Microfluidic Tools and Techniques for Artificial Cell Engineering

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Dirk van Swaay

Master of Engineering in Biomedical Engineering with a Year Abroad
Imperial College of Science, Technology & Medicine

born on 05.01.1980
citizen of Brazil and the Netherlands

accepted on the recommendation of

Prof. Dr. Andrew de Mello, examiner
Prof. Dr. Massimo Morbidelli, co-examiner

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To my family.
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Zusammenfassung


Kapitel 3 untersucht die Verwendung von Mikrofluidik zur Erzeugung von Protozellen mittels Koazervation. Es wurde ein iterativer Prozess bestehend aus Designing und Prototyping verwendet, um eine funktionierende mikrofluidische Plattform und ein kompatibles koazervierendes chemisches System zu entwickeln. Der verwendete Ansatz erzeugt eine fokussierte Strömung ohne die Verwendung von Düsen oder Kanalerweiterungen, wobei die benötigte hydrophobe Kanaloberfläche durch eine Beschichtung mit Perfluorocyl Trichlorsilan (PFOS) erhalten wurde. Das koazervierende chemische System der Wahl ist eine Kombination aus Poly (diallyldimethylammoniumchlorid) (PDDA) und entweder Carboxymethyl Dextran (CM-Dextran) oder Adenosintriphasphat (ATP).


Aufbauend auf den Analysen der beiden vorangehenden Abschnitte zeigt Kapi-
Zusammenfassung


In Kapitel 7 wird die Bildung von de facto künstlichen Zellen demonstriert. Koazervattröpfchen welche DNA und IVTT Komponenten einschließen werden hergestellt. Nach einer Inkubationszeit erzeugten diese künstlichen Zellen das fluoreszierende Protein mCherry. Sowohl Epifluoreszenz- als auch Konfokalmikroskopie wurden verwendet, um die erfolgreiche Genexpression nachzuweisen.

Abschliessend werden in Kapitel 8 die allgemeinen Schlussfolgerungen der Arbeit zusammengefasst und ein Ausblick für die künftige Forschung und Anwendung zur Verfügung gestellt.
Summary

The thesis describes the development of microfluidic tools and techniques for engineering artificial cells. For this purpose, a microfluidic flow focusing geometry is used to process a coacervating chemical system and form micro-droplets, which are then further developed into artificial cells. The thesis is organized into eight chapters.

Chapter 1 provides an introduction to the thesis and a description of the subsequent chapters. The chapter gives a general introduction to artificial cells, describes why they are becoming an important tool in the fields of biotechnology and synthetic biology and details the challenges associated with their engineering and application. Chapter 1 also provides a brief introduction to the field of microfluidics, its key characteristics, methods, and techniques, and outlines the main benefits associated with its application in artificial cell engineering.

In Chapter 2, the scientific literature describing the formation of liposomes and artificial cells using microfluidic methods is reviewed. Different methods for forming liposomes are evaluated, with particular attention being paid to liposome size and size distribution, stability, membrane composition and lamellarity, applicability and encapsulation efficiency. This review highlights that, to date, no single method is able to optimize all these parameters simultaneously.

Chapter 3 explores the application of microfluidic technology in the formation of protocells through the process of coacervation. Specifically, an iterative process of design and prototyping is used to develop a working microfluidic device and a compatible coacervate chemical system. The chosen device design employs a flow-focusing junction with no nozzle or channel expansion, and with channel surfaces rendered hydrophobic with perfluorooctyl trichlorosilane (PFOS). The coacervate chemical system of choice was a combination of poly(diallyldimethylammonium chloride) (PDDA) and either carboxymethyl Dextran (CMDextran) or adenosine triphosphate (ATP).

Chapter 4 addresses the measurement and analysis of droplet size and size distribution; key parameters in the production of artificial cells. Different methods for analyzing video and image data were evaluated. The analysis of still images of collected protocells using a custom MATLAB algorithm was the preferred approach due to its high accuracy, throughput and ability to make like-for-like comparisons between data obtained at different points in time.

Building upon developments in the two preceding chapters, Chapter 5 demonstrates the successful and controllable formation of coacervate droplets using a microfluidic device. In addition, studies demonstrate that DNA can be successfully encapsulated within such protocells. By forming two distinct populations of protocells (in parallel) containing different fluorescently labeled DNA oligonucleotides, it is shown that there is no exchange of DNA material between neighbouring droplets.

Chapter 6 addresses the issue of interfacing the macro- and micro-scales. A
novel connector used for injecting small-volume samples into microfluidic channels is described. The connector is used to interface external pumps and tubing with a microfluidic device, but also contains an easily accessible built-in chamber into which samples smaller than 20 \( \mu l \) can be introduced. This was a necessary development in this project that enabled the use of expensive and delicate reagents such as the in-vitro transcription and translation (IVTT) kit without the complications typically associated with loading samples into pumps, syringes, or tubing.

In Chapter 7, the formation of de facto artificial cells is demonstrated. Coacervate droplets were formed encapsulating DNA and IVTT components. These artificial cells were then shown to produce the fluorescent protein mCherry after a period of incubation. Both fluorescence and confocal microscopy measurements are employed to establish successful gene expression.

Finally, in Chapter 8, the general conclusions of the studies are summarized, and an outlook for future work and applications is provided.
Chapter 1

Introduction: towards engineering of artificial cells
Artificial cells are synthetic entities that mimic one or more functions of natural living cells. They can be made in a number of different ways. For example, a common method of creating artificial cells is to encapsulate isolated cellular systems into lipid membrane structures that form closed cell-like compartments known as liposomes. \[147, 51, 183, 184, 199, 263, 284\] Cellular systems such as membrane proteins, the components required for gene expression, or other biochemical pathways have all been studied in conjunction with artificial cells. \[8, 21, 104, 151, 225, 168\] For example, Merkle et al. and others have incorporated cytoskeleton proteins and structures inside liposome- or polymerosome-based artificial cells. \[168, 261, 255, 241, 200, 156\] Additionally, Noireaux et al. and others demonstrated successful expression of protein from DNA templates within artificial cells. \[183, 184, 157, 8, 104\] Artificial cells have also been used as a platform for studying membrane proteins and transmembrane transport. \[140, 64, 54\] Finally, there have also been studies replicating in vesicles the self-reproduction capabilities of living cells. \[234, 185, 132, 130\] Other methods, such as droplet-based encapsulation or even containment of biomolecules using solid structures patterned within microfluidic channels have all been used to form cell mimics. \[20, 121, 173, 232, 240\] "Top-down" approaches, entail the creation of a minimal cell by removing all components extraneous to life from an existing unicellular organism. \[85\] This was employed by Gibson et al. to introduce a foreign, chemically-synthesized genome into a minimal cell, thus creating an entirely new synthetic organism. \[83\] Regardless of approach however, the basic aim is to produce an artificial cell in which a minimal set of biological components can be contained, studied and eventually employed.

The principal benefit that artificial cells offer in regard to the study of cellular systems is a significant reduction in system complexity when compared to living cells. Whilst a living cell may contain thousands of interacting biological components and associated biochemical pathways, an artificial cell contains only components that have been introduced by design during its creation. From an investigative point of view, this reduction in complexity allows the experimentalist to study the behavior of different cellular components or systems in isolation and without interference from the rest of the cell. Moreover, artificial cells also have more practical applications. For example, artificial cells may be designed to function within chemical and physical environments that living cells could not tolerate, or to perform biochemical reactions with products that would normally kill living cells. \[199\]

The ability to form artificial cells has become a reality due to advances in two very different disciplines. The first is synthetic biology, which applies engineering principles to genetically modify an existing organism to perform new functions alien to its nature. \[92, 83, 9, 10, 32\] The second is the engineering of mesoscale chemical systems and soft matter chemistry, which enable the manipulation of liquid-phase materials to controllably form cell-size compartments such as liposomes, water droplets in oil, and other colloidal systems. \[33, 63, 121, 189, 190, 263\] In simple terms, artificial cells are formed by incorporating biological components and systems typically exploited in synthetic biology into such cell-size compartments. However, many technical challenges remain. In particular, the process by which cell-size compartments are formed is often incompatible with successful encapsulation of functional cellular components such as proteins, enzymes, or DNA. This is where microfluidic technology can be immensely helpful.

Microfluidics is a discipline in which fluids are manipulated in sub-millimeter
scale channels in order to perform chemical and biochemical reactions in a highly efficient manner.\[154, 268, 270\] By borrowing the manufacturing techniques typically employed in the semiconductor industry to fabricate micro-channels, a wide variety of lab-on-a-chip technologies have been developed. The reduced length scales inherent in microfluidic systems give rise to a unique set of advantages. For instance, the small channel dimensions result in a low Reynolds Number environment, in which flow conditions are laminar. This eliminates convective mixing, which allows for precise control of chemical reactions through diffusive mixing. Laminar flow conditions also almost always allow for the precise control of interactions between different fluid phases through carefully designed spatial confinement of fluid flow. This ability has been exploited to form, for example, nanoparticles or droplets within continuous flows, or in the study and manipulation of entire cell populations at the single cell level.\[250, 278\] In addition, the reduced volumes result in a significant reduction in the costs associated with the amount of sample used in experiments. Simultaneously, the effective increase in analyte concentrations due to spatial confinement combined with analytical methods such as optical and fluorescence microscopy result in increased analytical performance.\[211, 112, 110, 78\]

By making use of the properties inherent to microfluidic systems, new techniques can be developed to form cell-size compartments with successful encapsulation of functional cellular components. To a certain extent, a number of literature studies have already reported the use of microfluidic technologies to form artificial cells. As discussed in Chapter 2, a range of microfluidic techniques have been developed to form liposomes.\[259\] Artificial cells have also been formed using polymerosomes, which encapsulate biomaterials inside a polymer membrane, or even water-in-oil micro-droplets, albeit with limitations in function due to the non-aqueous nature of the external environment.\[34, 157, 156\]

Nevertheless, current strategies for forming artificial cells fail to replicate the highly crowded molecular environment typically encountered inside living cells.\[80, 151\] In the work presented in this thesis, artificial cells with a crowded molecular environment are formed using coacervate material. A coacervate is a structure formed by the self-assembly of water-soluble macromolecules into a separate phase suspended in water due to molecular charge interactions.\[49\] Because coacervates are typically formed by closely-packed poly-electrolyte molecules, their internal structure is thought to closely resemble the intracellular environment.\[48, 73\] DNA, poly-Lysine, and Poly(diallyldimethylammonium chloride) are only a few examples of many materials that form coacervates.\[50, 15\] Interestingly, it has also been proposed that DNA or RNA coacervates forming and self-replicating within porous rock cavities could have given rise to the function of DNA or RNA as a carrier of genetic information in living organisms.\[29, 17, 159, 186, 160, 123\] Because of these properties, coacervate materials may be used to form artificial cells that more closely resemble living cells.\[120, 272, 245, 228\]

The aim of this thesis is to develop tools and techniques that enable and facilitate the formation of artificial cells. In Chapter 3, a process of iteration and prototyping is described by which both a microfluidic device is developed and a compatible coacervate chemical system is identified. As an initial design, a conventional flow-focusing channel structure used for forming water-in-oil droplets was patterned in polydimethylsiloxane (PDMS) without any surface treatments beyond those performed in a typical soft lithography fabrication process. The chosen mi-
Microfluidic device design employs a flow-focusing structure without nozzle or channel expansion to form coacervate droplets in water. The coacervate chemical system is based on a combination of poly(diallyldimethylammonium chloride) and either carboxymethyl Dextran or adenosine triphosphate. Specifically, the bulk coacervate is dispersed as droplets in squalene oil, with and without surfactants, as well as in water. Due to the strongly interacting nature of the coacervate materials, the microfluidic channel surfaces are treated with perfluorooctyltrichlorosilane to prevent wetting and fouling of the channels.

In Chapter 4, a computational strategy for obtaining droplet size and size distribution information by analyzing microscopy images is described. The main challenge here is to overcome the difficulties associated with identifying droplet boundaries where contrast is low due to the small difference in the refractive index of the coacervate material and water. This challenge is compounded by the large volume of video and image data that must be processed. To this end, different techniques using both ImageJ (NIH, USA) image processing software and custom MATLAB (Mathworks, USA) programs are explored.

The outcomes of Chapters 3 and 4 are then leveraged in Chapter 5. Here, the formation of coacervate droplets using microfluidic tools is described in detail, making a careful comparison with conventional vortex methods that have been previously described in the scientific literature. It is observed that coacervate droplet populations formed using the microfluidic method exhibit a narrower size distribution and greatly enhanced stability when compared to those formed by the vortex method. In addition, Chapter 5 also describes the encapsulation of DNA within coacervate droplets. By simultaneously forming two distinct droplet populations containing different labeled DNA oligonucleotides and incubating them together, it is shown that there was no detectable exchange of DNA between adjacent droplets.

In Chapter 6, the issues associated with the introduction of small sample volumes into microfluidic devices are addressed. Specifically, a connector that interfaces external pumps and tubing with the microfluidic channels, but also incorporates a user-accessible chamber into which samples can be easily introduced, is designed and engineered. This solution is of particular relevance for the work described in Chapter 7, in which a commercial in-vitro transcription and translation (IVTT) kit is encapsulated within the coacervate droplets. Typical IVTT kit reaction mixtures often take up volumes smaller than 50 µl. Due to their fragile nature, as well as the necessity to maintain sterile conditions, the process of transferring the mixture from the test tube in which it was prepared to the microfluidic channel where it will be used is a critical part of the experiment. The connector described in Chapter 6 provided an effective solution to this problem.

Finally, the formation of artificial cells capable of performing transcription and translation of DNA into protein is described in Chapter 7. This is achieved by encapsulating the IVTT reaction mixture and a DNA plasmid encoding for the mCherry fluorescent protein inside the coacervate droplets. The presence of mCherry after incubation is verified by comparing fluorescence data obtained from the samples containing the plasmid encoding mCherry and control samples containing a plasmid encoding CALML3, a non-fluorescent protein. The results described in this chapter are a significant step towards developing artificial cells based on coacervate chemical systems, despite the facts that the amount of protein expressed is less than desirable and the presence of the IVTT reaction mixture has a destabilizing effect on
the coacervate droplets. Regardless, the progress described in this thesis represent significant steps towards the engineering of artificial cells.
Chapter 2

Microfluidic methods for forming liposomes

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Chapter 2: Microfluidic methods for forming liposomes

2.1 Introduction

Liposomes, or lipid vesicles, are self-assembled lipid structures in the shape of closed membrane capsules. (See Figure 2.1.) They can act as biomimetic compartments with a membrane that closely resembles that of living cells, encapsulating materials such as DNA, proteins, drugs, or other chemicals. They can be formed, manipulated, and modified in a variety of ways, and due to their similarity to cells and naturally occurring vesicles, they have been extensively studied. Tools for observing and characterising their properties are well developed, and moreover basic molecular dynamics simulations of their structure and behaviour have been refined in recent years.

First synthesised in a laboratory in the late 1960s, liposomes have become a standard tool in lipid and membrane science, drug delivery, compartmentalisation of biomolecules, as well as the formation of rudimentary artificial cells. Liposome-based artificial cells can be used to study cellular systems in isolation and in a simpler physical environment than in living cells. For example, Sackmann and coworkers, as well as other research groups, have studied the reconstitution of cytoskeletal components inside liposomes, while Noireaux et al. used liposomes to encapsulate an in-vitro transcription and translation reaction.

The usefulness of liposomes in such a wide range of applications dictates the im-

**Figure 2.1** (a) The structure of one POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) lipid molecule, showing the hydrophilic head and hydrophobic tail. POPC is a naturally-occurring lipid and is commonly used in the production of synthetic liposomes. (b) The heads and tails of the lipid interact to self-assemble into a membrane structure, and (c) a lipid vesicle.

Liposome structures have a wide range of applications in biology, biochemistry, and biophysics. As a result, several methods for forming liposomes have been developed. This review provides a critical comparison of existing microfluidic technologies for forming liposomes and, when applicable, a comparison with their analogous macroscale counterparts. The properties of the generated liposomes, including size, size distribution, lamellarity, membrane composition, and encapsulation efficiency, form the basis for comparison. We hope that this critique will allow the reader to make an informed decision as to which method should be used for a given biological application.
portance of developing robust production methods. Over the last three decades, a variety of macroscale methods have been developed for their production. These include extrusion through porous membranes, electroformation, freeze-drying, hydration or swelling, double emulsions (water-in-oil-in-water, or W/O/W), and budding to name a few. Macroscale techniques, using fluidic volumes on the order of millilitres to hundreds of microlitres, are the most common way to form liposomes. However, such methods afford limited process control and are often accompanied by features such as poor reproducibility and the inefficient use of materials and reagents. In order to address these difficulties, microfluidic technologies have recently been developed either as adaptations of macroscale methods or as completely new techniques. "Microfluidic" refers to methods in which fluid handling procedures are, in the current context, performed in a geometrically constrained volume, typically defined by sub-millimeter length scales and low Reynolds Numbers. The adoption of microfluidic techniques allows the integration of laboratory procedures into planar chips or other small devices, reducing reaction volumes and the associated cost of chemical and biological experimentation by several orders of magnitude, while simultaneously increasing throughput and analytical performance. This review will assess the merits of current techniques of liposome production and material encapsulation that are either entirely or partially performed within microfluidic devices, and appraise them according to application-specific criteria. As background for this discussion, a description of the desirable liposome and method characteristics follows.

2.1.1 Liposome and Method Specifications

Membrane composition and lamellarity. Vesicles have been made using many different surfactants besides phospholipids. The choice or combination of surfactants greatly affects the properties of the membrane, such as shape, thickness, stability, elasticity, and permeability, as well as compatibility with biological materials.

Naturally-occurring phospholipids such as L-α-phosphatidylcholine (Egg PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) have the advantage that they are more likely to be compatible with membrane-bound proteins. This is important since pore forming peptides such as α-Hemolysin allow for transport of molecules that would otherwise not be able to cross the membrane. This greatly extends the functionality of vesicles as bioreactors or artificial cells.

