

Aqueous droplet networks for functional tissue-like materials

Gabriel Villar

Balliol College
University of Oxford



A thesis submitted for the degree of
Doctor of Philosophy

Michaelmas 2012

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Abstract

An aqueous droplet in a solution of lipids in oil acquires a lipid monolayer coat, and two such droplets adhere to form a bilayer at their interface. Networks of droplets have been constructed in this way that function as light sensors, batteries and electrical circuits by using membrane proteins incorporated into the bilayers. However, the droplets have been confined to a bulk oil phase, which precludes direct communication with physiological environments. Further, the networks typically have been assembled manually, which limits their scale and complexity. This thesis addresses these limitations, and thereby enables prospective medical and technological applications for droplet networks.

In the first part of the work, defined droplet networks are encapsulated within mm-scale drops of oil in water to form structures called multisomes. The encapsulated droplets adhere to one another and to the surface of the oil drop to form interface bilayers that allow them to communicate with each other and with the surrounding aqueous environment through membrane pores. The contents of the droplets can be released by changing the pH or temperature of the surrounding solution. Multisomes have potential applications in synthetic biology and medicine.

In the second part of the work, a three-dimensional printing technique is developed that allows the construction of complex networks of tens of thousands of heterologous droplets $\sim 50\text{ }\mu\text{m}$ in diameter. The droplets form a self-supporting material in bulk oil or water analogous to biological tissue. The mechanical properties of the material are calculated to be similar to those of soft tissues. Membrane proteins can be printed in specific droplets, for example to establish a conductive pathway through an otherwise insulating network. Further, the networks can be programmed by osmolarity gradients to fold into designed shapes. Printed droplet networks can serve as platforms for soft devices, and might be interfaced with living tissues for medical applications.

For Caroline Dahl

Acknowledgements

I would like to thank the following people for their contribution to this work.

My family, who are responsible for my better qualities.

Hagan Bayley, for exemplifying creative scientific thought, and for enabling my development as a scientist. This work was made possible by his long-term vision and benefited from his consistent rigour in all things, from experimental details to the use of language.

The Bayley group, for their support. In particular, Andy Heron provided patient training, and was an essential collaborator on the work on multisomes. Alex Graham helped the droplet printing experiments progress rapidly. Ellina Mikhailova provided the α -haemolysin protein prepared by *in vitro* transcription and translation, and Qihong Li provided the protein from *Staphylococcus aureus*.

Dek Woolfson of the University of Bristol, for stimulating conversations during his visits to Oxford. Nicolas Villar of Microsoft Research and Kevin Valentine of the electronics workshop in Chemistry assisted with the driving electronics for the droplet printer. Alan Wainman of the Dunn School of Pathology assisted with confocal microscopy, and the Mark Wallace group in Chemistry provided a microscope objective. Martin Procter of Isis Innovation and Peter Silcock of J. A. Kemp provided the expertise necessary to develop patent applications from this work.

The author was supported by a studentship from the Life Sciences Interface Doctoral Training Centre, funded by the Engineering and Physical Sciences Research Council. Most of the work presented here was previously published by the author¹⁻³. All of the experiments, modelling and data analysis were performed by the author, with the exception of the droplet network in Fig. 4.6c, which was printed by Alexander D. Graham. The text and figures were produced by the author with editorial assistance from Prof. Bayley. The use of the first-person plural voice in this text is intended to acknowledge the above contributions.

Abbreviations & symbols

α HL	α -haemolysin
DAC	digital-to-analog converter
DHB	droplet-on-hydrogel bilayer
DIB	droplet interface bilayer
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DPhPC	1,2-diphytanoyl- <i>sn</i> -glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
E	Young's modulus
EDTA	ethylenediaminetetraacetic acid
ϵ	strain
g	standard acceleration due to gravity
γ_b	interfacial tension of lipid bilayer
γ_m	interfacial tension of lipid monolayer at oil-water interface
k	spring constant
M_w	relative molecular mass
P	permeability coefficient
ρ	density
SDS	sodium dodecyl sulphate
σ	stress
PAGE	polyacrylamide gel electrophoresis
PMMA	poly(methyl methacrylate)
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
θ_c	contact angle for a pair of droplets joined by a bilayer
T_m	melting transition temperature for a lipid bilayer
Tris	tris(hydroxymethyl)aminomethane

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CHAPTER 1

Introduction

1.1 Background

The biological cell membrane separates the contents of a cell from its surroundings. This barrier is responsible for the colocalization of genes and gene products, which ensures that the natural selection of a phenotype bestows an advantage on the corresponding genotype⁴. A cell must nevertheless exchange materials with the environment in a controlled way to grow, expel harmful species, sense the environment, communicate with other cells, and set up a transmembrane voltage.

Cells control transmembrane transport and signal transduction by using membrane proteins. Some pore-forming proteins, such as the staphylococcal α -haemolysin (α HL) pore, render the membrane permeable to a broad range of chemical species; while others, such as sodium channels, allow the passage of a particular species with exquisite selectivity. Some channels permit diffusion only under a transmembrane voltage in a specific range⁵, or upon binding a particular ligand⁶. Further, transporters and pumps exist that use a source of free energy such as photons, chemical reactions or concentration gradients to translocate a species against an unfavourable transmembrane voltage or concentration gradient⁷. Other membrane proteins effect an intracellular response to an extracellular stimulus not by transmembrane transport but by changing conformation, which activates further intracellular signalling events⁸.

The existing capabilities of membrane proteins for transport and transduction, and the novel functions that might be developed by protein engineering, suggest their technological potential as self-assembled, nanometre-scale devices. Crucially, the use of membrane proteins in an artificial system requires a substitute for the cell membrane, and the limitations of existing membrane surrogates have hindered the application of membrane proteins as elements of functional devices. We now describe the droplet interface bilayers that form the basis of the present work, and compare them to more common artificial platforms for membrane proteins.

