future researchers to understand how bilayer asymmetry affects therapeutic treatments transported through the cell membrane by constructing more biomimetic model membranes. In a collaboration with a local brewery, I also produced information about how protein in beer may influence the stability of hop oils using a microfluidic device. The method and framework we developed will be explored further and will likely inform how the industry controls protein and hop oil interactions during brewing.

4.2 Future work

Like any good research findings, this work created new questions as well as answering the ones we initially posed. The field of droplet-based microfluidic technologies is rapidly advancing, and understanding the surface properties of droplets is a critical part of that advancement, since researchers can control the behavior of droplets more precisely by manipulating droplet properties like dewetting and stability. The more precise the control, the more complex the droplet assembly that can be designed. As shown in this thesis, this allows us to create functional structures such as liposomes for biomedical research and emulsions with custom properties that can be used to understand the fundamentals of mechanisms (such as haze stabilization in beer) that affect industrial procedures.

There are several reasons why it is important to develop biomimetic research models like the asymmetric liposomes I made. Models that closely mimic human physiology and pathology are more likely to produce findings that translate to humans, allowing for a better understanding of the biology of healthy and diseased cells and tissues and how drugs interact with them. An efficient drug development process

requires a reliable model, ideally as close to a living cell as possible to reduce costs and time. To further investigate the biomimetic nature of asymmetric aqueous-phase liposomes, they should be compared with actual red blood cell membranes in a passive diffusion study. Future work on this project is underway.

First, a method will be developed to visualize the dewetting process using a fluorescent probe that is only soluble in the aqueous phase. Asymmetric and symmetric liposomes will both be produced and further characterized. Quinine, a drug known to treat malaria, is capable of passing through bilayers and will be used to validate the liposomes as a drug model by measuring the rate of diffusion into the asymmetric and symmetric liposomes over time using fluorescent microscopy. Then, more red blood cell membrane liposomes will be made and the rate of diffusion will be measured again and compared to the PC/PE and PC/PC liposomes, along with actual red blood cells. If the rate of diffusion of quinine into the liposomes is similar to the rate of diffusion into red blood cells in the literature, then we can continue making the model more biomimetic through the addition of cholesterol and membrane proteins.

A goal is to validate a model so that other drugs may be developed and tested in early trials before being translated to clinical trials, reducing the number of live cells needed. This could lead to the development of more effective treatments for diseases affecting red blood cells in the human body. The formation of red blood cell membrane mimics also indicates that other bespoke membrane mimics may be fabricated using similar methods, for example, to create a blood-brain barrier membrane for use in Alzheimer's disease research and treatment development. Overall, the development of biomimetic research models allows us to improve the validity of research findings, reduces the reliance on living models which in turn reduces the ethical considerations of using animal models, and potentially reduces the time and costs associated with the research and development of biomedical therapeutics.

Continued development of microfluidic techniques for industrial applications also can reduce the time and costs spent understanding the basic mechanisms behind known processes. Microfluidic devices had never been used to explore hop oil dynamics in beer, and as it is one of the four necessary components of beer, future work using the same methods to explore hop-oil emulsion stability could be done with other common biomolecules found in the brewing process. Another molecule originating in brewing grains, starch, will be studied next as it is enzymatically processed similarly to gluten and also contributes to O/W emulsion stability. Glycogen released from yeast cells post-fermentation may also destabilize emulsions in some instances and should be further explored.

The role of individual hop molecule structure was touched on in this research, but a robust panel of multiple hydrocarbons- and monoterpene-structured hop oils could be assessed for stability in beer, providing brewers with information about which hop oils to prioritize based on the final intended sensory profile of their products. We started with linalool and α-pinene and future work should include more monoterpenes such as myrcene and also include sequisterpenes such as humulene for a more robust hop profile. Most beer styles call for the addition of more than one type of hop oil, as raw hops contain many individual hop oils, so a combination of multiple terpenes commonly used in brewing (such as myrcene, linalool, pinene, and geraniol) should be tested together. As Brewers Clarex only targets proline-rich areas on gluten proteins, a different endoprotease could be used to target different protein motifs to further determine the role of protein in emulsion stabilization. This method could also be used to understand how cyclodextrins stabilize emulsions in beer as they have been shown to help maintain long-term O/W emulsion stability[94].