One aspect of membrane composition that must be considered when producing vesicles is the presence of residue solvents or oil inside the lipid bilayer as a result of the formation process. Most vesicle preparation methods involve the dissolution of lipids in an organic solvent (typically chloroform), with subsequent removal of this solvent. The solvent is usually removed by an evaporation, vacuum desiccation, or lyophilisation step prior to addition of the oil or buffer that will be used to prepare the liposomes. In some methods, water droplets are formed in oil and later transformed into liposomes by, for example, droplet transfer or by use of double emulsion templates (see sections 2.2.5 and 2.2.8). In the former, the oil may partition
into the liposome bilayer during transfer, while in the latter, the removal step may not be completely efficient.

The unidentified or uncharacterised presence of foreign material within the membrane introduces an uncontrolled experimental factor, which may compromise the quality or reproducibility of results if not properly accounted for. For example, a membrane-spanning protein may adopt a different conformation if an organic solvent is present inside the lipid bilayer and thus behave unnaturally. It has also been shown that residual solvent affects mechanical or physical characteristics of the membrane such as rigidity or elasticity, parameters that have been shown to affect protein behaviour. \[164, 26, 149, 25, 126\] Moreover, since some oils or solvents may pool in specific regions of a membrane (oils, for example, eventually float to the top),\[82\] protein behaviour may vary depending on whether it is located in the oil-containing portion of the membrane or not.

Another important aspect of membrane structure is its lamellarity, a term which defines the number of bilayers by which it is composed. A membrane composed of a single bilayer is unilamellar, while a membrane composed of many bilayers is multilamellar (see Figure 2.2). Unilamellar vesicles resemble living cells more closely both in structure and function, and are typically used in membrane protein studies.\[207, 167, 218, 18\] Multilamellar vesicles, on the other hand, are primarily used for encapsulating substrates to be released upon disruption of the membrane at a later time, such as in drug delivery, or when membrane properties other than stability have no effect, such as the use of magnetic-particle containing vesicles as MRI contrast agents.\[252\] It is also worth noting that existing methods cannot control the number of bilayers in multilamellar vesicles.

**Figure 2.2** The 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.

**Size and size distribution.** Many vesicle preparation methods result in polydisperse vesicles — that is, a vesicle population containing vesicles of different sizes.\[205\] Although different sized vesicles may have different applications, most applications require vesicle populations to be monodisperse.\[111\] This is because variability in size causes variability in other application-critical factors such as stability, the amount of material encapsulated or bound to the membrane, membrane curvature, or rates of transport across the membrane.\[52, 219, 169, 111\] As such,
monodispersity is a key criterion in determining the usefulness of a technique, and one of the main motivations for using microfluidic systems to form liposomes.

Vesicles can range in diameter from a few tens of nanometres to a few hundred micrometres. A commonly used classification system is summarised in Figure 2.2.

Stability. The stability of vesicles depends not only on size and membrane composition, but also on properties of the internal and external phases such as osmolarity, pH, salinity, and temperature.\textsuperscript{16, 86, 238} Since all of these properties are in some way affected by the method of formation, the method will also have a strong effect on vesicle stability. Physical instability of vesicles can manifest itself in the form of lysis, aggregation, coalescence, or budding (Figure 2.3, 265).

Vesicles are stable on timescales ranging from a few minutes to a few weeks. Indeed, Tan et al. reported unilamellar vesicles formed by their emulsion transfer process lasting for more than 26 days.\textsuperscript{244} Long-lasting vesicles allow for greater flexibility in their use after preparation, and thus greatly extends their breadth of applications. For example, liposomes used in drug delivery or as containers for MRI contrast agents typically need to be robust enough to survive on the skin or in the blood stream long enough to carry out their function, which may be anywhere from a few hours to a few days. Vesicles used for membrane protein studies or as artificial cells, on the other hand, must be able to withstand the experimental procedure. That may involve changing physical conditions, such as applying electric fields or chemical concentration gradients. Microfluidic techniques have the potential to create long-lasting vesicles due to a high degree of process control. For example, temperature, pH, salinity, osmolarity, fluid mechanical forces, and vesicle size can all be tuned very precisely using microfluidic tools. Moreover, all of these factors can be controlled during vesicle formation as well as during their subsequent manipulation or incubation.

If stability is a primary concern, techniques for enhancing vesicle lifetime after preparation have also been reported. For example, Angelini et al. improved stability by depositing PEGylated nanotubes on a membrane surface of charged multilamellar liposomes through charge interactions,\textsuperscript{11} while Li and Palmer used actin polymerisation along the internal surface of the membrane.\textsuperscript{139} The use of cross-polymerisable synthetic lipids to form the membrane, followed by initiation of polymerisation after liposome formation, have also been shown to greatly improve stability.\textsuperscript{134, 135, 276} Although modifications like these are not ideal in many applications, they do result in a significant improvement in liposome stability. Indeed, Lee et al. carried out stability tests on vesicles prepared by electroformation, and found that they were strong enough to withstand electroporation and the mechanical stresses of microfluidic systems without reinforcing treatment after formation.\textsuperscript{137}

Usability. It is important to consider usability when comparing technologies. All else being equal, it is often the simplicity or ease of use of a method that will decide how widely it is adopted. Microfluidic tools can often be difficult to set up or operate, especially those involving many fluid inputs, different fluid phases, or
complicated flow control. In this review, we evaluate the "usability" of each method by considering process setup and reusability, input-fluid handling, flow control, and whether the process can withstand fluctuations in operating conditions or alterations to operational parameters.

**Encapsulation efficiency.** Encapsulation efficiency is defined as the ratio of the concentration of material encapsulated to that found outside the vesicle after formation. Encapsulation efficiency can be inherently poor if the method does not separate the internal and external phases throughout the formation process. Stability also affects encapsulation efficiency, as ruptured or leaking vesicles release their contents into the external solution. One of the main purposes of encapsulation of materials into vesicles is to isolate them from other components in an experiment. A process with a low encapsulation efficiency clearly defeats this purpose.

Several methods exist for determining encapsulation efficiency. This is typically done by first separating loaded vesicles from the sample, followed by their disruption and then bulk quantification of the previously entrapped material by size exclusion chromatography or other separation techniques. NMR spectroscopy can also be used in some cases, with the advantage that it does not require lysis of the liposomes or removal of the untrapped sample from the external medium. Finally, individual vesicles can also be characterised using confocal single-molecule detection. It should be noted that finding the true encapsulation efficiency value is a non-trivial problem, due to the difficulty in distinguishing between internal and external material, as well as material adsorbed to the membrane.

In practice, the significance of the encapsulation efficiency of a method can be interpreted in different ways. The fact that all target material is contained within the liposome membrane does not necessarily mean it is functional or can be delivered. For example, the activity of a protein may be compromised during the formation process such that only a fraction of the sample is functional after encapsulation. Methods involving high temperatures, large or rapid temperature changes, phase changes, high shear stress, electric fields, or incompatible chemical environments may disrupt or inactivate fragile biological samples. Therefore, when choosing a method...
for a particular application, one should consider that descriptions of encapsulation efficiency do not necessarily account for the function of the material being enclosed.

The problem of low encapsulation efficiency can often be worked around by employing techniques such as dialysis, filtration, or column separation of the resulting vesicles after they are formed. Such methods can remove most if not all of the material that has not been encapsulated. However, this clearly adds to process complexity, wastes material, and may compromise vesicle stability.

Another option is to use micro-injection to introduce a desired material into an already-formed liposome. This requires the trapping of a liposome and careful disruption its membrane with a microneedle. The reliance on individual trapping and disruption of a vesicle membrane for injection of material is a major drawback in terms of throughput, however. Furthermore, due to the highly sensitive nature of the trapping and injection operations, it is likely that liposomes of different sizes, composition, or with different contents will respond differently to the procedure.

2.2 Microfluidic Liposome Formation

2.2.1 Electroformation and hydration

Electroformation is probably the most common method for vesicle production. The process was first described by Angelova and Dimitrov in 1986. It involves spreading lipids dissolved in an organic solvent such as chloroform on the surface of a planar electrode, evaporation of the solvent by vacuum desiccation to form a dry phospholipid film, immersing the coated electrode in an aqueous solution, and finally applying an electric field across the lipid film and surrounding buffer. The lipids interact with the aqueous solution and electric field by ”peeling off” the electrode surface in layers and self-assembling into giant but polydisperse, multilamellar vesicles. Later refinements of the method have allowed for unilamellar vesicles to be consistently produced.

One drawback of the first implementations of the electroformation method was the requirement for low salt concentrations in the buffer. Since most proteins require high salt concentrations in order to function properly, this made it very difficult to encapsulate active proteins using electroformation. Recent refinements to the technique have overcome this limitation and produced liposomes by electroformation using physiologically relevant salt concentrations. There is also a concern that the electric field applied during the vesicle formation process may disrupt proteins that contain charge. Though this may be the case, Girard et al. and Aimon et al. have shown functional proteins incorporated through electroformation.

The electroformation method has been successfully implemented within microfluidic channels by Kuribayashi et al. The process was a simple modification of the conventional method in which they coated an electrode with a film of dry lipids and then placed two slits made of silicone rubber over it. The slits were then covered with a second electrode plate, thus forming microfluidic channels through which
buffer could flow. The remainder of the process was identical to the conventional method, and resulted in a polydisperse population of liposomes.

In a further development, LeBerre et al. showed that vesicle size and size distribution could be controlled by artificially fragmenting the lipid film through patterning of the electrode surface with elevated microstructures (Figure 2.4). This limited the film segments to the size similar to that of the microstructures. Since these were small, fragments did not break apart when peeling, resulting in vesicles with surface areas equal to that of the patterned structures. This was a significant improvement over the standard electroformation method because it allowed for the formation of monodisperse vesicles.

Figure 2.4 The experimental setup for the electroformation of giant phospholipid vesicles on a silicon substrate (a), as demonstrated by Le Berre et al. (b) A section showing only the Silicon electrode, patterned with an array of micropillars 170 nm in height and with pitch sizes $p$ of 7, 15, and 60 $\mu$m. Adapted with permission from 136. Copyright 2008, American Chemical Society.

Hydration is another popular technique used for the formation of vesicles. The process is similar to that of electroformation, except that no electric field is applied. Instead, a vortex or controlled flow of aqueous buffer solution is passed over the coated surface. The shear stress acting against the lipid layers causes them to peel off, break, and self-assemble into liposomes. However, since there is no control over "peeling" and "fragmentation", the resulting vesicles are polydisperse and multilamellar. In addition, the hydration method is extremely sensitive to the type of phospholipids used and physical conditions such as the osmolarity of the buffer solution, temperature, and pH. For this reason, its use in applications where encapsulation is intended is limited, because the materials being encapsulated will often affect the formation process.
The use of hydration in a microfluidic device has been described by Lin et al. in the formation of microtubes, and under certain conditions, connected networks of microtubes and vesicles. This method was similar to that of Kuribayashi et al. in that the dry lipid film was formed on a glass slide before being covered by a block of polydimethylsiloxane (PDMS) with microfluidic channels patterned on the surface. The microtubes and vesicles were formed by continuous flow of an aqueous buffer solution over the lipid film. The resulting liposomes, however, were not unilamellar or monodisperse (Figure 2.5). Nonetheless, the ability to accurately control the buffer flow rate inside the microfluidic channel allowed them to find the optimum conditions for obtaining different types of structures — a notable advantage in comparison to the macroscale hydration method.

The processes of electroformation and hydration do not physically separate the solution that will eventually end up inside the vesicles from that which will be outside. Materials to be encapsulated in vesicles are dissolved or suspended in the aqueous solution in which the vesicles are formed. There is no way to isolate the contents of a vesicle from its external environment during the formation process. As a result, these two methods have a low encapsulation efficiency. This limitation is also present when the process is carried out inside a microfluidic channel. A second limitation, related to the usability of the procedures described by LeBerre et al. and Lin et al., is that every iteration of the process requires the deposition of a lipid film on a substrate followed by several hours of desiccation by vacuum before assembling the microfluidic channels. This prevents the continuous operation of the device, since lipids cannot be replenished during operation.

2.2.2 Extrusion

Extrusion of vesicles is typically carried out after their formation to modify properties such as lamellarity, size, and size distribution. The process was first described by Olson et al. in 1979. It consists of passing a lipid or vesicle solution multiple times through the pores of a polycarbonate membrane or other mesh of small apertures. Extrusion makes vesicles unilamellar and monodisperse, with diameters controlled by the size of the pores, the pressure applied during the extrusion process, and the number of times the solution is passed through the membrane. For example, Jousma et al. showed that a single pass through a membrane with a pore size of 200 nm resulted in liposomes with diameters of 320 ± 50 nm, while five passes yielded vesicles 143 ± 10 nm in diameter.

The basic concept has been applied on a microfluidic chip by Dittrich et al. in the actual formation of liposomes (Figure 2.6). The technique involved the use of a free-standing silicon nitride membrane, on which a lipid film coating was produced by desiccation, covering apertures with diameter of approximately 3.5 microns. Aqueous flow around the silicon-nitride membrane and through the apertures produced vesicles and microtubules, depending on flow speed. The resulting vesicles, however, were polydisperse. Nonetheless, this issue may be addressed if the method is modified such that the vesicles are passed through the membrane multiple times. Unfortunately, the authors provide no discussion of lamellarity or the encapsulation efficiency of their method. But since there is no safe isolation between the solution to be encapsulated and that in which the vesicles will be contained in, it is likely
Figure 2.5 Microtubule formation by hydration in a microfluidic channel, by Lin et al. (a) The DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) microtubules formed in a PDMS channel are stable and controllable. (b) The method sometimes also generated nanotube-vesicle networks. Vesicles are connected by nanotubules, allowing for material exchange between them. (c) A fluorescence image of a self-assembled network of lipid tubules and vesicles. Adapted with permission from reference 142. Copyright 2006, Elsevier.
that the encapsulation efficiency is low. By way of comparison, the work by Hope et al. reported an encapsulation efficiency of only 30% [95]. Similar to the microfluidic implementations of the electroformation and hydration methods, the coating of the free-standing silicon nitride membrane by phospholipid film adds complexity (in spite of vacuum desiccation not being necessary) and limits continuous use.

### 2.2.3 Flow focusing

The microfluidic flow-focusing method of vesicle production, demonstrated by Jahn et al. [108], has no analogous protocol on the macroscale. This elegant technique consists of a central flow of a phospholipid-containing alcohol solution being intersected on either side by an aqueous solution. As the three flows merge into a main microchannel, the alcohol diffuses into the aqueous solution. As the alcohol dilutes past a critical concentration, the lipids spontaneously self-assemble into liposomes. Figure 2.7 shows a schematic of the formation process. The vesicles produced have diameters between 50-150 nm, depending on flow rates, and are monodisperse [106, 107, 108]. It is noted that large vesicle sizes have not been formed using this method.

Microfluidic tweezing, described by Lin et al. [143] is somewhat similar to flow focusing, and is also only feasible within a microfluidic format. The technique was developed for the creation of membrane tubes, but it may be possible to adapt it for the formation of vesicles, or to form vesicles from the tubes [269]. The technique involves dissolving phospholipids in water inside a chamber that is connected to a T-junction through a small aperture. The centre channel of the T-junction is oriented away from the aperture. Two fast flows arrive into the T-junction from opposite
directions, and turn into the central channel, flowing away from the aperture. As the flows converge and turn, they pull the phospholipid solution through the aperture, forming membrane tubes.\[143, 269\] However, the vesicles resulting from this method are not monodisperse or unilamellar.

Importantly, the flow focusing method can be used for high-throughput vesicle production.\[99, 251\] The reaction solutions are prepared separately and stocked, and can be loaded into containers mounted onto the microfluidic device, allowing for continuous operation over long periods of time. There is no discussion of encapsulation efficiency in the work by Jahn et al. However, since membrane formation depends on a continuous, diffusive exchange of solvent into an aqueous phase, it is likely that some of the material to be encapsulated escapes to the exterior of the membrane before it forms. Furthermore, the solution that ends up inside the liposome also contains alcohol, with alcohol molecules being partitioned into the membrane. This means that the former may affect the behaviour of the encapsulated material of interest, and the latter will affect membrane properties such as stability.

### 2.2.4 Pulsed jetting

Pulsed jetting is a very elegant technique, first described by Funakoshi et al.\[82\] It mimics the action of blowing soap bubbles through a loop supporting a thin soap film. The method consists of creating a bilayer membrane by carefully contacting two lipid-stabilised water droplets,\[81\] and using a micro-nozzle or micro-pipette to shoot small jets of aqueous solution across it. The jetted volumes are wrapped by the membrane as they pass through, and as their momentum carries them further from the aperture, the membrane pinches off, forming a vesicle. Figure 2.8 provides a simple illustration of the process.

This method forms unilamellar, monodisperse giant vesicles, though under certain operation regimes, a smaller satellite vesicle is also formed.\[82, 230\] The size
of the vesicles produced can be controlled by the jet dispensing time. The encapsulation efficiency is reportedly also very high, with effectively all the material jetted across the membrane being encapsulated. Indeed, efficiency is only lost if liposomes rupture after forming. However, it is not clear whether all biological material can survive the high shear stress of the jetting process without damage. It is possible that, although encapsulated within the liposomes, some proteins or other biomolecules are not functional after formation.

The technique was used by Funakoshi et al. to compartmentalise both DNA and Jurkat cells (though no discussion was provided in regard to cell viability). More recently, Stachowiak et al. used the same technique to encapsulate 500 nm nanoparticles as well as the pore-forming protein α-hemolysin, and, in continuing work, refined the apparatus so that an inkjet system was used to control liposome formation (Figure 2.9). One problem with this technique is that a residual amount of the solvent used in the phospholipid solution is found inside the membrane, in between the monolayers. Kirchner et al. determined, using Raman spectroscopy, that the decane solvent used in membrane preparation is present throughout the membrane, forming a layer up to tens of nanometres thick. This is almost certain to affect the behaviour of some membrane-bound proteins and the passive diffusion of other materials across the membrane. Stachowiak et al. do not address this issue in their work, so it is not clear whether this problem can be
Figure 2.9 Blowing vesicles by pulsed-jetting, after Stachowiak et al. (a) A schematic of the device and apparatus assembly. A micropipette is inserted close to a lipid membrane, and a piezo-electric actuator is used to generate pulses of fluid. (b) The pulsed jet crosses the membrane, forming vesicles. (c) A micrograph of the resulting liposomes (scale bar: 100 µm, and (d) the resulting diameters of vesicles formed in seven trials. Reprinted with permission from reference 230. Copyright 2008, The National Academy of Science.

solved.[231, 230, 206] Although this problem has not been reported previously in other techniques, it is likely to be a factor in other vesicle formation methods.