Droplet interface bilayers

An aqueous droplet in a solution of lipids in oil acquires a lipid monolayer coat, and two such droplets brought into contact adhere to form a bilayer at their interface⁹ (Fig. 1.1a). The droplets can be reversibly attached to and detached from other droplets, and multiple droplets can be joined to form networks of aqueous compartments joined by single lipid bilayers⁹. Droplet interface bilayers are stable for days⁹. Droplets in some water-in-oil emulsions have been known for some time to form bilayers¹⁰, but the functionalization of these bilayers with membrane proteins or lipids is more recent^{9,11,12}. We now outline the existing variants of the system, and describe their use for basic studies and for the construction of functional devices.

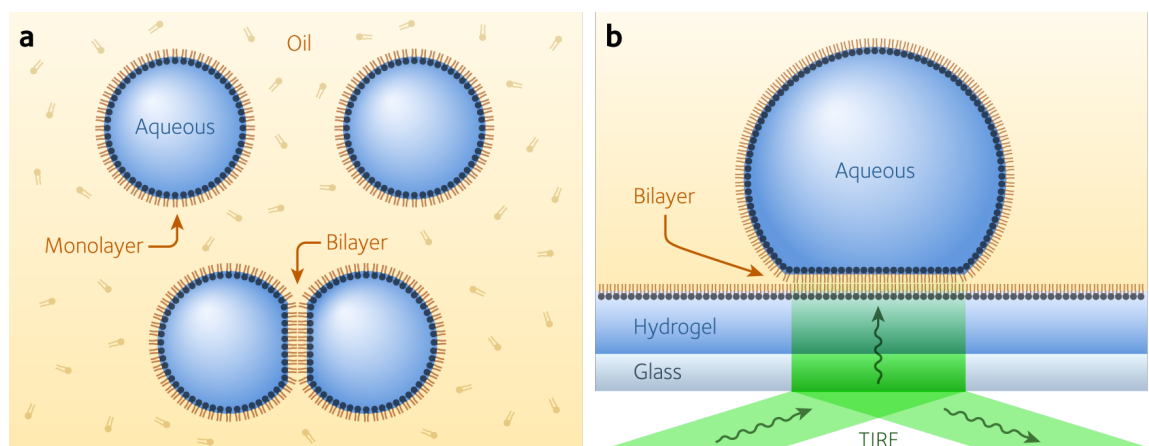


Figure 1.1 | Droplet interface bilayers. (a) Two aqueous droplets made in a solution of lipids in oil acquire a lipid monolayer, and adhere to form a bilayer at their interface. The droplets are typically hundreds of μm in diameter. (b) A bilayer can also be formed between an aqueous droplet and a hydrogel. The hydrogel can be applied as a thin coating on glass, which allows the bilayer and any membrane proteins to be monitored by total internal reflection fluorescence (TIRF) microscopy.

Aqueous, oil and lipid components. In the earliest variant of a droplet bilayer known to the author, the droplets protruded from larger chambers of aqueous solution¹³. Later studies confined the aqueous phases to narrow channels¹⁴, including in a microfluidic device¹¹. The use of isolated droplets (Fig. 1.1a) uniquely allows the construction of droplet networks, and requires only nanolitres of aqueous solution⁹. Although the composition of a greater aqueous volume is more readily manipulated¹³, material can be added to an isolated droplet by fusing it with another droplet¹⁵. This can be done by bringing the droplets into contact before both have acquired a lipid monolayer, or by voltage-^{16–18} or temperature-induced^{19,20} coalescence.

Lipid bilayers have been formed between droplets of aqueous solution and a variety of hydrogels, including agarose and polyacrylamide²¹. Importantly, the hydrogel can be applied as a thin coating on glass, which allows the bilayer and incorporated proteins to be monitored by total internal reflection fluorescence microscopy (Fig. 1.1b). The aqueous volumes can also be made to gel after bilayer formation by using photocurable polymers²².

The oil phase commonly consists of a hydrocarbon, such as *n*-decane¹¹, *n*-hexadecane⁹ or squalene^{15,23}. Shorter alkanes partition more strongly into the bilayer than longer alkanes, increasing the bilayer thickness^{23–25} (see also Section 2.2). Oils other than hydrocarbons, such as silicone oils^{1,10}, can be used to match the densities of the oil and aqueous phases and to promote the assembly of the lipid monolayer¹.

Natural and synthetic lipids have been used to form droplet interface bilayers that are stable and electrically insulating, to provide a suitable environment for a particular membrane protein¹⁵, or to study the behaviour of a membrane protein in a bilayer with a particular lipid composition²⁶. By including lipids in the aqueous phase in the form of vesicles, instead of in the oil phase, droplet bilayers can be formed in which the two leaflets have different compositions²⁶.

Droplet bilayers for basic studies. When a voltage is applied between two droplets joined by a bilayer, a single pore-forming protein inserted into the bilayer permits a measurable ionic current. The proteins can be expressed *in vitro*^{15,27} or *in vivo*^{15,28}. Membrane proteins from both the α -helix bundle and β -barrel classes have been studied in this way, including toxin pores^{9,29}, outer membrane