Further expanding the knowledge of how the fundamental biomolecules interact in solution allows brewers to make more consistent, stable products. As the brewing

industry is highly competitive, and consumers have so many choices available, brewers must make reliable products efficiently to succeed. Beyond brewing, protein-based surfaces on droplets can also influence the stability and sensory attributes of other emulsion-based food and beverage products, cosmetics, and pharmaceuticals. Understanding how proteins behave on the surfaces of droplets in emulsions allows for improved product formulations and the ability to create products that have greater performance characteristics, benefiting both the manufacturers and consumers.

Appendix A

Additional Information

A MicroTAS submission 2021

BESPOKE ASYMMETRIC LIPOSOMES TO MODEL PASSIVE DRUG TRANSPORT ACROSS RED BLOOD CELLS

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University of Victoria, British Columbia

ABSTRACT

Here we show the generation of lipid-based synthetic cells using a microcapillary microfluidic platform. We show for the first time a new method for the formation of liposomes where the lipids can be inserted into the aqueous phases for asymmetric liposome formation. We have also improved upon the biomimeticity of these synthetic cells through the use of a novel combination of mammalian lipids and membrane asymmetry as a step towards mimicking red blood cells.

KEYWORDS: Asymmetric, liposomes, red blood cells, microcapillary

INTRODUCTION

The aim of the following work was to use our versatile novel microcapillary microfluidic platform [1] to form reproducible asymmetric liposomes which are capable of red blood cell-like biomimeticity. In part due to their complexity, there is only a handful of prior work which creates asymmetric lipid bilayers using microfluidic technologies. The most common microfluidic approaches are the layer-by-layer technique [2], the pulsed-jet flow method [3] and the triangular post method [4]. That said, the micro-fabrication techniques required to design these platforms are highly complex and subject to variability. Prior work showing the facile microfluidic formation of symmetric liposomes using double emulsions in the literature involved putting the lipids in the oil phase [4]. We generate biomimetic asymmetric liposomes using a more facile and reproducible methodology.

EXPERIMENTAL

The microcapillary device for the liposome generation was assembled using three glass capillary sizes. A 0.20 mm inner diameter (ID) glass capillary was used for the inner phase. It was heat ligated to 0.25 mm ID Polytetrafluoroethylene (PTFE) tubing. 0.75 mm ID PTFE tubing was used for the middle phase. A 1.5 mm ID surface treated (in 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane for 15 min to generate a hydrophilic surface) glass microcapillary was used for the outer phase. Liposomes were formed in the same way for all experiments. First, the three solutions (outer aqueous, middle solvent and inner aqueous) were inserted into three 1 mL gastight glass syringes (Hamilton). For asymmetric liposomes, the inner phase consisted of 8 wt% polyethylene glycol (PEG), 2 wt% poly(vinyl alcohol) (PVA) with 5 $mgmL^{-1}$ phosphatidylethanolamine (PE). The middle phase consisted of a mixture of chloroform and hexane (50:50 v/v). The outer phase consisted of 10 wt% PVA, 0.5 wt% Pluronic F-68 and 5 $mgmL^{-1}$ phosphatidylcholine (PC). For the fluorescence experiments, 0.4% fluorescently tagged NBD-PC and Texas Red-PE were added to the outer and inner phases, respectively. The flow rate was controlled using neMESYS syringe pumps (Cetoni). Droplet