Two major shortcomings of this method are that it is non-trivial to set up, and very sensitive to both the operating conditions and the materials being used. Indeed, a micromanipulator stage is needed to position the micro-nozzle in the right location with respect to the membrane. Since the droplets that form the interface bilayer are created by manual pipetting, it is very difficult to reproduce their exact location. This means that the micro-nozzle must be repositioned every time the apparatus is used. Also, since liposome formation depends on the membrane being deformed by the momentum of a fluid jet, the whole process is highly dependent on the viscosity of the solutions, operating temperature, and membrane composition. As a result, each time the materials being used are changed (lipids, and internal and external phases), it is likely that the protocol must be modified in some way. Another possible problem with the pulsed-jetting method is the high shear stress experienced by the sample during the jetting process. This may disrupt large or delicate proteins or other biomolecules, limiting the applications of this technique. However, to our knowledge, the consequences of this shear stress have not yet been investigated.
2.2.5 Double emulsion templates

Vesicle preparation by solvent removal from double emulsion templates is a high-throughput method that produces monodisperse giant unilamellar vesicles with high encapsulation efficiencies. It was first described by Shum et al. [226]. Briefly, the method consists of the preparation of a lipid-stabilised water-in-oil-in-water emulsion in glass microcapillaries [258, 41] (Figure 2.10) or PDMS channels, [47] from which the oil phase is evaporated with the assistance of an organic solvent mixture of toluene and chloroform. [76, 226] As the oil phase is removed, the lipid monolayers at the internal and external oil-water interfaces come together into a bilayer, thus forming a liposome. Unfortunately, not all of the solvent is removed during the evaporation step. The toxicity of toluene and chloroform and their ability to dissolve membranes greatly limits the breadth of applications of vesicles formed using this method. However, it may be possible to remove the oil phase without the use of organic solvents: Lorenceau et al. used this same method without solvents to produce vesicle-like polymerosomes, where the membrane was composed of diblock copolymers. [150]

Figure 2.10 Double emulsion templates, by Shum et al. (a) A glass microcapillary device forming a phospholipid-stabilised W/O/W double emulsion. (b) The collected double emulsion as seen through a microscope. The droplets have an aqueous centre surrounded by a phospholipid suspension. Reprinted with permission from reference [226]. Copyright 2008, American Chemical Society.

A very similar method was developed by Tan et al., who used a microfluidic device and oleic acid to produce a lipid-stabilised water-in-oil emulsion, and transferred it into an aqueous mixture of ethanol and water. [243] The oleic acid phase dissolved in the ethanol, forcing the phospholipids to rearrange into a bilayer membrane, thus forming a vesicle. Such vesicles are unilamellar, monodisperse, of controllable size, and very stable — they were shown to last for more than 26 days — and the procedure is simple and fast to implement. Figure 2.11 provides an illustration of the method. More recently, the same group demonstrated the entire process performed inside a microfluidic device. [249] Since in this case the solvent used for removing the oleic acid from the membrane is ethanol, the method is much more biocompatible than those previously described.
Although they did not discuss encapsulation efficiency in detail, Tan et al. demonstrated that their method can successfully encapsulate nano-sized proteins, microbeads, and HeLa cells. They also stated that encapsulation efficiency is variable, and will depend on the flow rate during the emulsification process and on the concentration of alcohol in the external aqueous mixture when the droplets are transferred. Their method separates the internal aqueous solution from the external one throughout the initial stages of the process, and so it is likely that encapsulation efficiencies are good. The final stage is where leakage may occur, i.e., when droplets are transferred to the aqueous mixture of water and ethanol and the phospholipids rearrange into a bilayer. At this point, rupture and mixing of droplets with the external phase may occur before the lipids rearrange into a bilayer membrane. Loss of efficiency due to the emulsification step is likely to occur because the size of vesicles affects their stability, while the formation rate affects the lipid concentration at the oil-water interface. Hence, if the droplets are too small or form too quickly (not giving enough time for lipids to stabilise the oil-water interface), the resulting vesicles are more likely to rupture and release their contents into the external medium, thus lowering the overall encapsulation efficiency.

### 2.2.6 Ice droplet hydration

The method of "lipid-coated ice droplet hydration" was developed by Sugiura et al., and results in monodisperse, giant multilamellar vesicles, with a low encapsulation efficiency (around 30%). The technique uses a microfluidic device to form a monodisperse water-in-oil emulsion stabilised by span-80 and stearylamine. This is then cooled such that the water droplets freeze. The droplets are then removed from the supernatant, after which surfactants are replaced by phospholipids. Next, the oil phase is evaporated, while the water droplets are still frozen, and an aqueous phase is added. The vesicles can then be extruded through a polycarbonate membrane to form unilamellar vesicles. Figure 2.12 illustrates the procedure.

The low encapsulation efficiency and the fact that resulting vesicles must be
Figure 2.12 Vesicle preparation by ice droplet hydration in a microfluidic device, by Sugiura et al. (a) A schematic of the microfluidic device, and (b) the process of ice droplet hydration for surfactant replacement. Reprinted with permission from reference 236. Copyright 2008, American Chemical Society.
extruded in order to have unilamellar membranes are major drawbacks, especially since simpler methods exist which are less cumbersome and yield similar or better results.

2.2.7 Transient membrane ejection

An elegant method for forming vesicles that has only been demonstrated using microfluidic technology is that of transient membrane ejection, first demonstrated by Ota et al. (Figure 2.13). The technique consists of forming a lipid bilayer at a microchannel junction, and then disrupting that membrane to form vesicles. In this work, a laser is used to heat up a gas bubble in the aqueous solution on one side of the membrane. The resulting expansion of the bubble is enough to displace the fluid and push against the lipid bilayer to deform it. The bilayer then breaks off into liposomes in a way similar to the pulsed-jetting described in section 2.2.4. A modification of the technique by Kurakazu et al. uses pneumatic valves to decrease the volume of the channel instead. The main advantages of this method are that it is fully integrated within a microfluidic platform, and results in monodisperse unilamellar vesicles with tunable size. However, the membrane used to form vesicles is eventually depleted, and needs to be replaced. This is achieved by pumping a lipid-containing oil phase into the junction, where a lipid-stabilised oil-water interface forms. The oil phase is then pumped out by a second aqueous phase. As the two oil-water interfaces come into contact, a bilayer membrane forms. This depletion and replacement of the membrane imposes a complication: the user must deal with intermittent oil phases.

Figure 2.13 The transient membrane ejection method invented by Ota et al. (a) Water, oil containing lipids, and water are sequentially passed through a main microfluidic channel flanked by many small chambers. (b) The first water phase enters the chambers, pushing the air out through the PDMS. (c) As the lipid-containing oil passes through the main channel, it forms a lipid-stabilised oil-water interface at the entrance of the chambers, enclosing small volumes of water in them. (d) As the second water phase displaces the oil, the two lipid-stabilised water-oil interfaces come into contact at the chamber entrance, forming a lipid bilayer membrane. (e–f) A gentle flow generated from the chamber out into the main channel displaces the membrane, causing it to bud off into vesicles. (h) This gentle outward flow is generated by the controlled expansion of an optically generated microbubble. Reprinted with permission from reference 190. Copyright 2009, WILEY-VCH Verlag GmbH.
separating batches of liposome samples. This membrane replacement action (and resulting two-phase output) along with the pneumatic or light actuation result in significant usability shortcomings in this method.

Ota et al. state that the lipid membrane produced by their method contains oil residue between the two lipid layers, though the discussion they provide on this topic claims that they did not experience problems related to this issue.\textsuperscript{[190]} Nonetheless, the membrane pore protein $\alpha$-hemolysin was successfully incorporated into the membrane, and an \textit{in-vitro} gene-expression system based on \textit{E. coli} cell-extract was encapsulated and used to express green fluorescent protein.

Finally, it is likely that encapsulation efficiencies are high, since the internal and external aqueous phases are kept separate by the lipid membrane or oil phase at all times.

2.2.8 Droplet emulsion transfer

The droplet emulsion transfer method has been described previously on the macroscale by Pautot \textit{et al} (Figure 2.14).\textsuperscript{[194]} It consists of stabilising a water-in-oil emulsion (formed by vortexing or pipetting) with phospholipids, and then transferring the droplets to an aqueous medium. As the droplets cross the interface between the oil and aqueous phases, they pick up a second lipid layer, forming a unilamellar bilayer membrane.\textsuperscript{[195, 194, 91, 275]} This method was used by Noireaux \textit{et al.} to carry out vesicle encapsulated gene expression, and proposed as a first step towards artificial cells.\textsuperscript{[183]}

Since the original macroscale implementation of this method by Pautot \textit{et al.} carried out the emulsification process by vortexing, the resulting vesicles were not monodisperse.\textsuperscript{[194]} However, emulsification can be performed by other means. For example, the method has been significantly improved by using a microfluidic device to form the droplet emulsion, which is then collected and transferred in bulk across a pre-formed oil-water interface.\textsuperscript{[90, 97, 180]} The use of a well-established microfluidic droplet formation technique eliminated the polydispersity issues experienced in the macroscale implementation.

Another droplet emulsion transfer method, this time fully integrated into a microfluidic device, was described by Matosevic \textit{et al.}\textsuperscript{[101]} In this work, they formed lipid-stabilised water-in-oil droplets which were then displaced by a ramp-shaped obstacle into a co-flowing aqueous stream. When pushed from the organic into the aqueous phase, the droplets picked up a second monolayer of lipids at the oil-water interface (see Figure 2.15). This displacement stage, however, proved to be too disruptive, causing 95-99% of the droplets to burst as they crossed the interface. As a result, encapsulation efficiency (which should be close to 100% dropped to 83%), yield, and throughput were compromised. This method, however, seems very promising if the displacement step can be optimised to prevent droplets from bursting at the interface.

A major concern with the emulsion transfer method is the likely presence of oil residue inside the lipid bilayer after vesicle formation. Since the process involves the transfer of droplets through an oil phase, it is reasonable to suspect contamination...
Chapter 2: Microfluidic methods for forming liposomes

Figure 2.14 Droplet emulsion transfer method for vesicle formation, developed by Pautot et al. Water droplets are formed in a lipid-in-oil suspension, and then passed through a oil-water interface which is itself stabilised by phospholipids. As the droplets cross the interface into the aqueous phase, they pick up a second layer of phospholipids, forming a bilayer membrane, and thus become vesicles. Reprinted with permission from reference 195. Copyright 2003, The National Academy of Sciences.

of the final liposome membrane. As discussed in Section 2.1.1, the presence of oil in the lipid bilayer may have a significant impact in the behaviour of the membrane and associated proteins. It is therefore important to address this matter.

In their work first describing emulsion transfer, Pautot et al. performed a thin-layer chromatography assay on the final vesicles comparing them to pure lipids and pure oil samples, and could not detect the presence of oil in the vesicle sample.[194] This indicated that if any oil was present, it was less than 5% of the membrane (that being the sensitivity limit of their detection technique). They also suggested that squallene, a complex mixture of long-chain alkanes, is a good candidate for forming oil-free liposomes by emulsion transfer because it is immiscible in fully hydrated bilayers above the phase transition temperature.[164, 194]

However, Kubatta et al. showed that a large amount of oil was clearly visible in the membrane of vesicles formed by emulsion transfer.[124] It should be noted, though, that the vesicles were between 3 and 5 millimeters in diameter — much larger than typical liposomes. On the other hand, Abkarian et al. claimed that their vesicles formed by emulsion transfer decreased in volume in a time-scale comparable to electroformed ones when exposed to osmotic overpressure, and also that their membranes showed fluctuations with similar amplitude.[2] Additionally, they could not detect any membrane defects using light microscopy at 300 nm resolution, and they also managed to incorporate the pore-forming protein α-hemolysin.

It is clear that the matter of oil residue presence in the membrane of liposomes
Conclusions

Figure 2.15 Fully-integrated microfluidic droplet transfer method for vesicle formation, developed by Matosevic et al. Lipid-stabilised water-in-oil droplets are formed by flow-focusing of water into an oil stream at a microfluidic junction, and then forced across an oil-water interface by a ramp-shaped barrier. Similar to the macroscale method, the droplets pick up a second layer of lipids as they cross the interface, forming a bilayer membrane, and thus becoming liposomes. Scale bars: 100 µm. Reprinted with permission from reference 161. Copyright 2011, American Chemical Society.

formed by emulsion transfer is not resolved. Different implementations and different materials appear to yield different results, and when the issue is addressed, it is often not done satisfactorily. However, emulsion transfer can circumvent many of the difficulties encountered in other liposome formation methods, such as low encapsulation efficiency, unphysiological conditions, and polydispersity. Therefore, it would be of great utility if future work published with this method employed more sensitive techniques such as Raman spectroscopy or small-angle X-ray scattering (SAXS) to analyse the membrane of their liposomes. [68, 196, 119]

2.3 Conclusions

In the last ten years there have been many new developments in liposome formation technology. Most notably, macroscale methods have been transposed to the microscale while entirely new methods that are only possible with microfluidic technologies were also developed. This happened because of the many benefits associated with reducing the dimensions of a fluidic process down to the microscale, such as improved throughput and analytical performance, and a high degree of control over operational parameters.

While progress has been rapid, a method that is capable of addressing all the requirements of the various applications of liposome technology has yet to be realised. For each method there is a trade-off. For example, hydration, electroformation,
Table 2.1 Liposome formation method comparison

<table>
<thead>
<tr>
<th>Method</th>
<th>Lamellarity</th>
<th>Usability</th>
<th>Solvent in membrane?</th>
<th>Encapsulation efficiency</th>
<th>Size range</th>
<th>Size distribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electroform or hydration</td>
<td>Multi- and unilamellar</td>
<td>Medium</td>
<td>No</td>
<td>Low*</td>
<td>LUV-GUV</td>
<td>Polydisperse</td>
<td>129, 143</td>
</tr>
<tr>
<td></td>
<td>Unilamellar</td>
<td>Medium</td>
<td>No</td>
<td>Low</td>
<td>SUV-UV</td>
<td>Monodisperse</td>
<td>142, 136</td>
</tr>
<tr>
<td>Extrusion</td>
<td>Unknown</td>
<td>Easy</td>
<td>No</td>
<td>Low*</td>
<td>Monodisperse</td>
<td>Monodisperse</td>
<td>108, 143</td>
</tr>
<tr>
<td>Flow focusing</td>
<td>Unknown</td>
<td>Hard</td>
<td>Yes</td>
<td>High*</td>
<td>GUV</td>
<td>Monodisperse</td>
<td>82, 230</td>
</tr>
<tr>
<td>Pulsed jetting</td>
<td>Unknown</td>
<td>Medium</td>
<td>Unknown</td>
<td>High</td>
<td>LUV-GUV</td>
<td>Monodisperse</td>
<td>150, 233</td>
</tr>
<tr>
<td>Double emulsion templates</td>
<td>Unknown</td>
<td>Medium</td>
<td>Unknown</td>
<td>High*</td>
<td>GUV</td>
<td>Monodisperse</td>
<td>107, 269</td>
</tr>
<tr>
<td>Ice droplet hydration</td>
<td>Unknown</td>
<td>Medium</td>
<td>Yes</td>
<td>High</td>
<td>GUV</td>
<td>Monodisperse</td>
<td>231, 200</td>
</tr>
<tr>
<td>Transient membrane ejection</td>
<td>Unknown</td>
<td>Easy</td>
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<td>High*</td>
<td>LUV-GUV</td>
<td>Monodisperse</td>
<td>226, 76</td>
</tr>
<tr>
<td>Droplet emulsion transfer</td>
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<td>High*</td>
<td>GUV</td>
<td>Monodisperse</td>
<td>236, 131</td>
</tr>
</tbody>
</table>

* As discussed in Sections 2.2.1 and 2.2.4, it is possible that the electroformation and pulsed-jetting processes may disrupt the function of encapsulated material.
Conclusions

and extrusion result in excellent quality liposomes, but have very low encapsulation efficiency. Of those that achieve high encapsulation efficiencies, pulsed jetting and transient membrane ejection are difficult to implement, while double emulsion templates and ice droplet hydration are not very compatible with biological processes. Finally, droplet emulsion transfer, in spite of being a promising technique, has yet to be implemented in a reliable fashion.

Due to their qualities and drawbacks, different methods remain useful only in different application niches. For membrane protein biology membrane quality is paramount, while encapsulation efficiency or volume characteristics are less important. In such applications, electroformation and extrusion are well suited for vesicle production and hence their immense popularity. However, when liposomes are used as cell models, encapsulation efficiency and monodispersity are just as important as membrane quality. This demand has resulted in the invention of the other techniques, such as pulsed jetting, transient membrane ejection, and droplet emulsion transfer. Finally, the use of liposomes as enclosures for drugs or other chemical species tends to require specific membrane compositions or stability over defined periods of time, with different specific of demands depending on the intended use.

In all cases, however, microfluidic technologies for liposome formation are already establishing themselves as useful tools. Looking ahead, there are numerous opportunities for developing the processes that occur either upstream or downstream of liposome formation itself. For example, there is an array of droplet-based technologies available for manipulation, mixing, dilution, and concentration of the reagents to be encapsulated. Due to the similarity between liposomes and microdroplets or living cells, many established microfluidic technologies can also be adapted for use with liposomes downstream of their formation.