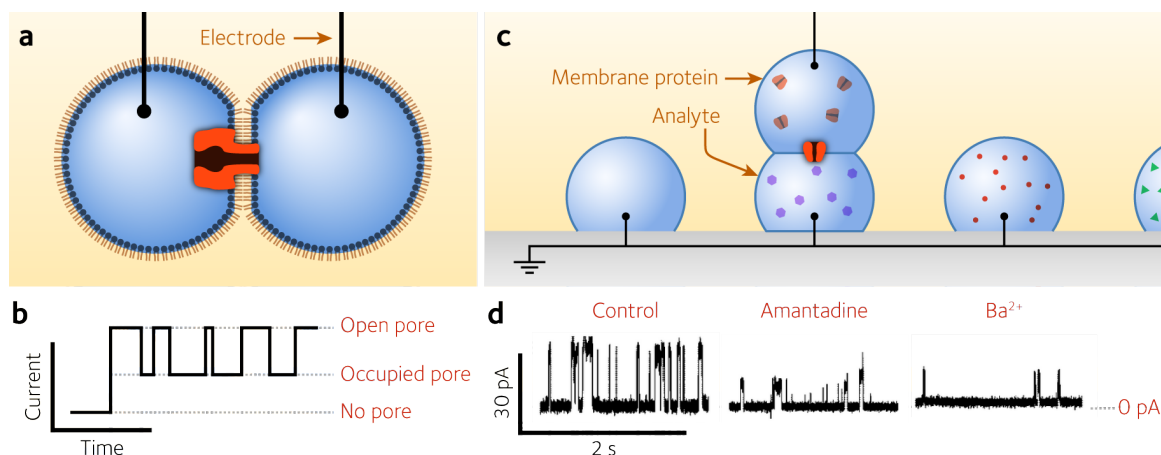


Figure 1.2 | Electrical recording across droplet bilayers. (a) Schematic of electrical recording across a droplet interface bilayer that contains a pore-forming membrane protein. The two electrodes are used to apply a voltage across the bilayer and to measure ionic currents. (b) Schematic of a typical electrical recording. The insertion of a protein pore produces an ionic current through the otherwise insulating bilayer. Interactions between the pore and species in the solution are evident as transient decreases in current. (c) Schematic of a screening experiment in which a droplet containing a membrane protein is brought into contact with each of several analyte-containing droplets in turn. In each case, the protein-analyte interaction is measured by electrical recording. (d) Selected recordings from a rapid screen of the viral potassium channel Kcv against nine potential blockers²⁷ as depicted in c. The control measurement shows the natural gating of the ion channel.

proteins²⁶, potassium channels^{15,27} and a proton pump⁹. Although α HL inserts spontaneously into the bilayer, some membrane proteins such as OmpG require a large voltage across the bilayer, and still others require a particular pH or salt concentration¹⁵. Membrane proteins can insert into a droplet bilayer from a purified, detergent-solubilized state¹⁵. Alternatively, entire membrane fragments with embedded membrane proteins can incorporate into the bilayer^{15,28}. Smaller pore-forming peptides^{15,22,30} and non-peptidic pore-forming molecules¹⁵ have also been inserted into droplet bilayers.

The interaction of a dissolved species with an incorporated membrane protein can modulate the measured ionic current, which allows the interaction to be monitored at the single-molecule level⁹ (Fig. 1.2a). Because droplet bilayer formation is reversible, a single sample of membrane protein can be screened rapidly against multiple analytes (Fig. 1.2b). This approach has been used to screen the viral potassium channel Kcv against several potential blockers²⁷, and to study the interaction of the α HL pore and various cyclodextrins^{9,21}. Droplet bilayers have also been used to study how the choice of lipids affects the function of membrane proteins. For example, asymmetric droplet bilayers were used to examine the gating behaviour of the outer membrane protein OmpG as a function of the charge on each leaflet of the lipid bilayer²⁶. Asymmetric droplet bilayers have also been used to show that the spontaneous exchange of lipid molecules between bilayer leaflets has a timescale of several days²⁶.

Functional droplet devices. Besides the utility of droplet bilayers for the basic study of lipid bilayers and membrane proteins, droplet networks are a promising platform for the construction of functional devices. Uniquely among artificial bilayer systems, in a droplet network multiple aqueous microcompartments communicate in a controlled way, and thereby display collective functions that

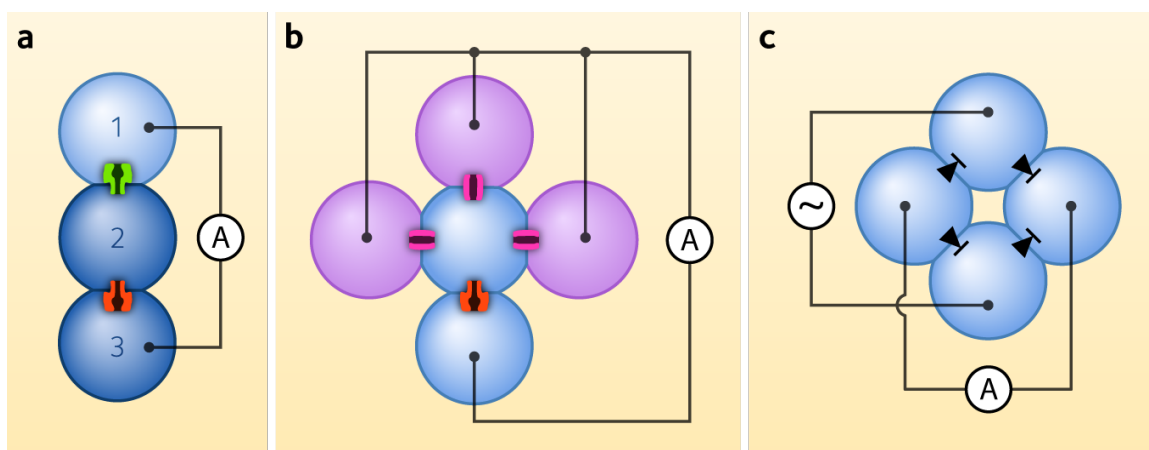


Figure 1.3 | Functional droplet networks. (a) An electrochemical battery⁹. Droplet 1 contained a ten-fold lower concentration of NaCl than droplets 2 and 3. The bilayer between droplets 1 and 2 contained the anion-selective N123R mutant of α HL, and the bilayer between droplets 2 and 3 contained the M113F/K147N mutant of α HL. The different rates at which Na^+ and Cl^- ions diffused into droplet 1 produced a voltage of ~ 30 mV between droplets 1 and 2. This voltage in turn drove an ionic current between droplets 1 and 3. (b) A transducer from illumination to ionic current⁹. The purple droplets contained bacteriorhodopsin, which under illumination pumps protons to produce an ionic current. The lower droplet contained the wild-type α HL pore. (c) A bridge rectifier³¹. Each bilayer contained copies of an α HL mutant in which a total of 49 residues in the barrel of the pore were replaced by positively charged arginines. The pore exhibited a rectification ratio of ~ 60 under the conditions of the study. The orientation of the pores in each bilayer is represented by the diode symbols.

are unattainable with simpler platforms. We review here three functional droplet network devices that employ natural and engineered membrane proteins.