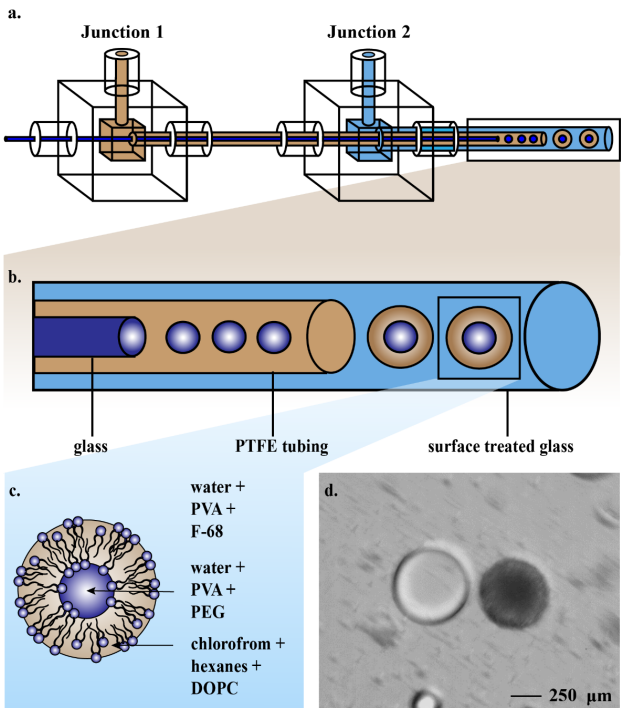

Figure 1: Liposome generation using a glass microcapillary device. a, Schematic of the microcapillary device used to generate liposomes. Dark blue indicates the inner aqueous phase, brown indicates the middle solvent phase and light blue indicates the outer aqueous phase. b, Schematic of the microcapillaries and droplet formation. The inner capillary was a glass capillary, the middle capillary was PTFE tubing and the outer capillary was a glass capillary which had been surface treated. c, Schematic showing the double emulsion composition. The inner phase was water, 2% w/w PVA and 8% w/w PEG, the middle phase was 12.5 $mgmL^{-1}$ DOPC lipid 50:50 chloroform:hexanes, and the outer phase was 10% w/w PVA and 0.5% w/w F-68 Pluronic surfactant. d, De-wetting process. The light droplet is the oil droplet and the dark droplet is the liposome.

formation was visualized using a stereomicroscope (SMZ800N, Nikon). Double emulsions were collected in a clean watch glass containing water. The double emulsion de-wetting was visualized on a Ti-U microscope (Nikon). Images were captured using a Phantom high-speed camera. Fluorescent images were captured using a Zeiss Confocal microscope.

RESULTS AND DISCUSSION

We used a novel microcapillary device [1] (Figure 1a) consisting of two junction boxes. The inner phase capillary (dark purple) was glass, the middle phase capillary (brown) was PTFE tubing, and the outer phase capillary (light blue) was glass (Figure 1b). For liposome formation, the inner phase contained PE) and the outer phase contained PC. PE and PC were selected as they are common eukaryotic lipids which are found in many different cells, in particular the inner and outer leaflets of red blood cells, respectively [5]. The schematic for the asymmetric double emulsion is shown in Figure 2a and for the asymmetric liposome is shown in 2b. We found that these liposomes de-wetted in approximately 15 min and produced liposomes reliably, as shown by the liposomes depicted in Figures 1d, 2c and 2d. Fluorescent imagery quantitatively proving asymmetry is shown in Figure 2e.

CONCLUSION

Here we show a simple microfluidic platform and surface treatment regime for the formation of asymmetric liposomes made with lipids in the aqueous phases. We also show that this platform can be used for the generation of asymmetric liposomes as a step towards the modelling of artificial red blood cells. Overall, this methodology has the potential to open up a route to the fabrication of synthetic cells capable of modelling the passive diffusion of clinically relevant drugs. We aim to quantify this transport for malarial drugs.