Already many downstream operations are being performed with liposomes. For example, Nishimura et al. have described sorting of vesicles by flow cytometry.\cite{Nishimura1996, Nishimura1997} Operations such as liposome merging\cite{Barlow2007, Bonilla2004, Wada2004} and splitting\cite{Ikeda2008, Ikeda2008, Ikeda2008} have also been demonstrated, as well as trapping,\cite{Wada2004, Ikeda2008} anchoring, and perfusing with reagents.\cite{Ikeda2008} Notably, Robinson et al. have developed a microfluidic platform in which a population of liposomes can be mechanically trapped into individual chambers, allowing single-liposome observation and manipulation.\cite{Robinson2009}

In conclusion, we have seen that liposome formation methods can be fully integrated into planar microfluidic platforms, and that such methods show distinct advantages over macroscale or hybrid approaches. The challenge will be to create an on-chip method of forming bespoke liposomes in high-throughput with high encapsulation efficiencies.
Chapter 3

Iterative design and prototyping of chemical systems and microfluidic devices
3.1 Introduction

The formation of artificial cells begins with the creation of cell-sized compartments within a given medium. One approach, as described in Chapter 2, is to form liposomes. However, the formation of liposomes with defined characteristics is in itself a difficult technical challenge. A simpler alternative is to create aqueous droplets within an immiscible (organic or fluorinated oil) medium, typically known as water-in-oil (W/O) droplets. Nevertheless, the external environment plays a crucial role in cell function, and thus such W/O droplets barely resemble biological cells due to the non-aqueous nature of the continuous phase. Accordingly, the approach taken in this thesis is to create so-called water-in-water (W/W) droplets. That is, aqueous droplets within an aqueous environment. Separation of the internal and external environments is achieved through phase separation induced by molecular charge interactions, a mechanism known as coacervation. Coacervate droplets bypass the need for a physical membrane barrier to preserve segregation of the intra- and extra-cellular media, and when compared to W/O droplets or liposomes, coacervate droplets more closely mimic cells due to their highly crowded and charged molecular environment.

Conventionally, a bulk coacervate is dispersed into droplets by (turbulent) vortex mixing with a second fluid such as water or oil. Such a dispersion process relies on shearing the coacervate phase by the continuous phase, which then breaks the coacervate into increasingly smaller droplets. In microfluidic channels, a similar “shear-based” process of segmenting one phase into another can be achieved using a flow-focusing strategy. Flow-focusing occurs when a stream of one fluid (the internal phase) is surrounded by a second fluid flow (the sheath). This is typically achieved either by use of concentric capillaries, where the internal phase flows out of a central capillary into a larger one containing the sheath flow; or through the use of planar microfluidic channels, where a central channel containing the internal phase leads through a nozzle into a larger channel, flanked on either side by channels containing the sheath flow. Figure 3.1 illustrates three different approaches. With flow focusing, segmentation of the internal phase occurs when the sheath fluid flows at a higher velocity than the internal flow, resulting shear at the interface between the two phases. In the present study, a planar microfluidic device was used for droplet formation. The main reason for this choice was the simplicity of setting up experiments with pre-fabricated planar devices compared to concentric capillary assemblies. Capillary-based systems, although simple to set up, require careful manual alignment of channels using support structures before use. Since devices could be easily fouled or clogged right at the start of an experiment, the speed with which planar devices could be swapped before the experiment resumed was a decisive advantage.

The principal difference between microfluidic flow-focusing and vortex mixing lies in the nature of the flow regime. Due to their differing length scales, vortex mixing is turbulent while microfluidic flow-focusing operates under laminar flow conditions. This difference gives rise to dispersions with different properties. Whilst dispersion by vortex mixing is simple to perform, it typically results in droplets with unacceptably large size distributions. In contrast, microfluidic flow-focusing usually confers the ability to control droplet size and also achieve narrow droplet size distributions. Moreover, and as will be shown in Chapter 5, the laminar flow conditions encoun-
Introduction

Figure 3.1 Three different approaches to forming droplets by microfluidic flow focusing. 
a) A capillary-based method demonstrated by Utada et al. where an inner fluid is focused through a round capillary into an outer fluid carried in a larger square capillary. A planar microfluidic device patterned in PDMS containing a disruption chamber within the flow-focusing junction nozzle. A different approach to flow-focusing in planar microfluidic devices employing a narrow nozzle followed by a channel expansion. Images reprinted with permission.

ted within microfluidic channels result in greatly enhanced stability of the formed coacervate droplets. However, successful phase segmentation using microfluidic flow focusing may only be achieved under certain conditions. These include the separation of the internal and sheath fluids (which may be achieved by exploiting phase separation or use of surfactants), controlling the shear rate (which is determined by the difference between internal and sheath flow velocities) and tuning the channel surface wettability by either phase. The latter is often the most complicated approach, requiring careful manipulation of channel surface properties by chemical surface modification. Accordingly, to achieve segmentation through microfluidic flow focusing, it is necessary to consider the interactions between the dispersed and continuous liquid phases, as well as the solid channel surfaces. Thus,
to identify compatible chemical systems and channel surface properties that were capable of forming droplets by microfluidic flow focusing, a series of pilot experiments was performed. This chapter describes this iterative prototyping process in detail.

3.2 Materials and methods

3.2.1 Device design and master mold fabrication

Microfluidic channel patterns were designed using AutoCAD 2011 (AutoDesk, USA). The drawing file was then sent to a commercial photolithography mask production service (Photolithography Services, UK) for printing on Mylar plastic film. Designs were arranged such that blocks of multiple microfluidic devices could be patterned onto a single 100 mm diameter silicon wafer.

Master molds were fabricated as follows according to the scheme shown in Figure 3.2. First, a 100 mm diameter silicon wafer was spin-coated with SU-8 photoresist (MicroChem, USA) and cured at 95°C. Spin speeds were adapted to control the final photoresist coating thickness according to manufacturer provided specifications. The photolithography mask was then used to transfer the microfluidic channel design pattern the photoresist by exposing with ultra-violet light in an MA6 mask aligner (Karl Süss, Germany). Subsequently, a post-exposure bake at 95°C was performed in order to complete the thermally-activated cross-linking of the exposed SU-8 photoresist. Next, the structures were developed using mr-Dev 600 Developer solution (Micro Resist Technology, Germany) to reveal the negative pattern of the microfluidic channels by removing the unexposed photoresist. Finally, after rinsing with isopropyl alcohol and thorough drying, the master mold was coated with 100 µl of chlorotrимethylsilane (ABCR Chemicals, Germany) by vapour deposition under vacuum for 1 hour. A completed master mold is shown in Figure 3.3.
3.2.2 PDMS device fabrication

Microfluidic devices were fabricated according to standard soft-lithography procedures. Briefly, Dow Corning Sylgard 184 polydimethylsiloxane (PDMS) silicone elastomer was mixed at a ratio of 10:1 base (dimethylsiloxane, dimethylvinyl-terminated, and dimethylvinylated and trimethylated silica) to curing agent (Tetra (trimethylsiloxy) silane), degassed in vacuum and then poured over an SU-8 master mold. After a second round of degassing in vacuum, the PDMS was left to cure in an oven at 70°C overnight. Once hardened, the PDMS was peeled from the master mold. Next, the excess PDMS around the edges of the block of devices was trimmed using a razor blade and all device input and output port vias cored. In parallel, a blank 100 mm silicon wafer coated with chlorotrimethylsilane was spin-coated with PDMS to form a 100-200 µm film. This was also left to cure in an oven at 70°C overnight. After curing, this film was not peeled from the wafer. The block of devices and the wafer-mounted PDMS film were then thoroughly washed with isopropyl alcohol and deionized water and dried under a stream of compressed air (with particular attention to channels and vias). As a final dust-removing step, both film and device channel surfaces were cleaned by application of Scotch adhesive tape (3M, USA).

Following cleaning, the channel side of the block of microfluidic devices and the thin PDMS film surfaces were activated using an oxygen plasma (K100X, Quorum Technologies, UK) for one minute. The two mating surfaces were then brought into
contact to seal the microfluidic channels. Immediately after sealing, a solution of 1H,1H,2H,2H-Perfluoro-1H,2H-trichlorosilane (PFOS) (ABCR Chemicals, Germany) in isopropyl alcohol was introduced into the channels, left to incubate for five minutes at room temperature, and subsequently removed by vacuum applied to the device input and output vias. Various PFOS concentrations were used, as described in the Results and Discussion. The block of treated devices was then left to stand for at least two hours before being peeled from the silicon wafer and diced into individual chips. These were then bonded to glass microscope slides using the same oxygen plasma bonding procedure and labeled with the date of manufacture.

3.2.3 Preparation of bulk coacervate

Bulk coacervate was prepared by mixing 100 mM of 8.5 kDa poly(diallyldimethylammonium chloride) (PDDA) with either 100 mM carboxymethyl-Dextran (CMDextran) or 100 mM adenosine triphosphate (ATP) dissolved in ultra-pure water and maintained at pH 8 using NaOH. All chemicals were purchased from Sigma-Aldrich and used as received. The CMDextran and PDDA solutions were mixed in molar ratios of 6:1, whereas ATP and PDDA were mixed in molar ratios of 4:1. In both cases, the coacervate phase was then separated by centrifuging the mixture for 20 minutes at 5000 rpm and isolated by removing the supernatant.

3.2.4 Preparation of DOPC dispersions in squalene

The phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, USA) was dispersed in oil by dispensing 1 ml of 25 mg/ml of DOPC in chloroform stock solution into a glass vial. The chloroform was evaporated under a gentle flow of nitrogen gas forming a film of lipid at the bottom of the vial. The lipid cake was dried under vacuum for a further 2 hours to ensure that all the solvent had been removed. After drying, 3 ml of squalene (Sigma Aldrich) was added to the vial over the lipid film to obtain a final lipid concentration of 8.33 mg/ml (10 mMol). The solution was then left to stir on a magnetic stirring hot plate at 40°C, for at least two hours to ensure complete dispersion of lipid in squalene. 5 mMol and 1 mMol lipid dispersions were prepared by diluting the 10 mMol stock dispersion. All DOPC-oil dispersions were stirred and heated at 40°C for at least 30 minutes before use to break down any lipid aggregates.

3.2.5 Preparation of silica-oil dispersions

Tetramethyl orthosilicate (TMOS) was purchased from Sigma Aldrich and used without purification. TMOS was dispersed in squalene at a concentration of 0.03 mg/ml and sonicated for 2 minutes to break up any large silica particles.

3.2.6 Experimental setup

Microfluidic devices were secured onto a motorized stage (Prior, UK) on an inverted microscope (Eclipse Ti-E, Nikon, Japan) equipped with high-speed camera (MotionPro Y5, Integrated Design Tools, USA). The inputs were connected via PTFE tubing to two separate pressure pumps (P-Pump, Dolomite Microfluidics, UK) containing the dispersed and continuous phases. PTFE tubing was also inserted into the
Results and discussion

Figure 3.4 (a) AutoCAD drawing of one flow-focusing device. (b) Enlargement of the flow-focusing junction, which is 40 µm wide at its narrowest point. (c) The same AutoCAD drawing rendered as a photolithography mask, and (d) detail of the flow-focusing junction. On the actual mask, black regions block transmission of light, while white regions are transparent. The sheath flow is injected through the left port; the focused phase (coacervate) is injected through the middle port; and the sample is collected from the right port.

outlet port for sample collection. The flow rates for both phases were individually controlled by the two pressure pumps.

3.3 Results and discussion

Initial attempts to use a microfluidic flow-focusing geometry to form coacervate droplets in water relied on a conventional flow-focusing junction with nozzle expansion (to form W/O droplets) as shown in Figure 3.4. In this design, the internal aqueous phase enters a flow-focusing junction, where it is surrounded by an oil sheath flow. This focused stream is motivated through a thin nozzle containing a disruption chamber before expansion into a wider exit channel. The rapid expansion and contraction experienced by the flow as it moves through the disruption chamber within the nozzle forces the Plateau-Rayleigh instability, necessary for segmenting the internal aqueous phase into droplets. In a set of pilot experiments, several different droplet and continuous phase systems were studied using this conventional flow-focusing geometry. The aim of these experiments was to establish which systems were most amenable to the formation of coacervate droplets in microfluidic channels.

The first prototype system involved dispersing a bulk coacervate phase into squalene. Although this was not the intended configuration, due to the oleic nature of the continuous phase, this experiment was deemed appropriate since, as a conventional W/O droplet system, it was simple to perform and yet provided some insights into in the behavior of the coacervate material within a microfluidic environment. The effects of the highly viscous and adherent nature of the coacervate fluid were of particular interest. Additionally, due to the porous nature of PDMS, it was not known whether dehydration of the coacervate would be a problem.

Bulk coacervate comprising 4:1 molar ratio of PDDA and ATP was dispersed into squalene. Figure 3.5 shows the break-up of coacervate and the resulting droplets after collection. As can be seen by the “tail-like” structure dragging behind the droplet (indicated by the white arrow in Figure 3.5a), the coacervate wets the PDMS channel surface after being focused into the squalene sheath flow. Accordingly, it was evident that the PDMS channel surface would need to be treated in order to prevent wetting by the coacervate droplets. Moreover, the droplets formed were not
Figure 3.5 Dispersion of coacervate into pure squalene oil. (a) A coacervate droplet immediately after formation at the flow-focusing junction, accompanied by a group of satellite droplets. (b-d) Collected coacervate droplets. The size variation confirms that droplet merging occurred after formation, most likely at the device outlet, where the large channel expansion allows droplets to come into contact. Wetting of channel surface by the coacervate droplets can also be observed.

Figure 3.6 Images of droplet formation at the flow focusing junction for dispersion of 4:1 molar ratio of PDDA-ATP coacervate into squalene with (a) with 1 mMol DOPC, (b) 5 mMol DOPC and (c) 10 mMol DOPC.

stable, and readily merged upon contact.

Before a surface treatment was developed, PDDA-ATP coacervate was also dispersed into squalene containing DOPC at concentrations of 1, 5, and 10 mMol, to investigate the effects of adding a surfactant (to prevent merging of coacervate droplets) to the mixture. Figure 3.6 shows that droplet were formed without any wetting of channel surfaces by the coacervate. Interestingly, coacervate droplets exhibited both shrinkage and deformation on collection, as shown in Figure 3.7. This is most likely due to dehydration of the coacervate by the squalene and DOPC present in the continuous phase. Dispersion of 6:1 molar ratio PDDA-CMDextran
Results and discussion

Figure 3.7 Droplets formed by dispersing 4:1 molar ratio PDDA-ATP coacervate bulk phase into squalene with (a) 1 mMol DOPC, (b) 5 mMol DOPC, (c) Droplets segregated by ultra-sonication, formed in squalene with 5 mMol DOPC.

Figure 3.8 (a) Droplet formation at the flow-focusing junction where 6:1 molar ratio PDDA-CMdextran was dispersed into squalene with 0.03 mg/ml TMOS. (b) Wetting of the coacervate droplet onto the surface of the microfluidic channel can be observed.

coaacervate into squalene containing 0.03 mg/ml TMOS as a surfactant was also attempted. In this case, slight wetting of channel surfaces by coacervate droplets was observed, as shown in Figure 3.8. Adding DOPC as a surfactant greatly increased the stability of droplets. Indeed, the droplet aggregates shown in Figure 3.7a could be broken down by sonication without disruption of individual droplets. However, the PDDA-CMDextran in squalene with TMOS system was not as stable, and exhibited some droplet merging after formation.

Subsequently, the problem of coacervate droplets wetting the PDMS was directly addressed. A fluorinated silane, PFOS, was used to coat channel surface due to the strongly omniphobic nature of its long fluorocarbon chain. PDDA-ATP coacervate was dispersed into pure squalene in microfluidic channels treated with undiluted PFOS, as well as 1 and 5 vol % PFOS solutions in isopropyl alcohol. Figure 3.9 shows the results for such an experiment. Although the undiluted PFOS completely prevents surface wetting by coacervate droplets, the treatment left a considerable amount of debris within the channels. Conversely, channels treated with 1% PFOS in isopropyl alcohol exhibited little wetting, while those treated with 5% PFOS showed no wetting at all. In either of these cases, there was no debris left behind by the surface treatment procedure. These results suggested that the 5% PFOS surface treatment should be used in subsequent experiments.

Finally, a 4:1 molar ratio PDDA-ATP coacervate was dispersed into deionized water using a microfluidic flow-focusing geometry with the 5% PFOS surface treat-
Figure 3.9 The effect of different PFOS concentrations on surface “cleanliness” and wettability during dispersion of bulk coacervate into pure squalene. Channels were treated with (a) pure PFOS, (b) 5% PFOS in IPA, and (c) 1% PFOS in IPA solutions. The arrow on the bottom right indicates wetting of the channel surface by the coacervate.

Figure 3.10 Dispersion of 4:1 molar ratio 8.5kDa PDDA-ATP coacervate into deionized water at different locations along the microfluidic channel (the precise location varied, depending on flow rates, coacervate viscosity, and temperature).

Figure 3.11 4:1 molar ratio 8.5 kDa PDDA-ATP coacervate droplets formed in water. The droplets have a wide size distribution but do not coalesce when packed.

As shown in Figure 3.10, the coacervate segmented into droplets without wetting the channel surface. However, the formed droplets exhibited a wide size distribution. Figure 3.11 shows a sub-population of the collected droplets. Droplets were also formed using 40 kDa PDDA (instead of 8.5 kDa). However, the break-up process for this heavier species was significantly extended in length, occurring inside the collection tubing, and out of sight. The droplets collected are shown in Figure 3.12.

Upon closer inspection, it became apparent that the nozzle and disruption cham-
Conclusion

Figure 3.12 Collected droplets from (a) 4:1 molar ratio 40 kDa PDDA-ATP (b) 6:1 molar ratio PDDA-CMDextran.

Figure 3.13 (a) Modified flow-focusing channel design without nozzle constriction, disruption chamber, or exit channel expansion. (b) Detail of the flow-focusing junction. (c) The flow-focusing junction in operation with PDDA-ATP coacervate. Note the absence of the wave patterns observed with devices using the previous design.

ber following the flow-focusing junction did not significantly influence the segmentation process, since the coacervate stream breaks into droplets much further down the channel. Moreover, the channel constriction and expansion was observed to induce oscillations in the focused coacervate stream. This resulted in an uneven distribution of material between droplets when the stream finally segmented, thus causing the wide droplet size distribution observed. As a result, a new flow-focusing geometry (without the nozzle constriction, disruption chamber, and channel expansion) was implemented. Additionally, due to the extended length over which segmentation occurs, the channel length was increased to over 10 mm to allow observation of the entire droplet formation process. The new design is shown in Figure 3.13. This design turned was shown to be highly successful, and the results obtained using it are described in Chapter 5.