One droplet network was built that converted a difference in chemical potential into an electrical voltage, thereby behaving as an electrochemical battery⁹ (Fig. 1.3a). The network included two droplets of different salt concentrations joined by a bilayer that contained an anion-selective mutant of the α HL pore. The different rates of diffusion of cations and anions through the pores produced a voltage across the bilayer; this voltage was used in turn to perform electrical measurements across another bilayer in the network. Another droplet network transduced illumination into an ionic current by using bacteriorhodopsin, a bacterial light-driven proton pump⁹ (Fig. 1.3b). When the droplet network was illuminated with a 1 mW green laser pointer, thousands of bacteriorhodopsin molecules in multiple bilayers produced a measurable current that propagated through the network. A droplet network has also been built to mimic a simple electrical circuit³¹ (Fig. 1.3c). In this case, a heavily mutated α HL pore was used that exhibited strong rectification of ionic current. The droplets were assembled such that the pores inserted in the configuration shown in Fig. 1.3c. The network behaved as an ionic bridge rectifier circuit, which outputs a current of a single polarity for both polarities of input voltage.

Comparison with other bilayer platforms

We now briefly compare droplet interface bilayers with the most widely used types of artificial lipid bilayers.

Liposomes. A unilamellar liposome is an approximately spherical lipid bilayer that encloses a single aqueous volume³², and multilamellar liposomes consist of multiple such bilayers arranged

concentrically. Liposomes have been developed extensively for drug delivery³³ and synthetic biology³⁴, among other applications³⁵. Although a cell membrane is more closely related topologically to a liposome than to a droplet bilayer, droplet networks compare favourably for the construction of functional devices. Each bilayer between the distinct aqueous compartments in a droplet network can host different membrane proteins, which allows networks to demonstrate collective properties not readily engineered into a single liposome. To achieve similarly controlled communication between multiple liposomes would require the use of membrane proteins that span two bilayers, such as gap junctions³⁶, instead of the much wider range of single-membrane proteins. Further, whereas membrane proteins in a droplet can insert only into bilayers formed by that droplet, it would be more difficult to direct the insertion of double-membrane proteins into specific pairs of liposomes.

Planar bilayers. A planar lipid bilayer, also known as a black lipid membrane, covers a small circular aperture between two bulk compartments of aqueous solution^{12,37–39}. In contrast to a liposome, the solutions on both sides of a planar bilayer are readily accessed³⁹. Planar bilayers have been widely used to study protein channels and pores, and their interaction with species in solution, on the single-molecule level by electrical recording^{40–42}. However, a planar bilayer has a number of drawbacks as a device platform: the bilayers are mechanically sensitive and rupture easily⁴³, the aqueous volumes are orders of magnitude greater than those required for droplet bilayers, and the apparatus is not easily adapted to construct a network of planar bilayers.

Supported bilayers. A lipid bilayer can be supported by a solid substrate to improve its stability, and to allow the study of incorporated membrane proteins by various surface-analysis techniques. Although this configuration might be well suited for the use of certain membrane proteins in sensing applications^{42,44}, the solid support can interfere with the function of membrane proteins in the bilayer, and the obstructed access to one side of the membrane^{44,45} precludes the construction of bilayer networks.

Dispersed bilayers. Other constructs include bicelles and nanodiscs, which are nanometre-sized lipid bilayer discs bounded by a circumferential ring of lipid or protein, respectively. Such structures are useful for the determination of membrane protein structure by nuclear magnetic resonance or crystallographic methods^{46,47}, but are of limited use for the construction of functional devices. Because the suspended lipid discs do not separate two compartments, the action of any pumps, channels or pores in the bilayers would be largely inconsequential.

1.2 Summary of the present work

We have reviewed the construction of droplet networks as devices that employ membrane proteins to achieve a specific function. Although droplet networks are well suited to this end, two principal factors limit the utility of droplet networks in technology and medicine. First, the droplets have been confined to a bulk oil phase, which precludes direct communication with physiological environments. Second, defined networks must be assembled manually, which limits their scale and complexity. These obstacles are addressed by the present work.

Ch. 2 presents the encapsulation of defined droplet networks within small drops of oil in water to form structures called multisomes. The encapsulated droplets can communicate with each other and with the surrounding aqueous environment through membrane pores in interface bilayers. Further, the contents of the droplets can be released by changing the pH or temperature of the surrounding solution. Multisomes enable prospective applications for droplet networks in synthetic biology and medicine.

Ch. 3 presents a three-dimensional printing technique developed for the construction of large, complex networks of heterologous droplets $\sim 50\ \mu\text{m}$ in diameter. Droplet networks can be printed in bulk oil or water. The droplets form a self-supporting material analogous to biological tissue, being composed of discrete aqueous microcompartments that can communicate through membrane proteins. We derive the mechanical properties of the material, which are similar to those of soft tissues. Membrane proteins can be printed in specific droplets, for example to establish a conductive pathway through an otherwise insulating network. Further, the networks can be programmed by osmolarity gradients to fold into designed shapes. Printed droplet networks can serve as platforms for soft devices, and might be interfaced with living tissues for medical applications.