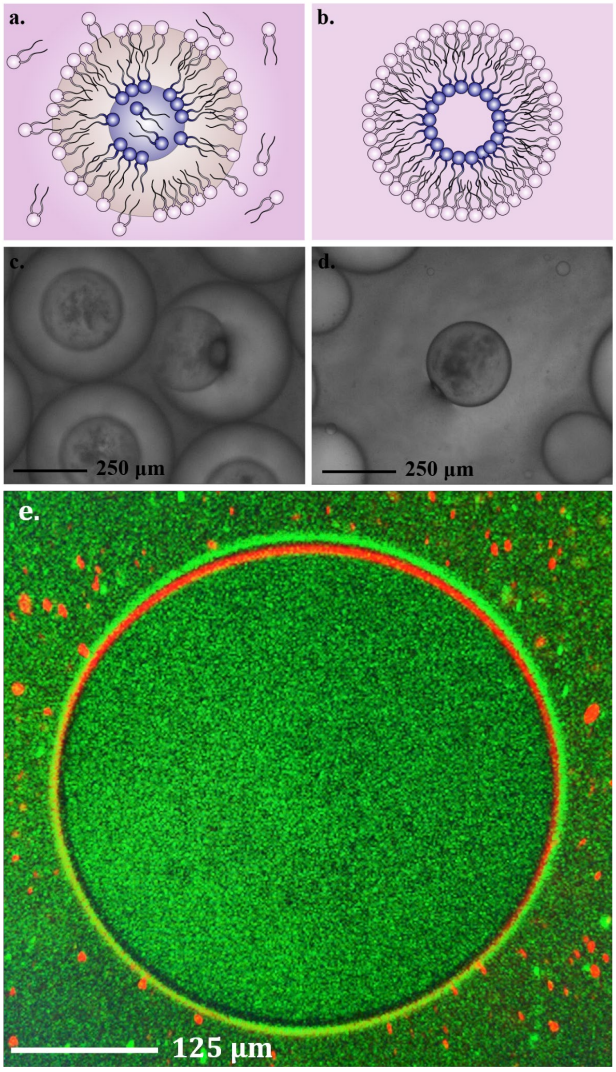

Figure 2. Asymmetric aqueous-phase liposomes. Pink denotes the outer aqueous solution, brown denotes the middle solvent phase and purple denotes the inner aqueous phase. a, Schematic for the double emulsion formation with the asymmetric PE and PC mammalian lipids contained in the inner (purple) and outer (pink) aqueous phases, respectively. b, Schematic for the asymmetric liposome formation after de-wetting of the solvent phase. c, Monodisperse double emulsions prior to de-wetting. d, Liposome after de-wetting of the solvent phase. e, Confocal fluorescent image showing asymmetric, PC (green) and PE (red).

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B MicroTAS submission 2022

MICROBREWIDICS: WHAT STABILIZES HOP OIL EMULSIONS IN BEER?

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ABSTRACT

Here we use a microfluidic platform to explore the role of proteins on the stability of hop oil emulsions in craft beer. The terpene α-pinene is found in high concentrations in hops (*Humulus lupulus*) and was used to form oil-in-water emulsions with a light-style ale. Industrial brewing enzymes (Brewer's Clarex and amyloglucosidase) were used to digest the emulsions to determine stability and hence what the stabilising molecule is in these emulsions.

KEYWORDS

Proteins, brewing, hop oil, oil-in-water emulsions, stability

INTRODUCTION

Beer is a complex mixture of many compounds contributed by raw materials, as well as by-products created during yeast fermentation [1]. A recent emergence in brewing is the "hazy" style, characterized by intense hop aromatics, low bitterness, and high turbidity. Turbidity is comprised of an excess of haze-active proteins, starches, and tannins from brewing grains [1] that may be responsible for the emulsification of aromatic terpenes from hops [2], though the detailed mechanism is not known. Here we study emulsion properties by observe oil-in-water mixture stability over time while influencing the suspected emulsifying agents. Miniaturizing these experiments using microfluidic technologies holds promise in food science for determining key emulsion constituents, employing beer as the aqueous phase and α-pinene (a common terpene found in brewing hops [3]) as the oil phase to explore the role of proteins inherently found in beer on these emulsions. We hypothesize that the stabilizing agent of terpenes in beer has a proteinaceous component, which we tested by examining the emulsion stability effects of commercial brewing enzymes Brewer's Clarex and amyloglucosidase, which break down gluten and starch respectively. We created terpene emulsions using our microfluidic platform and digested them with different concentrations of the enzymes, monitoring droplet number and volume over time as a metric of emulsion stability. This is a novel approach for studying emulsion formation and stabilization in beer, with the goal of helping brewers gain more precise control over the brewing process to improve consistency, flavour, and product development.

EXPERIMENTAL

Microfluidic devices were designed using AutoCAD (Figure 1) and fabricated using standard photolithography for the SU8 master and soft lithography for the PDMS devices. The hydrophobic surface of the PDMS devices was modified to support oil-in-water emulsions by surface-treating the channels with a solution of polyvinyl alcohol (PVA) post-plasma bonding. The PVA solution was manually flowed through the channels, and they were then dried and placed in an oven for 2 days prior to use. The aqueous phase was 78 Kolsch beer, de-carbonated. The oil phase was α-pinene. Emulsions were formed using a Nemesys syringe pump, gas-tight glass syringes, and the emulsion was collected for off-chip analysis. Brewer's Clarex and amyloglucosidase were added to the terpene and beer emulsions off-chip at two concentrations of enzyme (50% and 25% v/v) and three control experiments were performed (Figure 2). Images were processed using ImageJ.