3.4 Conclusion

In summary, the iterative process by which the final coacervate chemical system and microfluidic device design were defined has been described. It was found that both PDDA-CMDextran and PDDA-ATP coacervates could be successfully dispersed in
water without fouling of microchannel surfaces, if channels had been previously treated with 5% PFOS in isopropyl alcohol solution. Furthermore, it was also found that conventional flow-focusing designs employing nozzle constrictions, disruption chambers, or exit channel expansions were detrimental to the formation of droplet populations of narrow size distribution. Instead, a straight channel following the flow-focusing junction that possesses no variation in cross-sectional profile provided the best performance. Further experimental results using this combination of chemical system and device design are explored in Chapter 5.
Conclusion
Chapter 4

Measurement and analysis of droplet size
4.1 Introduction

This chapter describes the instrumentation, measurement techniques and the analysis of acquired data for determining droplet size distributions. One of the primary goals of the current work was to form coacervate droplet populations possessing narrow size distribution. Different coacervate size analysis methods were explored in validating this capability. Both video and still image data were analyzed using open source image processing software (ImageJ, NIH, USA \cite{16, 202} and custom MATLAB (Mathworks, USA) programs. Moreover, small-angle light scattering (Mastersizer 2000, Malvern Instruments, UK) measurements of collected droplets were used to supplement the analysis.

As described in Chapter \cite{3} flow-focusing microfluidic devices were mounted on an inverted microscope (Eclipse Ti-E, Nikon, Japan) equipped with a high-speed camera (MotionPro Y5, Integrated Design Tools, USA) and a high-power white light source (HPLS 200, Thor Labs, USA). Using this system, visualization and imaging of the formation of coacervate droplets within microfluidic channels could be performed in real time. Of particular importance to successful imaging was the use of a long working distance objective with 40x magnification (CFI S Plan Fluor ELWD ADM 40X, Nikon, Japan), which enabled the capture of high-resolution images for analysis. The wide gap between the objective lens and its focal plane allowed focusing through the 1.2 mm thick glass slides and 100 - 200 µm PDMS layer that constitutes the bottom of the microfluidic channel. Use of the high-speed camera also enabled collection of videos with shutter speeds of less than 10 µs which, when coupled with the high-power white light source, effectively eliminates motion blur effects which can be problematic when operating at high volumetric flow rates. This capability, in turn, enabled frame-by-frame image analysis of video data. Black insulation tape was placed over input and output tubing to protect samples loaded with fluorescent dye from bleaching through over-exposure by the bright light source. Figure 4.1 shows a microfluidic device with two parallel flow-focusing channels mounted onto the microscope.

Droplets were collected in clear-bottom 96- or 384-well plates. Plates were then mounted onto the microscope for further imaging. Samples stored in well plates were also incubated at room temperature for extended periods of time (up to 80 days). Imaging of collected droplets was performed using a second inverted microscope system (Eclipse Ti-U, Nikon, Japan) equipped with a wide-field camera (CoolSNAP HQ2, Photometrics, USA), an LED white light source (LDB100F, Prior, UK), and motorized stage (HLD117, Prior, UK).

4.2 Video analysis using ImageJ

Initially, particle size information was extracted from video data using ImageJ image processing software. High-speed videos (1000 frames per second) of droplets inside the microfluidic channel (just before the outlet via) were recorded and processed with the following ImageJ operations.

First, the scale dimensions are converted from pixels to microns, i.e.

\texttt{run("Set Scale...", "distance=657 known=300 pixel=1 unit=microns global");}
Video analysis using ImageJ

**Figure 4.1** A microfluidic device with two parallel flow-focusing junctions mounted onto an inverted microscope. The arrows indicate a) the external aqueous phase input tubing, b) the coacervate input tubing protected from the light source by black insulation tape, and c) the output tubing used for sample collection.

Next, nine out of every ten frames in the video are discarded, to avoid duplicating measurements on the same droplet, i.e.

```java
run("Reduce...", "reduction=10");
```

The video is then cropped to define the appropriate region of interest, i.e.

```java
makeRectangle(0, 32, 2336, 248); run("Crop");
```

Subsequently, a bandpass filter is used to reduce noise and enhance contrast.

```java
run("Bandpass Filter...", "filter_large=40 filter_small=20 suppress=Horizontal tolerance=5 autoscale saturate process");selectWindow("video.avi"); setAutoThreshold("Default");
```

An automatic threshold operation is then used to produce a black and white image.

```java
setAutoThreshold("Default stack"); run("Convert to Mask", "method=Default background=Light calculate");
```

This is followed by a “fill holes” instruction to turn droplet outlines into solid objects.

```java
run("Fill Holes", "stack");
```

Finally, the built-in ImageJ particle analysis function is employed to detect and measure the surface area of droplets in each frame.

```java
run("Analyze Particles...", "size=2500-Infinity pixel circularity=0.00-1.00 show=Outlines display exclude clear stack");
```

Example output images for each step in this process are shown in Figure 4.2. As is clearly shown in Figure 4.2, not all droplets are processed using this method. In this image, the two large droplets to the left hand side are excluded since they contact the edges of the image. Since the analysis measures particle size by counting the
number of pixels within a particle, taking into account droplets that are “cropped” by the edges would yield erroneous information. However, it can also be seen in Figure 4.2 that the threshold image used in the particle analysis function contains many smaller objects that are not present in the final data. Some of these are accurate representations of small (satellite) droplets, while others are artifacts of the filtering and threshold operations (where only a portion of a droplet is detected). Finally, some droplets were too faint in the original image to be detected, due to their location outside the focal plane. Figure 4.3 shows the histogram of droplet size data obtained using this procedure. The presented ImageJ analysis procedure was deemed to insufficiently reflect reality and accordingly an alternative droplet analysis strategy was sought.

4.3 Video analysis using MATLAB

A key problem encountered when analyzing video data with ImageJ was a lack of flexibility in tuning specific image processing operations. As a result, a new analysis procedure using MATLAB was developed. MATLAB, being an interpreted computer programming language, allows for complete control over each step of the analytical process. In addition, MATLAB contains an image processing toolbox that provides a rich library of image processing functions. A range of analytical functions were written to perform the droplet size analysis, with the full code for each function being provided in Appendix A.
Video analysis using MATLAB

Figure 4.3 Frequency histogram summarizing results obtained from video analysis using ImageJ. The extracted mean droplet diameter is 8.58 µm with an RSD of 11% (n = 1027).

As a first step, the function `avibgremove.m` was written to remove the background contained in each frame. This was performed by constructing a background image where each pixel was either the mode, mean or median value (decided by the operator) of that same pixel across all frames in the video. This background image was then subtracted from each frame in the video. The resulting video output contained a black background over which only pixels that had changed between frames were represented with the same intensity as that in the original video. Figure 4.4a shows a single frame from the original video, and Figure 4.4b shows that same frame after background removal. (Droplets are hard to see against the black background due to low contrast.)

Next, the video was inverted such that black pixels became white, and white pixels became black, using the `avibwinvert.m` function (Figure 4.4c). Frames were then processed using a threshold operation (with the threshold being set either automatically or by the user) through the function `avithreshold.m` to produce a binary (black and white) image (Figure 4.4d). This was then re-inverted using `avibwinvert.m` (Figure 4.4e), before a series of “erosion” and “dilation” operations were performed (Figure 4.4f) using the `avibwsmooth.m` function. These had the effect of smoothing out the edges of droplets as well as getting rid of small object artifacts. Additionally, because the droplets in the resulting black and white binary image were consistently smaller than those in the original video after the threshold operation, the function `avibwsmooth.m` also performed an extra dilation step in order to restore them to a size that more closely matched the raw data.

In order to validate this process, individual frames in the final black and white binary video (Figure 4.4g) were compared by qualitative visual inspection to corresponding frames in the original video (Figure 4.4h) in order to ensure that the droplets detected matched closely in size with those in the original. Finally, the `avianalyseparticles.m` function was used to detect individual droplets and calculate their area in pixels. The results of this procedure were then plotted in a histogram as shown in Figure 4.5. It is important to note that this sequence of operations successfully captures out-of-focus droplets that exhibit little contrast against the
Figure 4.4 Frames showing the output of each step of the video analysis performed using the custom MATLAB program. (a) The original frame (b) after background removed, (c) after black/white inversion, (d) after thresholding, (e) after black/white inversion and finally (f) after erosion and dilation. The white arrows in (a) indicate stationary artifacts that are part of the image background, and thus excluded by the analysis program.

Figure 4.5 Histogram of PDDA-CMDextran coacervate droplet size distribution obtained from the video analysis performed using the custom MATLAB program. The mean droplet size was 4.71 µm with an RSD of 34% (n = 56768)

background. It is also noted that many objects appearing in the original frame seem to be excluded in the final output image, such as those indicated by the white
arrows in Figure 4.4a. These objects are in fact stationary and part of the background, and thus successfully removed by avibgremove.m.

Although this method of analysis proved far more successful than that associated with the use of ImageJ, it was not without problems. Each video to be analyzed was captured under slightly different conditions, due to variations in device geometry and lighting conditions between experiments. Since function parameters such as the threshold value and the auxiliary inputs to avibwsmooth.m (which define the extent of erosion and dilation operations) were user defined instead of automatically calculated, a number of video processing attempts had to be made for each video in order to find a combination of function input parameters that yielded a black and white binary video that could be successfully validated as described in the previous paragraph.

This approach was problematic due to the extended timescales required to process a single video file (at least 40 minutes) and the fact that a single video would often require ten or twenty iterations of "parameter tweaking" before the results were deemed accurate. As a result, it was decided that this method too cumbersome to be employed in the routine analysis of the hundreds of video files obtained during the current work.

4.4 Droplet sizing through small-angle light scattering

To address the difficulties associated with extracting droplet size information through analysis of video data, a more direct method was assessed. Specifically, droplet samples were collected after formation and analyzed using the method of small-angle light scattering (Mastersizer 2000, Malvern Instruments, UK). An example of the results obtained from this analysis can be seen in Figure 4.6. Although simple to implement and fast, the use of this method did pose some issues for measuring coacervate droplets. First, because light scattering depends on the refractive index, effective control measurements of particles of known size and size distribution would have to be performed with particles of the same coacervate material as the experimental sample. This posed a “chicken and egg” problem, where a sample of droplets with known size and size distribution would have to be produced for instrument calibration before other similar samples could be accurately measured. Nonetheless, although the lack of calibration prevented the measurement of absolute values, relative size and size distribution could be obtained for comparison between different samples of the same material. Moreover, performing the light scattering measurement involved introducing the sample into a flow cell in which the carrier fluid (deionized water) was in continuous turbulent flow. And, since it was already known that coacervate droplets could be formed under turbulent flow conditions, there was a possibility that droplets in the sample would break down into smaller ones or be otherwise modified during the measurement process. As a result, given the inability to measure absolute values and the possibility that the sample could be altered during measurement, it was decided that small angle light scattering was not the ideal method for determining coacervate droplet size and size distribution.
Figure 4.6 Particle size distribution obtained from small angle light scattering measurements of PDDA-CMDextran coacervate droplets. (a) A control sample with droplets prepared by the vortex method. Mean droplet diameter is 12.9 µm, with 10th percentile diameter of 4.2 µm and 90th percentile diameter of 27.0 µm. (b) Droplets prepared using the microfluidic method. Mean droplet diameter is 10.2 µm, with 10th percentile diameter of 5.5 µm and 90th percentile diameter of 15.9 µm. Note that these values are not absolute due to the lack of instrument calibration from a control sample with known size distribution.

4.5 Image analysis in MATLAB

Due to the difficulties associated analyzing video data, combined with the uncertainty inherent in the light scattering measurements, it was decided to perform droplet size analysis on still images of collected droplets instead. To this end, a new MATLAB program was written to iteratively process multiple still images and detect circular objects within them. The program consisted of just two functions, both detailed in Appendix A. The first, findcircles.m, detects circles within an image, computes their radius and indexes their location. Since droplets are spherical in shape, they appear as circles in the images, and thus the diameter of the circles detected by this function represent the diameter of the corresponding droplets. However, since the droplets appear in the images as dark rings against a light background, the standard MATLAB function imfindcircles.m used in the custom findcircles.m often detected two circles per droplet: one inside and one outside the dark ring. This problem was addressed by excluding the smaller of any two overlapping circles, under the assumption that the outer circle was more representative of the actual droplet diameter.

The second function, imageprocess.m, performs auxiliary operations such as iterating through different directories, opening image files, sending image data to
findcircles.m, saving analytical results, and plotting histograms. Figure 4.7 shows an example of an image with detected circles overlaid, while Figure 4.8 shows the resulting droplet size histogram. This analysis was validated through a visual inspection of processed images, by checking that the overlaid circles consistently matched their corresponding droplets in diameter. Through this inspection, it was found that less than 7% of circles were significantly larger or smaller than their corresponding droplets. However, since these errors occurred in either direction in similar proportions, their combined statistical effect is likely not very significant. Therefore, the performance of this program was considered adequate and sufficient for its intended purpose.

Analyzing still images of collected droplets also enabled a direct comparison between images of the same sample of droplets obtained at different time points. This was not possible with in-channel video data, because that could only be obtained at the time of droplet formation for any given sample. However, because droplets were collected into 96- or 384-well plates, they often settled into multiple layers and packed too densely for successful still image analysis. Therefore, only a limited number of images acquired of droplets scattered around the edges of the main population were amenable to analysis. As a result, still image analysis required a compromise in the number of droplets that could be analyzed in each experiment. Typically, analysis of a single video encompassed tens or even hundreds of thousands of droplets, while analysis of still images included at most hundreds or thousands of droplets. Nonetheless, reliable statistics could still be obtained from the smaller number of droplets measured in the still image analysis. Finally, although each image file required approximately 2-5 minutes of processing time, analysis could still be completed in a much shorter time than with videos, especially when performed using the Parallel Processing Toolbox available in MATLAB.

4.6 Conclusions

In summary, this chapter has described the assessment of a variety of methods for droplet size analysis. Video analysis proved to be too time-consuming. That said, if the issues with parameter optimization and performance are resolved the defined approach could be very useful in future experiments, especially where on-line measurements are used as part of a feedback control mechanism. A analytical method based on small-angle light scattering was also investigated, but could not be guaranteed to give reliable results due to the turbulent flow conditions in the instrument’s flow-cell. Analyzing still images of collected droplets using MATLAB, on the other hand, gave reasonably accurate results with the added benefit of the ability to compare like-for-like data obtained from the same sample at different points in time. In conclusion, the analysis of still image data offered the best compromise between accuracy, speed, and flexibility compared to the other methods.
**Figure 4.7** Circles computed and overlaid over coacervate droplets on an image analyzed with a custom MATLAB program. The program has detected 250 droplets in this image. By visual inspection, 17 circles appear to be significantly larger or smaller than the corresponding droplet, which corresponds to an error rate of approximately 7%.

**Figure 4.8** Histogram of the size distribution obtained from the still image analysis of 4753 PDDA-CMDextran droplets performed using a custom MATLAB program.
Conclusions
Chapter 5

Microfluidic formation of membrane-free aqueous coacervate droplets in water

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Dirk van Swaay, Dora Tang, Stephen Mann, Andrew deMello.
In the present study, we report the formation of coacervate droplets from Poly(diallyl dimethylammonium chloride) (PDDA) with either adenosine triphosphate or carboxymethyl Dextran using a microfluidic flow-focusing system. The formed droplets exhibit improved stability and narrower size distributions for both coacervate compositions when compared to the conventional vortex formation techniques. Additionally, we demonstrate the simultaneous formation of two distinct coacervate droplet populations within the same microfluidic device, each containing different DNA oligonucleotides. Our results show that the observed improvements in droplet stability and size distribution may be scaled with ease. In addition, and most significantly the ability to encapsulate different materials into coacervate droplets using a microfluidic channel structure allows for their use as a cell-mimicking compartments.

5.1 Introduction

The ability to compartmentalize different materials within small volumes is essential to the development, study, and application of chemical and biological systems. Under normal circumstances, this is achieved by use of flasks, tubes, vials, and other containers of different sizes and materials. In recent years, a range of microfluidic technologies have been shown to be advantageous in realizing chemical and biological compartmentalization, and in addition are accompanied by significant improvements in analytical performance, experimental control and increased throughput. Of particular note has been the encapsulation of chemical or biological materials in sub-nanoliter aqueous droplets contained within fluorinated oils. To date, the function of the continuous phase in water-in-oil droplet systems has typically been restricted to the simple separation of material. In contrast, the external medium is fundamentally important in a biological context, where compartmentalization is achieved by means of a membrane structure that separates the intra- and extra-cellular aqueous environments. However, a physical boundary that is selectively permeable and capable of containing chemical or biological components may also be realized without the need for a membrane. A coacervate droplet – a phase-separated structure of self-assembled water-soluble macromolecules suspended in water – can also provide compartmentalization in an analogous manner to that seen in cells, albeit without the need for a membrane. Coacervates assemble via charge interactions between large molecules, forming a highly viscous and crowded molecular environment that mimics the interior of living cells. For this reason, coacervate droplets have been proposed as likely viable forms of artificial cells, with practical applications in biotechnology and molecular biology. However, previous studies with coacervates have formed droplets by vortex-mixing of coacervate and water phases. The turbulent flow conditions present in this method result in droplets with very wide size distribution. Moreover, droplets obtained in previous studies were unstable, and merged when brought into contact for prolonged periods. In the present study, we demonstrate use of microfluidics to rapidly and controllably form large populations of coacervate droplets of narrow size distribution, capable of encapsulating DNA molecules, and which exhibit structural integrity for at least six days at room temperature without the need for surfactants or other stabilizing agents.
5.2 Materials and methods

Droplets were formed using a microfluidic flow-focusing geometry (Figure 5.1a) that re-disperses "bulk" coacervate as droplets in water. Accordingly, the system is most correctly described as generating "water-in-water" (W/W) droplets. Microfluidic channels were fabricated in polydimethylsiloxane (PDMS) using conventional soft lithography techniques. Briefly: a master mold was prepared using SU-8 photoresist (Microchem, USA) spin-coated to a height of 40 µm on a silicon wafer (Silicon Materials, Germany), onto which a mixture of Sylgard 184 PDMS elastomer mixture (Dow Corning, USA) was poured and allowed to cure for 24 hours at 70 °C. The PDMS replica was then peeled and diced into individual devices, and the inlet and outlet vias cored. Devices were then washed with isopropanol alcohol and deionized water, dried thoroughly in a stream of nitrogen, and, using an oxygen plasma treatment (K100X, Quorum Technologies, UK), bonded to a PDMS membrane prepared by spin-coating PDMS onto a silicon wafer silanized with trichloromethyl silane (Sigma-Aldrich), thus sealing the microfluidic channels. In order to prevent wetting of the channel walls by the coacervate, the channels were filled with a solution of 5% 1H,1H,2H,2H-Perfluorooctyltrichlorosilane (PFOS) (ABCR Chemicals, Germany) in isopropyl alcohol immediately after bonding, and were left to stand for 5 minutes before flushing with air. The sealed and treated devices were then bonded to a glass microscope slide for facile handling and operation.