Ch. 4 details experimental materials and methods used in Chs. 2 and 3. This includes the compositions of the solutions used, procedures for the various types of measurement, and details of the printing apparatus and procedure.

Ch. 5 provides mathematical derivations for various results in Chs. 2 and 3. This includes a thermodynamic analysis of multisomes, and physical models of the mechanical, electrical and self-folding behaviour of printed networks.



CHAPTER 2

Multisomes

2.1 Introduction

Aqueous droplets can be joined by interface bilayers and functionalized with membrane proteins to construct functional devices (see Ch. 1). However, droplet networks are constrained to a bulk oil phase, and therefore are not readily deployed in physiological environments. Here, we form structures called multisomes in which networks of aqueous droplets with defined compositions are encapsulated within small drops of oil in water (Fig. 2.1). The encapsulated droplets adhere to one another and to the surface of the oil drop to form interface bilayers. The droplets can communicate with each other and with the surrounding aqueous environment through membrane pores in the bilayers. Further, the contents of the droplets can be released by changing the pH or temperature of the surrounding solution. The multicompartment framework of multisomes mimics a tissue^{48–50} and has potential applications in synthetic biology and medicine^{12,51}.

2.2 Production

Multisomes were produced manually in three steps (Fig. 2.2). A drop of oil containing dissolved lipid was first placed in bulk buffer. The lipid used was 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC). This lipid is commonly used to form droplet interface bilayers because it yields stable and insulating bilayers over a wide range of experimental conditions^{9,11}. Droplets of buffer were then pipetted into a bulk solution of the same lipid in oil. After ~ 5 min, a micropipette was used to transfer the aqueous droplets into the oil drop. The encapsulated droplets sank to the bottom of the oil drop and adhered to one another, thereby forming *internal* bilayers, and to the surface of the oil drop, forming *external* bilayers that separated the encapsulated network from the bulk aqueous solution. The resulting structures (Fig. 2.3) were stable for at least 24 h.

Incubation time and lipid concentration. It was essential to wait several minutes (the *incubation* time) before encapsulation: if transferred too soon into the oil drop, the aqueous droplets fused with each other and with the external aqueous phase. The incubation period allows lipids to form a well-packed lipid monolayer around the oil drop and each of the aqueous droplets⁹.

The adsorption of lipids^{52,80} and surfactants^{53,54} at an air-water interface has been widely studied. In our system, the lipids are expected to exist in the oil as monomers at low concentrations, and to form inverse micelles at high concentrations⁸³. For an amphiphile that at high concentrations forms micelles rather than liposomes, as the bulk concentration C of the amphiphile is increased,

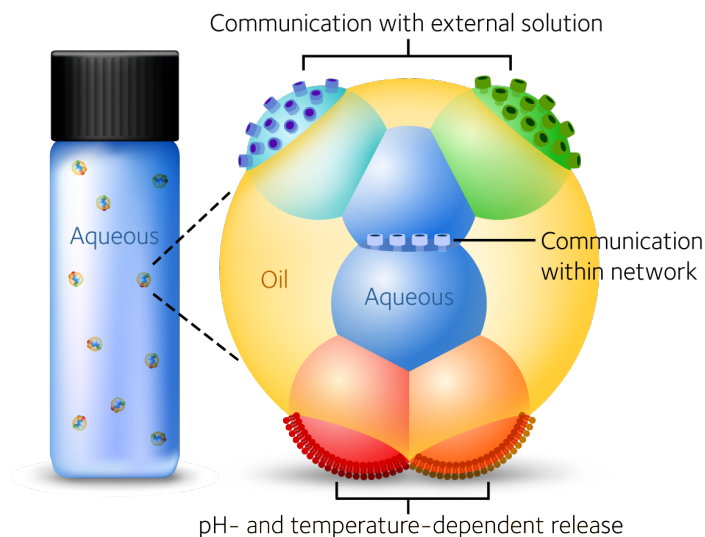


Figure 2.1 | Illustration of a multisome. Aqueous droplets encapsulated in an oil drop are connected by lipid bilayers, which allow the droplets in the network to communicate through protein pores. Pores in bilayers at the surfaces of the aqueous droplets that protrude from the oil drop enable the network to communicate with the bulk solution. Multisomes can release the contents of encapsulated droplets by pH- or temperature-induced rupture of bilayers.

the equilibrium surface density Γ of the amphiphile approaches saturation⁵³. The surface density Γ_0 that corresponds to a well-packed monolayer can be determined from the monolayer interfacial tension, γ_m : as C increases, γ_m decreases at first gradually, and then rapidly to a minimum value that typically corresponds to a tightly-packed monolayer. In our experimental conditions, the area per lipid molecule is expected to be $\sim 70 \text{ \AA}^2$ (ref. 55). In some systems, such as sodium dodecyl sulphate (SDS) at a water-air interface, the amphiphile reaches the critical micelle concentration (CMC) before the monolayer is well packed⁵⁴. Micelle formation may then prevent further adsorption onto the interface⁵⁴, although not in all cases⁵³.

Therefore for an amphiphile to form a tightly-packed monolayer from solution, C must be high enough for Γ to reach Γ_0 ; enough time must be allowed for Γ to equilibrate to Γ_0 ; and, depending on the lipid, the CMC in the solvent must be high enough to allow Γ to reach Γ_0 . Although no data was available on the CMC of DPhPC in the oil mixture used here, or on the kinetics of DPhPC adsorption to an oil-water interface, it was found that at a lipid concentration of $0.1\text{--}0.2 \text{ mg ml}^{-1}$ an incubation time of a few minutes was sufficient for droplets to form stable and insulating bilayers when brought into contact.