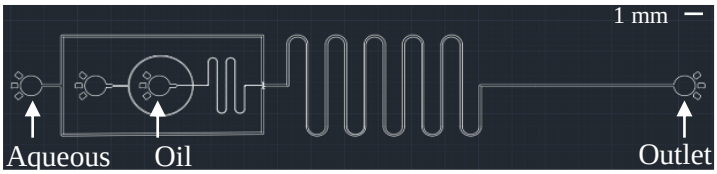

Figure 1: Device design.

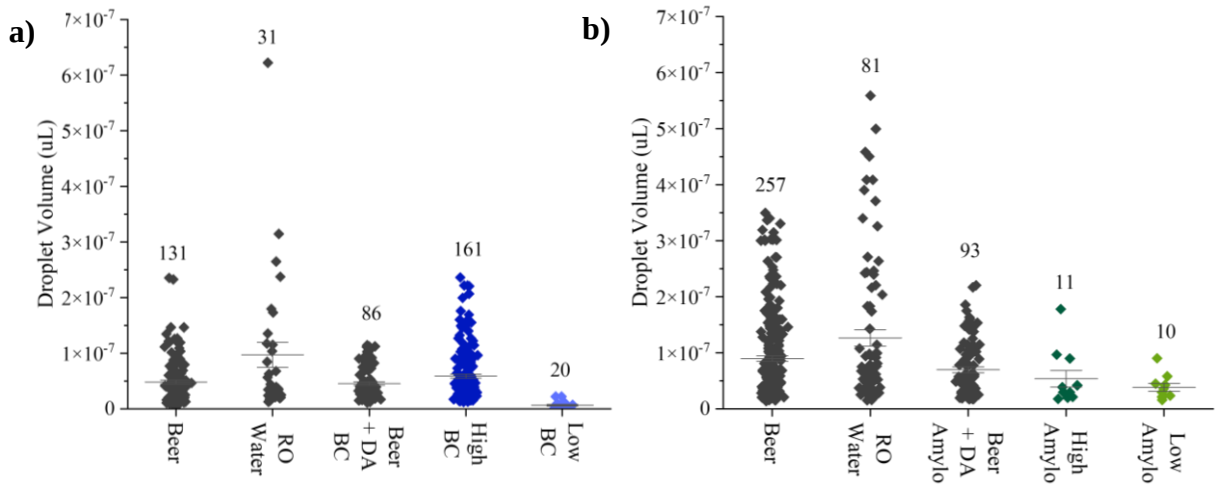

Figure 2: Emulsion stability quantified based on droplet volume and droplet number for emulsion digestion with a) Brewer's Clarex (BC) and b) amyloglucosidase (amylo). Each graph shows the digestion of emulsions formed from three control aqueous phases (dark grey) and two enzyme phases (high dose=dark blue or green, low dose=light blue or green). Control experiments varied the content aqueous phase to solely beer, reverse osmosis (RO) water or beer with deactivated (DA) enzyme.

RESULTS AND DISCUSSION

Droplet volume and count measurements showed that a high Brewer's Clarex concentration offered high droplet stability and consistent droplet volume production when compared to a low or a deactivated concentration of the enzyme (Figure 2a). Deactivated Brewer's Clarex provided a higher droplet stability and volume than a low concentration of the active enzyme, which may indicate that another unknown ingredient in Brewer's Clarex (or a digestion side product) was providing a stabilizing role. At a low concentration of Brewer's Clarex, the active enzyme was likely at the correct dosage to digest the targeted proteins but not at a high enough dosage to provide a stabilizing effect. Amyloglucosidase at high and low active concentrations served to strongly decrease droplet stability and volume in the emulsions formed (Figure 2b).

CONCLUSION

We show for the first time that a proteinaceous component influences hop oil emulsion stability in beer, which narrows down our search for the exact mechanism of emulsion stabilization in beer. Further research is ongoing to determine what else stabilizes hop oil emulsions in beer, as it is likely a multifaceted set of conditions including temperature and other classes of compounds. This research is completely novel in the field and provides brewers with information that enables the targeted design of terpene emulsions in beer.

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