The coacervate to be dispersed into droplets was prepared at by mixing 100 mM of 8.5 kDa Poly(diallyl dimethylammonium chloride) (PDDA) with either carboxymethyl Dextran, dissolved to 100 mM in Milli-Q water (18.2 MΩ/cm) and maintained at pH 8 using NaOH; or adenosine triphosphate (ATP) at 100 mM also at pH 8. All chemicals purchased from Sigma-Aldrich and used as received.
carboxymethyl Dextran and PDDA solutions were mixed in molar ratios of 6:1, whereas ATP and PDDA were mixed in molar ratios of 4:1. In both cases, the coacervate phase was then separated by centrifuging the mixture for 20 minutes at 5000 rpm and isolated by removing the supernatant. For fluorescence experiments, dye-labelled complementary oligonucleotides (Eurofins Genomics, Germany) were mixed into the coacervate during preparation, diluted 6000 times from an initial stock concentration of 16.7 µM.

5.3 Results and discussion

The bulk coacervate phase was then injected into the microfluidic channel, where it was focused into a stream of deionized water. The flow of both coacervate and water streams were controlled by pressure pumps (P-Pump, Dolomite Microfluidics, UK) set from 80 to 320 mbar and 100 to 400 mbar respectively. As the coacervate stream stretched into a filament within the water stream, it broke up into droplets. This break-up process was imaged using a high-speed camera (MotionPro Y5, Integrated Design Tools, USA) mounted on an inverted microscope (Eclipse Ti-E, Nikon, Japan) and illuminated with a high power white light source (HPLS 200, Thor Labs, USA). The coacervate droplets were then collected into 96- or 384-well plates for further imaging and incubation.

Because the coacervate mixture is composed primarily of long-chained polyelectrolyte molecules, the resulting fluid is viscoelastic or non-Newtonian in nature.\[272\] Moreover, the interface between the coacervate and water phases has very low surface tension (42 +/- 0.2 mN/m for 1:4 PDDA-ATP coacervate). Both properties have important implications for the break-up mechanism of the bulk fluid into droplets. Contrary to simple micro-droplet systems based on immiscible fluids such as water and fluorinated oils, which rely on surface tension and the Plateau-Rayleigh instability for breaking the continuous stream into droplets,\[198, 203, 69\] breaking the coacervate stream within a laminar flow requires the continuous shearing of the material into a filament until it is able to segment. As the material is stretched, the long polymer molecules align themselves in parallel to the filament. When the filament finally breaks, the polymers recoil, and the segment “curls up” into a spherical droplet.\[1\] This process is shown in its different stages in Figure 5.1b-d and in the Electronic Supplementary Information (Video S1). It is significant to note that this breakup process takes considerably more time, and occurs over much longer lengths (millimeters rather than microns), than observed for the breakup of immiscible phases.\[274, 212, 192\]

Segmenting the viscoelastic coacervate fluid within a laminar flow is crucial in enhancing the stability of the resulting droplets. This is most likely due to the more organized recoiling of the polymer molecules during the collapse of the filament segments into their final spherical shape. Droplets formed in this manner do not merge readily when they come into contact, even over prolonged periods, as shown in Figure 5.2a. Long-term incubation studies indicate that populations of closely packed droplets in water persist with little or no merging for more than six days at room temperature. This is in stark contrast to droplets of the same material formed under turbulent flow (i.e. formed by vortex-induced mixing of coacervate and water), which readily coalesce (Figure 5.2b). Importantly, our coacervate droplets are stable without recourse to surfactants or membranes.
Results and discussion

Figure 5.2 (a) Two images of PDDA-CMDextran coacervate droplets prepared in the microfluidic channel taken six days apart. Although the images are not from exactly the same location in the sample, it can be seen that droplets remained in contact but little or no merging throughout this period. (b) Two images of PDDA-CMDextran coacervate droplets prepared using the vortex method, taken 5 seconds apart. The red dotted circles highlight four droplets that merge into larger ones during this short period. All scale bars are 25 µm.

Figure 5.3 (a) Two images of PDDA-CMDextran coacervate droplets formed (a) in the microfluidic channel and (c) using the vortex method, with respective histograms (b and d) showing the size distribution for each population. All scale bars are 25 µm.

Figure 5.3 A comparison of the size distribution of PDDA-ATP coacervate droplets formed (a) in the microfluidic channel and (c) using the vortex method, with respective histograms (b and d) showing the size distribution for each population. All scale bars are 25 µm.

Figure 5.3 shows a comparison of PDDA-ATP samples prepared using either microfluidic flow-focusing or by turbulent vortex mixing. Figure 5.4 shows the equivalent for PDDA-carboxymethyl-Dextran samples. We used a custom MATLAB (Mathworks, USA) program, provided in the ESI, to perform size analysis of droplets in the extracted images to obtain average size distributions for different populations. It can be seen that dispersion of the coacervate using a microfluidic flow-focusing geometry results in a significantly narrower droplet size distribution...
Figure 5.4 A comparison of the size distribution of PDDA-CM-Dextran coacervate droplets formed (a) in the microfluidic channel and (c) using the vortex method, with respective histograms (b and d) showing the size distribution for each population. All scale bars are 25 µm.

When compared to populations produced using the vortex method, PDDA-ATP droplets obtained through our microfluidic method were on average 4 µm in diameter, with a relative standard deviation (RSD) of 17%. By comparison, PDDA-ATP droplets formed by the vortex method had mean diameter of 13 µm and an RSD of 62%. Likewise, droplets from the PDDA-carboxymethyl-Dextran coacervate population had a mean diameter of 8 µm with an RSD of 17% when prepared by the microfluidic method, and a mean diameter of 6 µm with a 45% RSD when prepared by vortexing. Since experiments involving droplets often incorporate some kind of volume-dependent measurement, it is generally desirable to obtain a homogeneous population wherever possible. For example, fluorescence intensity of individual droplets is expected to be proportional to their volume. Therefore, the narrower size distribution achieved by the microfluidic method eliminates the wide variation in fluorescence intensities between droplets that is typically encountered in polydisperse populations. As such, the narrower size distribution of coacervate generated using our microfluidic method is a significant advantage.

Using a device containing two parallel flow-focusing channel structures that converge close to the exit (Figure 5.5a), two distinct populations of droplets could be formed simultaneously, each containing complementary single-stranded DNA oligonucleotides that were labeled with either fluorescein or Cy5. Due to the channel convergence at the outlet, the two populations were able to mix while exiting the device. The mixed droplets were then loaded into 96-well plates for storage and imaging studies. Following collection, fluorescence images of the droplets (using filter sets for both dyes) were measured. For fluorescein, we used 475/28 nm excitation and 560/40 nm emission filters along with a 518 nm dichroic mirror; for Cy5 we used 626/40 nm excitation and 655 nm long-pass emission filters with a 648 nm dichroic mirror. (All filters were purchased from AHF, Germany). The two filter sets were selected to avoid leakage of excitation and emission light while imaging both dyes within the same sample. Imaging was performed with an inverted
Results and discussion

Figure 5.5 (a) A schematic diagram of the parallel flow-focusing microfluidic channel structure. Bulk coacervate containing Cyanine 5 and fluorescein labelled single-stranded DNA oligonucleotides are simultaneously focused into streams of water in the top and bottom channels respectively. The two parallel streams then converge at the outlet to the right. (b) A brightfield micrograph of PDDA – ATP coacervate droplets containing the different labels collected from the parallel device. (c) A composite image of both Cyanine 5 (red) and fluorescein (green) fluorescence imaging channels showing the two separate populations of droplets coexisting without merging. (d) and (e) show the single channel images, Cyanine 5 and fluorescein, respectively, combined to form the image in (c). All scale bars are 25 µm.

microscope (Eclipse Ti-U, Nikon, Japan) with wide-field camera (CoolSNAP HQ2, Photometrics, USA). Exposure and acquisition was controlled using Micro Manager software.[66] Figure 5.5b-e shows the results of fluorescence imaging experiments with PDDA-ATP (see Figure 5.6 for PDDA-carboxymethyl-Dextran) and demonstrates that the droplets from both populations remain distinct over the timescale of the experiment, i.e. Cy5-containing droplets did not produce a signal when using the fluorescein filter set and vice versa. This state was preserved for a period in excess of six days. A control experiment where droplets containing both dyes were formed (Figure 5.7) demonstrates that if Cy5- and fluorescein-containing droplets had merged, the resulting droplets would be visible when using both filter sets.

It is important to note that droplets formed using the parallel flow-focusing channels also exhibit a narrower size distribution and improved droplet stability when compared to those formed using the vortex method (Figure 5.8). This confirms that the advantages conferred by the single-channel device are preserved when the process is scaled. The ability to simultaneously produce distinct droplet populations with similar characteristics is another unique capability of the microfluidic method that cannot be reproduced using other techniques. This unique capability opens
Figure 5.6 (a) A brightfield micrograph of PDDA – CMDextran coacervate droplets containing the different labels collected from the parallel device. (b) A composite image of both Cyanine 5 (red) and fluorescein (green) fluorescence imaging channels showing the two separate populations of droplets coexisting without merging. (c) and (d) show the single channel images, Cyanine 5 and fluorescein, respectively, combined to form the image in (b). All scale bars are µm.

Figure 5.7 Coacervate droplets containing both Cyanine 5 and fluorescein labelled single-stranded DNA oligonucleotides was focused into a stream of water and collected for imaging. (a) A brightfield micrograph of PDDA – ATP coacervate droplets containing both labels. (b) A composite image of both Cyanine 5 (red) and fluorescein (green) fluorescence imaging channels showing that droplets exhibited fluorescence in both channels. (c) and (d) show the single channel images, Cyanine 5 and fluorescein, respectively, combined to form the image in (b). All scale bars are µm.

up opportunities to perform studies involving transport as well as chemical and biological interactions between droplets loaded with different materials.
5.4 Conclusions

In summary, we have shown that stable membrane-free aqueous coacervate droplets can be formed in water using a microfluidic flow-focusing channel structure, and that this technique offers unique advantages compared to the traditional vortex method. The excellent stability of the droplets, which could be preserved without merging at room temperature for periods longer than six days, can only be achieved through the laminar flow break-up process afforded by the low Reynolds Number (Re \( \leq 2100 \)) regimes encountered in microfluidic channels. The approach additionally offers the ability to form distinct coacervate populations of differing chemical composition in parallel, without detriment to coacervate stability or size distribution. This opens up the possibility to study interactions between droplets loaded with different materials. Current studies are focused on the encapsulation of cellular components such as the gene expression and protein synthesis machinery, and the viability of coacervate droplets as artificial cell systems.
Chapter 6

A chip-to-world connector with a built-in reservoir for simple small-volume sample injection

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We present a novel connector that allows for easy handling and injection of sample volumes between 1 and 20 µl. All tubing connections between external pumps and the microfluidic device are established before the sample is introduced into a sealable reservoir built into the connector. This approach allows for multiple injections of small sample volumes without the need to dismantle the chip-tubing assembly. We demonstrate that the connector reservoir seal can withstand pressures of up to 6 bar, that opening or closing the reservoir does not dislocate the sample by more than 35 nl, and that the connector can be used for injecting samples into both miscible and immiscible carrier fluids.

6.1 Introduction

One of the most common practical challenges encountered when working with microfluidic systems is the realization of a robust interface between the device and the outside world. A variety of connection systems have been developed or adapted for use with chip-based microfluidic devices. These include small hollow steel cylinders connecting plastic tubing to input holes punched into elastomeric substrates, pressure-seal chromatography connectors, D-subminiature connectors, Fit-to-Flow interconnects, gaskets and manifolds, or simply direct insertion of tubing straight into the device. However, regardless of the connection system, the handling of fluid volumes of the order of 20 microlitres or less using standard connectors and tubing remains a challenge. This is because in order to efficiently inject such a small volume into a microfluidic device, one cannot simply load the sample into a syringe or pressurised vessel and then transfer it through several millimetres or centimetres of tubing. Such an approach is constrained by the accumulated dead volume of tubing, connectors, containers, and syringes, meaning that either the sample volume must be significantly larger than what is actually injected, or that a carrier fluid must be used to push the sample through.

A common procedure for introducing small-volume samples into a microfluidic channel involves loading the sample into the device-end of narrow bore tubing, which is then inserted into the chip. Unfortunately, this approach often results in the introduction of gas bubbles, or premature injection of the sample into the channels due to pressure fluctuations during insertion. In the worst case, the sample may be completely lost if the end of the tubing suffers accidental vibration prior to connection. An alternative approach is to pipette a droplet of sample directly over the channel inlet and draw it into the microfluidic system by applying vacuum at the outlet. However, this method also introduces bubbles into the device if any air is trapped between the sample and the inlet, and also greatly limits flow control possibilities due to the fact that suction must be applied at the outlets and at least one inlet must be left open to the atmosphere. It goes without saying that handling multiple small samples becomes exceptionally difficult in situations where this approach is taken. Furthermore, while the sample stands open to air over the inlet, it is vulnerable to contamination, evaporation, or dislocation. It should be noted that an extremely effective approach to handling small volume samples is to integrate sample reservoirs within the chip substrate itself, and implement integrated pumping systems, such as those based on ”Quake valves” or electrokinetic...
Herein we propose a new approach to fluidic interconnection (Figure 6.1) that provides a simple and direct interface between external tubing and a microfluidic device, is compatible with conventional syringe and pressure pumps, and allows the user to introduce samples by pipetting into a sealable reservoir built into the connector. The connector has three openings: one that connects to the chip, one to interface with tubing and external pumps, and one for introducing sample. All tubing and pump connections are made prior to sample introduction, de-coupling the process of sample loading from fluidic network assembly. The sample reservoir is sealed by a sliding cap, which minimises pressure oscillations during opening and closing and thus avoids premature sample injection. Importantly, if the reservoir is capped while overflowing, air bubbles are not introduced into the chamber. Finally, it should be noted that the re-sealable sliding cap can be reopened for the introduction of additional sample without requiring any tubing disconnection. Figure 6.2
Chapter 6: A chip-to-world connector with a built-in reservoir

6.2 Interconnect fabrication

The connector is manufactured using standard mechanical workshop milling techniques (AutoDesk Inventor 2013 technical 3D drawing available in ESI). The main body of the connector and the sliding cap are milled from steel or polyether ether ketone (PEEK). Hollow steel pin connectors (Instech Laboratories, USA) with a 1 mm outer diameter are used to link the built-in reservoir to the microfluidic channel inlet. The pins are fixed into a 0.98 mm hole drilled through the connector into the reservoir. In the current study, we use either hollow steel pin connectors or Vici 6-40 threaded PEEK fittings (Vici AG, Switzerland) to interface with external tubing, though the design may be modified for compatibility with other commercially available connector systems. When a Vici 6-40 fitting is used to connect 1/16th inch outer diameter tubing, a 4 mm hole is drilled and tapped with 40 threads per inch. The reservoir inside the connector has a maximum volumetric capacity of 20 µl. It is closed by a manually-operated sliding cap, which is also milled from steel or PEEK, whilst the seal is provided by a 1 mm thick Viton rubber O-ring (Georg Rutz AG, Switzerland) with a 2.5 mm inner diameter. The O-ring is stretched and inserted into a 1 mm wide groove with a 3 mm inner diameter and a depth of 0.8 mm milled into the bottom of the sliding cap. Since the connector can be fabricated from a variety of materials, the surface characteristics of the reservoir and internal vias can be tailored to different requirements. Even though stainless steel is generally compatible with many chemical and biological applications, its surface can, for example, be electroplated with gold or other metals or grafted with PEG for improved biocompatibility. Moreover, due to the flexible design and simple manufacturing process, multiple inputs can be integrated into the same connector block, allowing several input flow streams to be handled simultaneously. In the current work, we show the use of up to three inputs (Figure 6.3).

Figure 6.2 Schematic of connector operation. (a) Connect all tubing, pumps, and microfluidic device according to experiment. (b) Fill the device with a carrier fluid. (c) Open the connector lid, and pipette the sample into the reservoir. (d) Close the connector lid. (e) Pump the sample into the microfluidic channels.
6.3 Interconnect testing and operation

The pressure tolerance of the connector was tested by connecting it to sealed tubing at one end, and to a compressed air source at the other. Air pressure was then increased while the connector was submerged in water and monitored for the emergence of air bubbles. For repeated use testing, the cap was completely removed and reinserted before the pressure tolerance was reassessed. It was found that the seals within the connector were able to withstand at least 6 bar of pressure without leaking. This is well beyond the requirements of most microfluidic applications, which typically operate in pressure regimes of 2 bar or less. It was also found that even after 250 cycles of opening and closing the reservoir, the sliding cap seal did not leak under 6 bar of pressure. This open/close cycle test was repeated with five different O-rings, with similar results, demonstrating that the quality of the seal provided by the connector assembly is well suited for most microfluidic applications.