Formation of lipid structures other than monolayers. In some solutions of lipid in oil, such as lecithin in decane⁵⁶ and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in dodecane⁵⁷, a water droplet will acquire a multilamellar lipid film at its surface rather than a single monolayer. The lipid film swells with water, causing the spontaneous growth of water droplets in the oil phase at the surface of the larger droplet, which grow to up to a few μm in diameter⁵⁷. There was no evidence that this occurred for the oil solutions and incubation times used in this work. However, with incubation times of ~ 30 min in solutions with $\sim 90\%$ silicone oil in hexadecane, aqueous droplets acquired a thin rigid film that prevented the formation of a bilayer between two such droplets. This

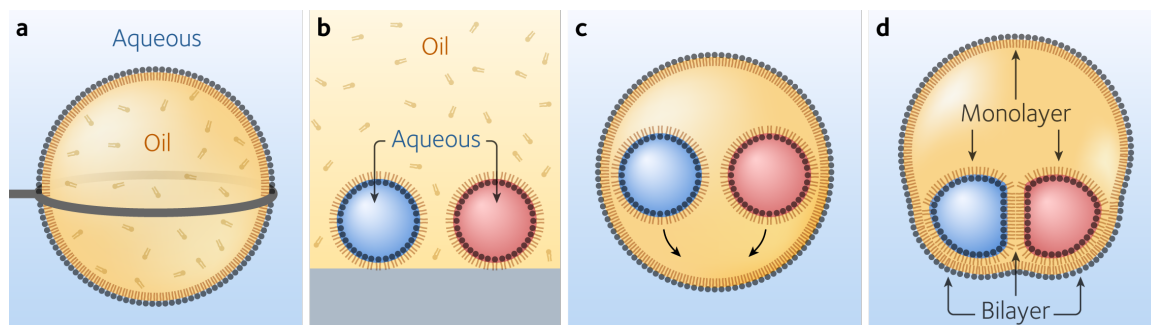


Figure 2.2 | Production of multisomes. (a) A drop of oil containing dissolved lipids is suspended in bulk buffer. The oil drop is held by a wire loop to allow extended study (see Ch. 4). (b) Aqueous droplets are pipetted into a bulk solution of lipids in oil, and allowed to incubate for ~5 min. (c) The aqueous droplets are transferred into the oil drop using a micropipette. (d) After ~1 min, the aqueous droplets adhere with each other and with the surface of the oil drop to form internal and external bilayers, respectively.

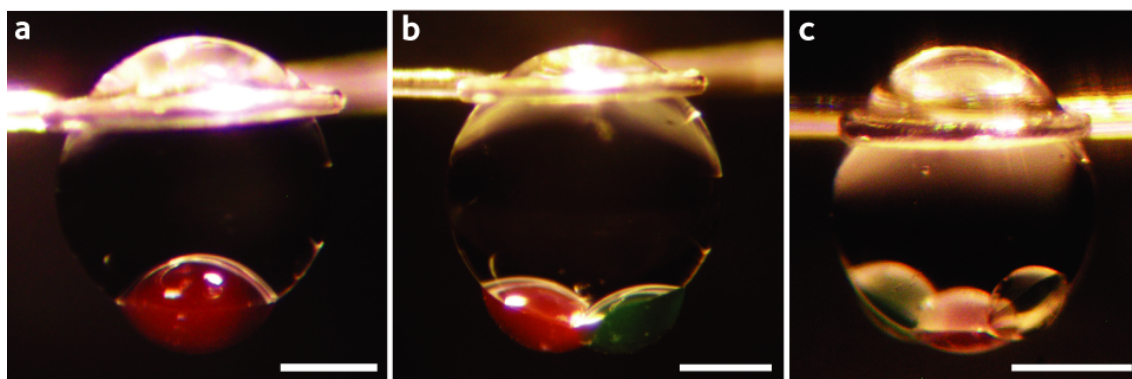


Figure 2.3 | Photographs of multisomes. Each multisome contained (a) one, (b) two or (c) three aqueous droplets, which were dyed red or green. Scale bars: 400 μm .

film might consist of a multilamellar lipid structure with microscopic aqueous droplets as described above.

Choice of oil

In most of the foreseen applications, multisomes should be approximately neutrally buoyant in the aqueous environment, so the oil should be chosen to approximately match the density of water. We used a silicone oil chosen accordingly for its density of 1.01 g cm^{-3} , mixed with a small proportion of hexadecane to improve bilayer stability during electrical recording (see Ch. 4). The choice of oil also affects the minimum incubation time required to form stable multisomes. Whereas our oil mixture required an incubation time of $< 5 \text{ min}$, multisomes made with 1-bromododecane (density 1.04 g cm^{-3}) were not stable even with longer incubation times and higher lipid concentrations, possibly due to adsorption of 1-bromododecane molecules at the water-oil interfaces.

Finally, aqueous droplets made in a solution of lipids in silicone oil are more strongly adhesive than in hexadecane. This is in agreement with the finding that the energy of bilayer formation is greater in solvents with larger molecules^{25, 58–60}. This effect is due to two contributions: one due to

the interactions of the solvent with the lipids, and one due to the van der Waals attraction of the aqueous phases across the bilayer.

Interaction of solvent with lipids. For an alkane to enter the outer regions of a lipid bilayer, it must straighten and interdigitate between the lipids in order to avoid exposing the hydrophobic region to the aqueous phase, which is energetically unfavourable^{59,60}. Longer alkanes have more conformational degrees of freedom, and therefore suffer a greater loss of internal entropy when straightened. Further, shorter solvent molecules straighten more readily and therefore distribute more evenly throughout the bilayer, whereas longer molecules are more confined to the bilayer centre. The entropic costs of straightening and confinement therefore favour the exclusion of large solvent molecules from the bilayer^{59,60}. This is the dominant contribution to the free energy of bilayer formation for bilayers made in large-molecule solvents such as squalene^{25,60}, and is therefore expected to dominate in our system. For solvents with very large molecules, the greater monolayer adhesion that results from the exclusion of solvent from the bilayer can be seen as a depletion interaction^{10,61,62}.

Interaction of aqueous phases. Because of the preferential exclusion of large solvent molecules from a bilayer discussed above, bilayers made in solvents with larger molecules are thinner^{59,60}. The energy per unit area of the van der Waals interaction between the aqueous phases across the bilayer is proportional to $-h^{-2}$, where h is the thickness of the hydrophobic region^{58,63}. The aqueous phases are therefore more strongly attracted across thin bilayers, and this interaction contributes to the free energy of adhesion of two monolayers to form a bilayer. For bilayers made in small-molecule solvents such as decane, the exclusion of the solvent from the bilayer offers a relatively small decrease in free energy, and the van der Waals interaction is instead the dominant contribution^{25,58,60,63}.

Related structures

Multisomes are topologically related to other lipid structures. A multisome with a single inner droplet is equivalent to a liposome with an interstitial oil phase between the bilayer leaflets. A multisome with multiple encapsulated droplets can be compared with a vesosome⁶⁴—a vesicle that contains multiple, smaller vesicles—and a multivesicular liposome⁶⁵, which is a foam-like aggregate of aqueous compartments joined by lipid bilayers. Unlike the vesicles inside a vesosome, the aqueous compartments in a multisome are separated from each other and from the bulk aqueous solution by single lipid bilayers. Further, whereas vesosomes and multivesicular liposomes are poorly controlled structures produced by bulk methods, multisomes are assemblies of defined structure that can employ membrane proteins to form functional devices.

2.3 Thermodynamics

Before bilayer formation, the encapsulated droplets and the oil drop minimize their interfacial energies by adopting spherical geometries. After bilayer formation, the geometry of the multisome represents a compromise between the favourable adhesion of apposing monolayers to form bilayers, and unfavourable distortions that expose a greater monolayer or bilayer surface area. By considering all the possible geometries of a multisome, and calculating the total interfacial energy of each geometry, it is possible to create a free energy landscape for the formation of a bilayer. Such a calculation is performed in Ch. 5 for a multisome with a single inner droplet, and the results are outlined below.

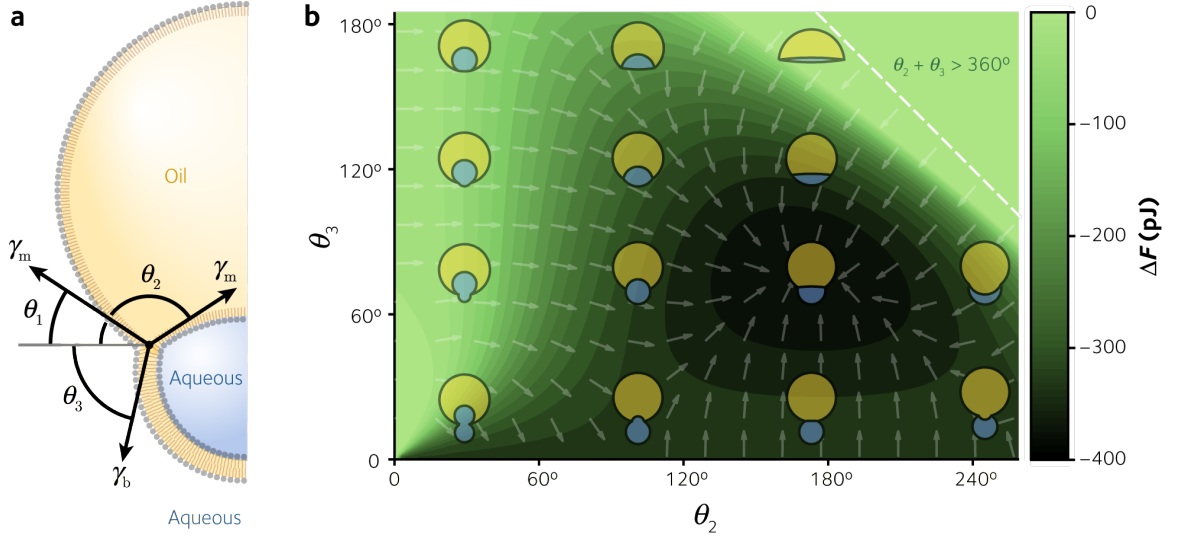


Figure 2.4 | Free energy landscape. (a) Schematic of a multisome with a single inner aqueous droplet, showing the definition of the contact angles θ_i relative to the horizontal. The arrows labelled γ_m and γ_b represent the monolayer and bilayer interfacial tensions, respectively. (b) Free energy of bilayer formation for an encapsulated droplet, as a function of the contact angles θ_2 and θ_3 . The dashed line indicates the boundary to the unphysical region where $\theta_2 + \theta_3 > 360^\circ$. The landscape was computed assuming an oil drop radius of $R_1 = 400 \mu\text{m}$, an aqueous droplet radius of $R_2 = 200 \mu\text{m}$, a monolayer interfacial tension of $\gamma_m = 1 \text{ mN m}^{-1}$, and a ratio of bilayer to monolayer interfacial tensions of $\gamma_b/\gamma_m = 0.68$. Arrows indicate the direction of steepest descent. The geometry of the multisome is depicted at various points in the landscape, including the state of minimum free energy at $(\theta_1, \theta_2, \theta_3) = (33^\circ, 173^\circ, 77^\circ)$.