The occurrence of sample dislocation during the opening and closing of the reservoir was also investigated. The connector reservoir and tubing were completely filled with mineral oil, clamped down, and connected to a graduated 5 µl glass capillary (minimum graduations of 15 mm per 1 µl) via flexible silicone tubing. As the capillary was connected, oil flowed into it, forming an oil-air (O/A) interface inside. The reservoir was then opened and the loose end of the capillary raised in order
Figure 6.4 A schematic of the sample displacement test setup. The connector is linked to a 5 µl graduated glass capillary via flexible silicone tubing at the output, and to sealed plastic tubing at the input. The system is filled with oil such that an oil-air interface is formed inside the glass capillary, and the capillary raised so that there is no movement of fluid while the reservoir is left open. To measure sample displacement, the position of this interface is observed during opening and closing of the sliding cap. For clarity, the clamping system used to prevent unwanted movement of the connector and tubing is not shown.

to balance gravitational and capillary forces acting on the fluid. An illustration of the process is provided in Figure 6.4. When the O/A interface was stationary, the sliding cap was closed and opened repeatedly (10 cycles), and the position of the O/A interface observed during each operation. The distance moved by the O/A interface during opening or closing was never more than 500 µm, corresponding to a maximum sample displacement smaller than 35 nl. These data show that the connector is effective at preventing untimely injection of the sample into the microchannels, since the dead volume inside the connector pin is far greater than 35 nl. Importantly, this means that the interconnect is ideally suited to applications where injection timing and sequence are important. It should be noted that typical disturbances such as bending of the tubing normally dislocate volumes greater than 35 nl.

Finally, to demonstrate sample injection, we inserted the connector into a simple straight-channel silicone elastomer microfluidic chip. The whole system, including tubing, channels, and connector reservoir, was flooded with carrier fluid. A 5 µl sample of aqueous food colouring was then pipetted into the reservoir, the cap closed, and the sample injected into the channel via a syringe pump at 10 µl/min. As depicted in Figure 6.5(a), sample injection was completed in precisely 30 seconds when mineral oil containing 2% Span80 (Sigma, USA) was used as a carrier fluid, which is to be expected for a 5 µl sample flowing at 10 µl/min. When water was used as the carrier fluid, however, both the leading and trailing ends of the sample plug were diffuse into the carrier fluid. As shown in Figure 6.5(b), the injection lasted much longer than 30 seconds due to sample dilution.

6.4 Conclusions

We have demonstrated a robust chip-to-world connector that allows on-demand injection of small-volume samples (1-20 µl) into microfluidic devices while still allowing for external pumps to be connected via conventional tubing. The connector allows
for sample loading to be performed after all tubing assemblies have been made, thus eliminating the risk of sample loss or premature injection. The connector is inexpensive to manufacture, while its simple operation and reusability allow for multiple samples to be injected sequentially. Due to its flexible design, connector blocks with multiple tubing-reservoir-inlet interfaces can be manufactured and operated independently.

**Figure 6.5** A timed sequence of images taken of the connector mounted on a simple straight-channel silicone elastomer microfluidic chip and being used to inject a 5 µl sample of food colouring at 10 µl/min. The sample is injected with mineral oil containing 2% Span80 (a) and water (b) as carrier fluids. It takes exactly thirty seconds to inject the entire sample when using an oil carrier, while the start and end of the sample plug are both diffuse when using a water carrier. Movie files are available in the electronic supplementary information.
Chapter 7

Coacervate-encapsulated \textit{in-vitro} gene expression

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7.1 Introduction

Artificial cells are synthetic entities that possess a basic set of components able to reproduce or mimic natural cell function, such as compartmentalisation, gene expression, metabolism, or replication. Because of their ability to exploit individual biological machines, artificial cells have proven useful in a range of applications in biotechnology. In recent years, a growing number of approaches for producing artificial cells have been developed. These include the use of liposomes, polymerosomes, and even picolitre volume containers structured within microfluidic devices. A common theme explored in all these approaches is the encapsulation of a cellular extract, used for in-vitro gene expression, by some form of physical barrier such as a lipid or polymer membrane, or a porous but rigid wall. For example, Noireaux et al. demonstrated the extended gene expression capabilities of an in-vitro gene expression system (IVTT) encapsulated within lipid vesicles. Similarly, Martino and colleagues created polymerosomes in which proteins were synthesised and incorporated within the hydrophobic membrane or released into the external medium. Using a lab-on-a-chip approach, both Sinti et al. and Karzbrun et al. independently demonstrated the expression of proteins within compartments in microfluidic devices. Finally, gene expression within water-in-oil droplets have been extensively used as a platform for directed evolution based on in-vitro gene expression.

In the present study, we demonstrate that the microfluidic methods developed in Chapters 3 and 5 for the production of coacervate droplets in water can be directly applied to the formation of membrane-free artificial cells. In contrast to approaches that encapsulate biological materials using a physical barrier (such as a membrane) coacervate droplets achieve encapsulation by phase-separation induced by molecular charge interactions. We exploit the ability of poly-Lysine (Plys) and carboxymethyl Dextran (CMDextran) to form a separate coacervate phase when mixed together in water under specific conditions as a platform to compartmentalise all the components necessary for transcribing and translating DNA into protein. As a proof of concept, we demonstrate this capability by expressing the fluorescent protein mCherry in such membrane-free coacervate artificial cells. It is of central importance to note that this could only be achieved through adoption of microfluidic tools, since coacervate droplets formed via conventional vortexing methods are not sufficiently stable for the extended periods required to sustain both transcription and translation reactions.

7.2 Materials and methods

A commercially available in-vitro transcription translation (IVTT) kit (Expressway Cell-Free E.coli Expression System, Invitrogen) composed of S30 E.coli cell lysate, reaction buffer, proprietary T7 polymerase mix, feed buffer and amino acid mixture, was used to perform in-vitro gene expression. The IVTT kit was prepared according to manufacturer instructions, under sterile conditions and on ice. Briefly: 20 µl of reaction buffer, 25 µl of feed buffer, 4.5 µl of a 50 mM amino acid solution, 1 µl of proprietary T7 polymerase mix and 1 µg plasmid were added to 20 µl of S30 E.coli extract. This IVTT reaction mixture was then combined with different polymer
or coacervate mixtures according experimental needs. The pEXT5-NT/CALM3 plasmid encoding for the non-fluorescent CALM3 protein, provided with the IVTT kit, was used as a control. The plasmid pEXT5-NT/mCherry (prepared by Dr. Dora Tang at University of Bristol) was used to express mCherry fluorescent protein.

### 7.3 Results and discussion

First, the ability of coacervates to incorporate the IVTT reaction mixture and perform transcription and translation of a plasmid into protein was assessed. Coacervate was prepared from carboxymethyl Dextran and polyLysine mixed at a 4:1 monomer molar ratio, at pH 8. This mixture was then centrifuged at 5000g for 10 minutes to allow separation of the denser coacervate phase from the aqueous supernatant, which was subsequently removed. The remaining bulk coacervate was then added to the IVTT reaction mixture at a volumetric ratio of 4:3. This IVTT/coacervate mixture, containing the pEXT5-NT/mCherry, was then homogenised, incubated at 18 °C and agitated at 180 rpm for 24 hours. Figure 7.1 demonstrates that the mixture remains homogeneous (i.e. shows no evidence of phase-separation) throughout the entire incubation period, indicating that the coacervate material is successful in incorporating the IVTT reaction mixture.

Next, IVTT/coacervate droplets containing pEXP5-NT/mCherry plasmid in 0.02 M HEPES buffer were generated using the conventional vortex method (see Chapter 3). The resulting droplets were unstable, and coalesced within minutes after formation and before imaging could be performed (Figure 7.3a). Since the IVTT reaction takes place over a period of 6 hours, it was not possible to express mCherry in IVTT/coacervate droplets produced by the vortex method.

Next, we formed IVTT/coacervate droplets using the previously developed flow-focusing microfluidic channel geometry (Chapter 5). Details regarding the fabrication methods used and device characteristics are described in Chapter 3. Since the volume of the IVTT/coacervate reaction mixture typically less than 50 µl, the interconnect described in Chapter 6 was used to allow reproducible injection of the sample into the microfluidic system. Using this microfluidic technique, bulk IVTT/coacervate could be redispersed as droplets into a stream of 0.02 M HEPES buffer. After formation, droplets were collected in a clear-bottom 384-well plate for imaging. A sterile environment was maintained using a nearby flame throughout

![Figure 7.1 Images of the IVTT kit, containing the pEXP5-NT/mCherry plasmid, and bulk coacervate (carboxymethyl-Dextran and poly-Lysine, 4:1 molar ratio) being mixed and incubated in an Eppendorf tube at 3:4 volume ratio. a) The IVTT mixture sitting above the bulk coacervate prior to mixing. b) The two components form a single homogeneous phase after mixing. c) The mixture remains homogeneous after incubation for 24 hours. Note the change in colour from colourless to light red, as expected from the expression of mCherry fluorescent protein.](image-url)
both the sample loading and collection steps.

As shown in Figure 7.2, IVTT/coacervate droplets containing both pEXP5-NT/mCherry and pEXP5-NT/CALM3 plasmids were successfully produced using the defined microfluidic method. Moreover, although many larger droplets could be seen to wet the surface of the collection vessel after a few hours, droplets formed using the microfluidic method remained phase separated from the surrounding HEPES buffer for periods of at least 40 hours after formation (Figure 7.3b-c). Such timescales were deemed to be more than ample time to afford the expression of both mCherry and CALM3 proteins within the IVTT/coacervate droplets.

Fluorescence measurements of IVTT/coacervate droplets (expressing mCherry) formed using the microfluidic flow-focusing method were performed using an inverted
Results and discussion

A microscope (Eclipse Ti-U, Nikon, Japan) equipped with a wide-field camera (Cool-snap HQ2, Photometrics, USA). Acquisition was controlled using Micro Manager software (REF). Image analysis was performed using Volocity® 3D image analysis software (Perkin Elmer, USA) to extract information regarding droplet size, mean fluorescence intensity per droplet surface area, and mean background fluorescence intensity. The background signal was then subtracted from the signal originating within the droplets and plotted against droplet surface area in pixels. This was performed for measurements taken approximately 1 hour after droplet formation, and again after 16 hours of incubation at 18 °C and agitation at 180 rpm. Finally, a linear fit was applied to the data and the gradients (representing the change in intensity per pixel) compared, with an increase in the gradient signifying a corresponding increase in the total fluorescence per droplet. Performing the analysis in this manner allows direct comparison of images originating from different samples. As can be seen in Figure 7.4, such an analysis of fluorescence images shows that there is small but significant increase in fluorescence over an incubation period of 16 hours in the sample expressing mCherry compared to the sample expressing the CALM3 control. Specifically, coacervate droplets containing the pEXP5-NT/mCherry plasmid exhibited a mean pixel intensity per droplet surface area of 3.20×10⁻⁵ ± 5.94×10⁻⁶ au (arbitrary units) at 1 hour, and 7.71×10⁻⁵ ± 1.22×10⁻⁵ au at 16 hours, while droplets

Figure 7.3 IVTT/coacervate droplets produced using the vortex method coalesced into a buk phase before imaging could be performed (a). The same IVTT/coacervate dispersed into droplets using the microfluidic flow-focusing method is shown in (b) right after formation and in (c) after incubation at 18 °C and shaking at 180 rpm for 40 hours. Scale bars are 25 µm.

Figure 7.4 Fluorescence measurements of gene expression in IVTT/coacervate droplets after 16 hours incubation at 18 °C and with agitation at 180 rpm for 40 hours. a) A micrograph showing several droplets including an insert showing the fluorescence signal detected using filters for mCherry. The scale bars are 25 µm in length. b) Variation of the mean pixel fluorescence intensity per droplet surface area as a function of pixel number for droplets expressing mCherry, with a gradient dI/dP = 3.20×10⁻⁵ ± 5.94×10⁻⁶ au (arbitrary units) at 1 hour (red), and 7.71×10⁻⁵ ± 1.22×10⁻⁵ au after 16 hours of incubation (black), c) illustrates a similar plot for droplets expressing CALML3, with dI/dP = 2.13×10⁻⁵ ± 1.22×10⁻⁵ au at 1 hour and 2.34×10⁻⁵ ± 4.99×10⁻⁵ au at 16 hours.
Figure 7.5 Confocal measurements of protein-expressing IVTT/coacervate droplets. a) Brightfield images of a sub-population of droplets producing mCherry and b) the CALML3 control; c) fluorescence images of sample producing mCherry and d) CALML3 control; and e) fluorescence emission spectrum of the sample producing mCherry and f) CALML3 control. Excitation was performed at 561 nm. All scale bars are 50 µm.

containing the pEXP5-NT/CALM3 plasmid exhibited $2.13 \times 10^{-5} \pm 1.22 \times 10^{-5}$ au at 1 hour and $2.34 \times 10^{-5} \pm 4.99 \times 10^{-5}$ au at 16 hours.

Although this increase in fluorescence signal in the pEXP5-NT/mCherry sample is almost certainly a result of the expression of mCherry, since our results show that the IVTT/coacervate droplets exhibit significant autofluorescence at similar wavelengths to mCherry, a change in signal for reasons other than mCherry fluorescence cannot be entirely ruled out. In order to establish definitively that the fluorescence variations originate from the mCherry protein, spectral measurements of droplets were performed using a confocal microscope (SP8, Leica, Germany). Using a 10x magnification objective lens (Nikon, Japan), both pEXP5-NT/mCherry and pEXP5-NT/CALM3 samples were excited using a 561 nm laser (Leica, Germany) and a spectral scan performed at emission wavelengths between 600 and 750
nm with a bandwidth of 5 nm and an increment of 3 nm using hybrid detectors. Figure 7.5 shows brightfield and fluorescence confocal images of the two samples, as well as the associated spectra.

Combined, these results show for the very first time that IVTT/coacervate droplets produced using our microfluidic flow-focusing technique are capable of encapsulating the transcription and translation reactions required to express protein from plasmid DNA. It is noted, that the fluorescence signals originating from coacervate droplets producing mCherry were only moderately increased when compared to background fluorescence observed from the CALM3 controls. It is possible and even likely that a significant proportion of the produced proteins were unable to fold properly due to the charged and crowded environment of the coacervate. Alternatively, it is also possible that the small-molecule components of the IVTT kit, such as amino acids and ions, were able to diffuse out of the coacervate phase and into the external aqueous environment.

7.4 Conclusions

In summary, we have shown that the defined microfluidic method for forming coacervate droplets can be used to compartmentalise reagents necessary for in-vitro transcription translation. The enhanced droplet stability afforded by the laminar flow conditions encountered during formation in the microfluidic channels allows droplets to maintain their integrity over extended periods of time, and most importantly throughout the duration of the transcription and translation reactions. This results in the successful production of mCherry fluorescent protein from a DNA plasmid template. Despite this initial success, further optimisation of the IVTT reaction within the coacervate phase is need to improve achievable protein yields. The possibility of component leakage from coacervate droplets may be addressed through the incorporation of a selectively permeable membrane around the coacervate droplets. Although this would require one or more steps to be added to the droplet formation process, it should not alter the nature of the encapsulated coacervate and IVTT kit mixture. Another issue that is currently being addressed is the wetting of coacervate droplets on the bottom surface of the collecting 384-well plates. Although such wetting did not significantly affect the reactions contained within the droplets, it may pose a problem in experimental situations where droplets need to be moved after incubation. That aside, the defined approach provides for a crowded molecular environment, a unique property not found in other artificial cell constructs. Such a situation more closely resembles the environment inside living cells and opens the possibility of exploring molecular crowding as a controllable experimental parameter in artificial cell models. Taken together, the results presented here are a significant step forward in the development of artificial cell platforms.
Chapter 8

Conclusions and outlook
The work presented herein has described the development of microfluidic tools and techniques for the controlled formation of artificial cells based on coacervate materials. Specifically, a flow-focusing microfluidic channel structure possessing hydrophobic surfaces, along with a compatible coacervate chemical system, were developed through an iterative prototyping process. These were then successfully utilized to encapsulate DNA and *in-vitro* transcription and translation (IVTT) reaction components within coacervate droplets, with the successful production of mCherry fluorescent protein from a plasmid DNA template.

Although such developments represent a significant step towards the engineering of artificial cell mimics, the expression of protein within the resulting artificial cells was not optimal. This aspect represents a key future challenge in creating a robust “cell synthesis” platform. In particular, studies should focus on understanding the precise reasons why transcription and translation reactions are sub-optimal. It is likely that leakage of ions and other small molecules from the coacervate droplets, due to the absence of a membrane, is a significant contributory factor in this respect. Since the IVTT reaction mixture is highly sensitive to salt balance and concentration, leakage of smaller components from the coacervate matrix into the external aqueous phase will interfere to some extent with gene expression. This hypothesis could be tested by encapsulating IVTT-coacervate droplets within membranes, based on liposomes or polymersomes. Alternatively, the IVTT-coacervate could be dispersed as droplets into squalene, as performed (without the IVTT mixture) in initial experiments described herein. Another possible explanation for the poor performance of IVTT reactions is that the coacervate material itself interferes with the IVTT components. The highly charged polyelectrolyte molecules comprising the coacervate may interact with or adsorb to molecular components of the IVTT mixture, perhaps by affecting RNA polymerase binding and transcription of DNA, or by altering the conformation of ribosomes or their interaction with transfer-RNA molecules. The possibility of coacervate interference would be best studied by comparing the performance of a pure IVTT reaction with that containing the coacervate material without forming droplets.

Beyond determining the factors that affect gene expression, there is still scope to optimize the droplet-bound IVTT reaction itself. For example, it is well established that parameters such as temperature, the concentration of DNA, dilution factor of the IVTT mixture, and the amount and concentration of reaction nutrient components such as ribonucleotides, amino acids, and ATP affect both the extent and rate of the IVTT reactions. Although, beyond the scope of the work presented herein, it would be desirable to find the optimal conditions (or design rules) for which specific IVTT reactions can take place within coacervate droplet systems.

Incorporating a membrane into an IVTT-coacervate droplet is also a potential solution to the droplet stability problems. In such a scenario, the membrane would surround the coacervate droplet and present a comparatively less interactive lipid surface to the external environment. This has already been achieved by Tang et al. in other work, albeit only with coacervate droplets prepared by the vortex method and without encapsulation of IVTT reagents. Another potential solution is to assay longer chain polyelectrolytes than the ones used in herein. It is likely that longer chains would “entangle” more extensively, making it harder to dismantle the droplet by intercalating other molecules within the coacervate matrix.
In addition to addressing the problems encountered in the present studies, there is also a wide scope to focus on new concepts. For example, the artificial cells could be used as a platform to study directed evolution of biochemical systems and pathways. Interestingly, the communication between artificial cells could also be studied through the use of assays for detecting the transport of different molecules between droplets. Indeed, it may also be possible to study the effects of molecular crowding on processes such as peptide assembly at the ribosome or the folding of resulting proteins. It should not be forgotten that there are also other materials whose potential to form coacervate-based artificial cells could be explored. In particular, DNA stands out as a compelling option due to its potential as a primordial form of life. Other polypeptides or polysaccharides may also be interesting alternatives.

The incorporation of lipid membranes (resembling those of living cells) around coacervate droplets presents a number of inviting possibilities. For example, membrane proteins could be expressed within the droplets. This is particularly attractive because many energy-generating systems such as ATP-synthase rely on membrane-bound proteins. Similarly, functions such as cell locomotion and surface adhesion are performed by proteins incorporated into the lipid bilayer with moieties projecting into the extra-cellular environment. By expressing cyto-skeletal proteins within the artificial cells, it may be possible to study how the structures they form interact with the membrane or external objects and surfaces. In such studies, the fact that the coacervate phase within the membrane would more closely match the viscosity and other physical properties of the cytosol of living cells would be a key advantage and would likely yield different results when compared to similar work already performed using liposomes.

There are also possibilities to build upon and improve on the studies described here from an engineering perspective. Although the MATLAB video analysis method was too cumbersome to be employed in the present work, it may be possible to improve such an approach to a point where it could be used as part of a feedback control mechanism to modulate the formation of coacervate droplets in real time. This would allow for the exploration of the effects of flow conditions on the size, size distribution, and stability of the resulting droplets.

The collection of coacervate droplets is also an area that can be refined in future studies. In the present work, droplets were collected into clear-bottom 96- or 384-well plates. Since the plate bottom substrates were plastic and thicker than 650 µm, it was not possible to use high-resolution objectives during microscopy, since these typically possess a working distance less than 170 µm. In addition, due to the fact that the coacervate droplets adsorbed to glass, it was not possible to collect them onto thin glass cover slips that would have allowed high-resolution imaging. The value of being able to perform higher resolution and more sensitive optical measurements cannot be understated in this respect. As described in Chapter 7, the fluorescence signal detected in the artificial cell samples expressing mCherry fluorescent protein was relatively low. This may have been in part due to the fact that imaging was performed using a long working distance 40x objective, with relatively low light-gathering capability. Improving the imaging setup to use a more capable objective lens is likely to yield significant improvements in light recovery. Achieving a higher optical resolution would also be of immense practical value in further studies involving membranes that incorporate membrane or cytoskeletal proteins.
In closing, the work presented in this thesis aimed to develop novel techniques and tools necessary for successfully forming artificial cells. Although the study and application of artificial cells is itself a nascent discipline, with vast scope for future work, the results obtained here represent significant initial strides in exploring this new field.
Appendix A

Supporting information for Chapter 4: Measurement and analysis of droplet size
A.1 Function: avibgremove.m

% This function calculates the background image of an input video based on the single pixel average intensity for each pixel across the entire video. The inputs are:
% input - video to be analysed
% average - type of average (0 = mode, 1 = mean, 2 = median)

function output = avibgremove(input,average)

% Determine number of frames
nFrames = size(input,2);

% Determine video dimensions
vidWidth = size(input(1,1).cdata,2);
vidHeight = size(input(1,1).cdata,1);

% Define colormap
map = colormap(gray(256));

% initialise stack array for speed
stack = zeros(vidHeight, vidWidth, nFrames);

% Convert video data structure to stack of images
for n = 1 : nFrames
    stack(:,:,n) = input(n).cdata;
end

% Pre-allocate array for speed
backgroundPixel = zeros(vidHeight,vidWidth);

% Loop calculating pixel average
parfor i = 1 : vidHeight
    for j = 1 : vidWidth
        if average == 0
            backgroundPixel(i,j) = mode(stack(i,j,:));
        elseif average == 1
            backgroundPixel(i,j) = mean(stack(i,j,:));
        elseif average == 2
            backgroundPixel(i,j) = median(stack(i,j,:));
        else
            error('avibgremove:argChk', ...
                  'Wrong input for average: choose 0 (mode), 1 (mean), or 2 (median).');
        end
    end
end

% Create data structure for processed output video
processed(1:nFrames) = struct('cdata',zeros(vidHeight,vidWidth,1,'uint8'),'colormap',map);

% Remove background - Subtract average from original
parfor n = 1 : nFrames
    processed(n).cdata = input(n).cdata - uint8(backgroundPixel);
end

% Display background image
% imshow(uint8(backgroundPixel));

output = processed;
end
A.2 Function: avibwinvert.m

% This function performs a black/white inversion of an input video. The inputs are:
% input = video to be processed

function output = avibwinvert(input)

% Determine number of frames
nFrames = size(input,2);

% Define colormap
map = colormap(gray(256));

% Determine video dimensions
vidWidth = size(input(1,1).cdata,2);
vidHeight = size(input(1,1).cdata,1);

% Create output movie data structure
processed(1:nFrames) = struct('cdata',zeros(vidHeight,vidWidth,1,'uint8'),'colormap',map);

% Copy data from input structure to an image stack, invert grayscale, and copy back to an output structure
for n = 1 : nFrames
    stack(:,:,n) = input(n).cdata;
    invstack(:,:,n) = imcomplement(stack(:,:,n));
    processed(n).cdata = invstack(:,:,n);
end

output = processed;
A.3 Function: avithreshold.m

% This function accepts the data structure created by the avibgremove.m
% function. It is part of the image processing flow. thresMult allows a
% small scalar adjustment to the calculated threshold.

function output = avithreshold(input,thresMult)

% Find number of frames
nFrames = size(input,2);

% Define colormap
map = colormap(gray(256));

% Determine video dimensions
vidWidth = size(input(1,1).cdata,2);
vidHeight = size(input(1,1).cdata,1);

% Copy data from input structure to an image stack
for n = 1 : nFrames
    stack(:,:,n) = input(n).cdata;
end

threshold = graythresh(stack)*thresMult; % calculates a global threshold value for stack

% initialise bw array for speed
bw = zeros(vidHeight, vidWidth, nFrames);

parfor n = 1 : nFrames
    bw(:,:,n) = im2bw(stack(:,:,n),threshold); % create bw image of frame based on threshold
end

% Create output movie data structure
movthres(1:nFrames) = struct('cdata',zeros(size(bw,1),size(bw,2),1,'uint8'),'colormap',map);

% Convert binary to uint8 with 1 set to 255 => binary to grayscale extremes
for n = 1 : nFrames
    movthres(n).cdata = uint8(bw(:,:,n)*255);
end

output = movthres;
end
A.4 Function: avibwsmooth.m

% This function erodes and dilates the thresholded video. The inputs are the data  
% structure produced by the avithreshold() function, and the number of  
% pixels to use in imclose() and erode/dilate functions (clval and erval).

function output = avibwsmooth(input,clval,erval,dilval)

% Convert avithreshold() output.cdata to 3D matrix  
nFrames = size(input,2);

% Determine video dimensions  
vidWidth = size(input(1,1).cdata,2);  
vidHeight = size(input(1,1).cdata,1);  

% initialise stack array for speed  
stack = zeros(vidHeight, vidWidth, nFrames);

for n = 1 : nFrames
    stack(:,:,n) = input(n).cdata;
end

% Add image processing code here  
threshold = graythresh(stack);

% initialise bw array for speed  
bwout = zeros(vidHeight, vidWidth, nFrames);

parfor n = 1 : nFrames  
    bw = im2bw(stack(:,:,n),threshold); % create bw image of frame based on threshold  
    bw = imclose(bw,strel('disk',clval));  
    bw = imerode(bw,strel('disk',erval));  
    bw = imdilate(bw,strel('disk',dilval));
    bwout(:,:,n) = bw;
end

% Define colormap  
map = colormap(gray(256));

% Create output movie data structure  
movsmoothed(1:nFrames) = struct('cdata',zeros(vidHeight,vidWidth,1,'uint8'),'colormap',map);

for n = 1 : nFrames
    output(n).cdata = uint8(bwout(:,:,n)*255);
end

end
A.5  Function: avianalyseparticles.m

% This function performs a particle analysis on a black and
% white video by calculating particle connectivity and then
% determining the center, radius, diameter, area, and volume
% of each particle detected.

function avigeometry = avianalyseparticles(input)

% Convert avithreshold() output.cdata to 3D matrix
nFrames = size(input,2);

% Determine video dimensions
vidWidth = size(input(1,1).cdata,2);
vidHeight = size(input(1,1).cdata,1);

% initialise stack array for speed
stack = zeros(vidHeight, vidWidth, nFrames);

% copy data from structure to array
for n = 1 : nFrames
    stack(:,:,n) = input(n).cdata;
end

% calculate stack threshold
threshold = graythresh(stack);

% initialise bw array for speed
bw = zeros(vidHeight, vidWidth, nFrames);

parfor n = 1 : nFrames
    bw(:,:,n) = im2bw(stack(:,:,n),threshold); % create bw image of frame based on threshold
end

% initialises vector to store particle area data
partA = [];

% Perform image connectivity and particle analysis in parallel
parfor n = 1 : nFrames
    % Compute object index
    cc = bwconncomp(bw(:,:,n), 8);

    % Extract individual particle data
    partData = regionprops(cc,'basic');
    partA = horzcat(partA,[partData.Area]);

% Calculate particle geometry
partR = sqrt(partA/pi);
partD = 2*partR;
partV = (4/3)*pi*(partR.^3);

% Create particle geometry data structure
avigeometry = struct('Area',partA,'Radius',partR,'Diameter',partD, ...
    'Volume',partV);
end
A.6 Function: findcircles.m

% This function detects, locates, and measures the radius
% of circular objects in an image. The image may be black
% and white, grayscale, or colour. The inputs are:
% image = the image to be processed
% radiusRange = the range of radii to be detected
% EdgeThreshold = the level of ‘blurriness’ of acceptable edges

function [centers, radii] = findcircles(radiusRange, image, EdgeThreshold)

centers = [];
radii = [];
circleData = struct('Center', centers, 'Radius', radii);

for i = 1 : length(radiusRange)
    [centers, radii, ~] = imfindcircles(image, radiusRange(i,:), 'EdgeThreshold', EdgeThreshold);
    circleData.Center = vertcat(circleData.Center, centers);
    circleData.Radius = vertcat(circleData.Radius, radii);
end;

[centers, radii] = RemoveOverLap(circleData.Center, circleData.Radius, 10, 2);

% RemoveOverLap.m function available at:
% http://www.mathworks.com/matlabcentral/fileexchange/42370-circles-overlap-remover

% RemoveOverLap.m function requires snip.m function, available at:
A.7 Function: imageprocess.m

% This function is used to detect circular structures in a series of images. It scans through % directories according to defined file and directory name patterns, opens the image files, % and sends them to the function "findcircles.m" for individual processing. The output of % "findcircles.m" is then saved to the directory containing the image. % This function has no inputs.

% Specify number of files in sequence
n = 1;

% Specify the file to be processed
nameStart = '40x objective'; % initial non-changing part of file name
seqDigits = '%06d'; % number of digits in sequence indexing
nameEnd = '.tif'; % final non-changing part of file name

% Specify range of particle radii
radiusRange = [5, 10; 10, 20; 20, 40; 40, 80; 80, 160; 160, 240; 240, 300; 300, 400; 400, 500;];

% Specify edge detection threshold (in case of blurry images)
EdgeThreshold = 0.01;

% Note: When tweaking the detection parameters above, keep in mind that % this program disregards one of two circles that overlap with more than 10 % pixels. To change this, edit line 14 of findcircles.m - more information % about this behaviour can be found in RemoveOverLap.m.

% Folder handling
rootFolder = pwd;
templateFolder = '2013.02.27 - 8.5kDa PDDA+ATP in water_';
folderIndex = '%03d';
start = 21;
finish = 24;
aggRadii = [];
for k = start:finish
    foldername = [templateFolder num2str(k,folderIndex)];
    cd(foldername);
    centers = [];
    radii = [];
    for i = 1:n
        % Select next file to be processed
        filename = [nameStart num2str(i-1,seqDigits) nameEnd];
        image = imread(filename);
        [c,r] = findcircles(radiusRange,image,EdgeThreshold);
        centers = vertcat(centers,c);
        radii = vertcat(radii,r);
    end;
    save('analysis.mat','centers','EdgeThreshold','radii','radiusRange');
    aggRadii = vertcat(aggRadii,radii);
    hist(aggRadii,20);
    cd(rootFolder);

end;
Appendix B

Supporting information for Chapter 7: Coacervate-encapsulated \textit{in-vitro} gene expression

Published in parts as:

Disclaimer: Authors Dirk van Swaay and Dora Tang contributed equally to the published work. In the interest of completeness, and to aid comprehension of the work described in Chapter 7, the present appendix describes work performed by Dora Tang and/or the remaining authors without the participation of Dirk van Swaay.
B.1 Expression of pBAD/mCherry in \textit{E. coli}

mCherry was expressed from the pBAD/mCherry plasmid (gift from Dafydd Jones, University of Cardiff) by transforming 1 µl of plasmid DNA into 50 µl of Top10 competent cells. The vector was added to the competent cells in ice and incubated for 30 minutes, heat-shocked at 42 °C for 30 seconds and then placed in ice for 2 minutes. 1 ml of SOC media was added to the cells, which were then incubated for 1 hour at 37 °C with agitation at 180 rpm. The cells were rescued and plated onto LB agar/carbenicillin medium and left at 37 °C overnight. A single colony was selected and added to 6 ml LB broth media and incubated at 37 °C with agitation at 250 rpm until the OD (optical density) at 600 nm was greater than 1 (approximately 12 hours). The saturated cells were then added to pre-warmed media (1 litre, 37 °C) with 1 ml of 50 µg/ml of carbenicillin and incubated at 37 °C at 250 rpm. 0.5 g of arabinose (Sigma Aldrich) was added to the cell culture once the OD reached 0.6, and then further incubated at 37 °C and 250 rpm for 30 hours. The cells were then harvested by centrifugation at 4 °C and 4000g for 25 minutes. The cells were lysed in ice by ultra-sonication after re-suspension in 30 ml of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNAse 1 and 10 mM CaCl\textsubscript{2}) and 1 ml of 100x PMSF and then centrifuged at 4 °C for 30 minutes at 20000g. The protein was purified by loading onto a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, UK), washing the column with wash buffer and then eluting with 100-500 mM imidazole buffer.

B.2 mCherry characterisation

mCherry protein purified from \textit{E. coli} was characterised using fluorescence spectroscopy using a Fluoromax 4 fluorimeter (Horiba Scientific, Japan). Typically, the protein was diluted to 1.5 µM with ultra-pure water and 200 µl was loaded into a 1 cm path-length quartz cuvette. The sample was excited at 587 nm with a 5 nm bandpass. The emission was collected at right angles to the excitation between 600 nm and 750 nm with a 1 nm increment. Emission spectra for samples containing only mCherry, mCherry with 100 mM CMDextran and 30 mM ATP, and mCherry with 100 mM PDDA and 100 mM Polylysine are shown in Figure B.1.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figureB1.png}
\caption{Fluorescence emission spectra (excitation 587 nm) of “lysed” supernatant after in vitro gene expression system was incubated with pEXP5-NT/mCherry plasmid at 18 °C for 6.5 hours with a) no additional component, b) 100 mM CMDextran (black) and 30 mM ATP (blue), and (c) 100 mM PDDA (black) and 100 mM Polylysine (blue).}
\end{figure}
B.3 Construction of pEXP5-NT/mCherry

The mCherry gene was cloned and amplified from pBAD/mCherry and inserted into the pEXP5-NT/CALML3 plasmid using ligase-independent cloning to yield the plasmid pEXP5-NT/mCherry. Primers were designed to facilitate replacement of CALML3 gene with the mCherry insert whilst retaining the TEV-cleavable N-Terminal hexahistidine tag. Primer sequence as follows:

```
CCTGACGCTTTTTATGCACACTCTCTACTGTTTCTCCATACCCGTTTTTTGGGCTAACAG
GAGGAATTAAACCATTGGAAGGCAAGGGAGAGGATACATGCCCATTATCCAAGGAGTT
CATGGCGTCTCAAGGTGCCACATGGGAGGCTCCGTAACGCCACGAGTTCGAGATCGAGG
GCGAGGGGGGCGAGGCGCCGCCCCTACGAGGGCACCAGCCACACGCAAGCTTAAGGTGACCAAG
GGTGGCCCATCCCTGCCTGCTGGACATCGCTGCTCCCTCATCTGTACGCTGTAGCTCCAA
GCCCTAGTGAAGCAAGCGGCGACATCCCAGACTACTGAGCTGCGCTTCCGGAGGG
GCTTCTAAGTGGGAGGCGGTGATGAACCTTGAGGAGGCGGCGTGTAGGTACGGTACCAGAC
GACTCTCTCCGAGCGGCGGGGTTACATCTTACAAAGGTGAACTGAGGACGGCGCCGTTCC
CCTCGACGGGCGGCGTAATGCGAGAAGAAGCAGATGGGCTGGGAGCCCTCCGCGGC
GAGTGTACCGAGGACGGCGGCTGAGGCTAAGACCACCTACAAGGCGCAAGAGCGGCTGCA
GCTGCGGCGCGCTCAACAGCTCAACATCGATGAGTGGACATCCTACCCACAAAGGAGCT
ACACCCTAGTGAACAGTAACGAGCGGCGCCGAGGCGGCCACTCCACGGCAGCAGAC
GAGCTGTACAGGGGAGGCGCACCACATCATCACCATTAAAGCTCGAGATCGACCCGG
TACACATGGAATTCGGTTGCTTTGTTGCGGATGAAGAGAATTTTCACGCTTG
ATACAGATTAATCGAGAACCAGAGCGGTCTGATAAAAACAGAAATTTGCCTGGCGCCAG
TAGAGGCGGTTGCTCACCCTGACCCAGACGTGGAACGCTAACGAAAGTGGAAACCCCGTACG
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pEXP-NT/mCherry was sequenced and reinserted into BL21 competent cells by transformation for amplification. The BL21 cells were isolated by centrifugation and smeared onto an agar plate containing carbenicillin and incubated overnight at 37 °C. A single colony was removed from the agar and incubated in LB broth with carbenicillin overnight at 37 °C. The cells were harvested and the plasmid was extracted from the E. coli cells and purified using MINIPREP® (Qiagen, UK) resulting in a final plasmid concentration of 219 ng/µl.
Bibliography