Results

The interfacial tensions of a monolayer and bilayer, γ_m and γ_b respectively, are measures of the energetic cost of creating a unit area of each kind of interface. The free energy change for the formation of a specified geometry is therefore given by the changes in monolayer and bilayer areas relative to the initial state, weighted by the tension of each interface. The structure of a multisome was parameterized by three contact angles (Fig. 2.4a). In Ch. 5 we show that the free energy of formation of a multisome with contact angles $(\theta_1, \theta_2, \theta_3)$ is given by

$$\Delta F = 2\pi\gamma_m \left[r_1^2 (1 + \cos \theta_1) + r_2^2 (1 + \cos \theta_2) + \frac{\gamma_b}{\gamma_m} r_3^2 (1 + \cos \theta_3) - 2(R_1^2 + R_2^2) \right], \quad (2.1)$$

where R_1 and R_2 are the radii of the oil drop and inner droplet, respectively, before bilayer formation, and r_i are the radii of curvature of the three spherical caps corresponding to the contact angles θ_i , and can be evaluated from θ_i , R_1 and R_2 . One contact angle is constrained by conservation of the volumes of the oil drop and inner droplet, and the other two angles remain free variables. We found that keeping θ_2 and θ_3 as the free variables facilitated the visualization of the free energy landscape.

Equation 2.1 was used to evaluate ΔF for all combinations of θ_2 and θ_3 , using the values $R_1 = 400 \mu\text{m}$, $R_2 = 200 \mu\text{m}$, $\gamma_m = 1 \text{ mN m}^{-1}$ (refs. 66,67) and $\gamma_b/\gamma_m = 0.68$. The value of γ_b/γ_m was obtained from measurements of droplet interface bilayer contact angles (see Ch. 5). The resulting free energy landscape $\Delta F(\theta_2, \theta_3)$ is shown in Fig. 2.4b. The minimum of the landscape represents the multisome geometry of lowest energy, which for the chosen parameters lies at $(\theta_1, \theta_2, \theta_3) =$

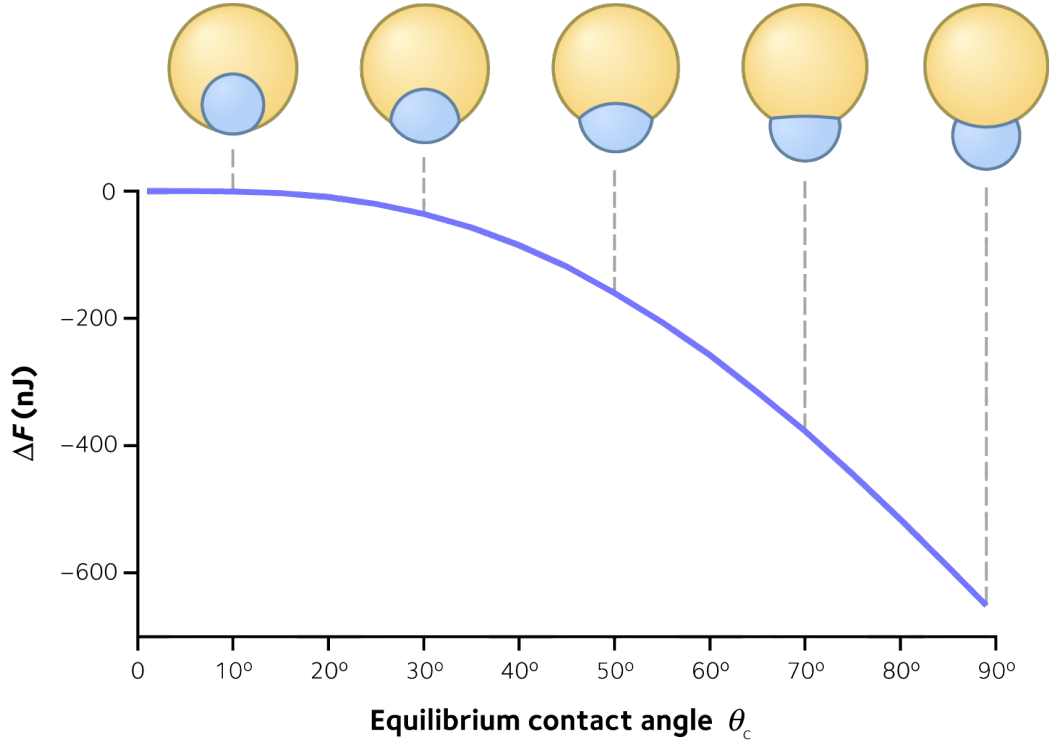


Figure 2.5 | Geometry and free energy of formation of a multisome in different oils. The equilibrium geometry of a multisome and the free energy of bilayer formation ΔF were calculated for a range of values of γ_b/γ_m that corresponded to the full range of θ_c . The calculations assumed an oil drop radius of $R_1 = 400 \mu\text{m}$, an aqueous droplet radius of $R_2 = 200 \mu\text{m}$ and a monolayer interfacial tension of $\gamma_m = 1 \text{ mN m}^{-1}$. The equilibrium geometries of the multisome are shown for $\theta_c = 10^\circ, 30^\circ, 50^\circ, 70^\circ$ and 89° . For the multisomes presented here, $\theta_c = 70^\circ$.

($33^\circ, 173^\circ, 77^\circ$). Relative to the state before bilayer formation, the calculated equilibrium geometry has 76% as much monolayer area, and 5% more interfacial area if the bilayer is counted as two interfaces. The multisome in this state is only metastable, because fusion of the inner droplets with the bulk aqueous phase would further decrease the free energy of the system.

Energy landscape as a function of composition. The values of γ_b and γ_m in general depend on the combination of oil and lipid used to make the multisome. Inspection of the formula for ΔF shows that for a given ratio γ_b/γ_m , the absolute value of γ_m affects only the energy scale of the free energy landscape, but not its shape.

The equilibrium geometry of a multisome depends only on the ratio γ_b/γ_m , and on the ratio of the oil drop and aqueous droplet volumes. The equilibrium geometry and free energy of bilayer formation can be calculated as a function of γ_b/γ_m by repeating the above procedure for different values of γ_b/γ_m , and in each case finding the minimum of the free energy landscape (Fig. 2.5). Here we express the ratio of the interfacial tensions in terms of θ_c , the equilibrium contact angle between the monolayers and bilayer, which is readily measured for planar²⁵ and droplet interface bilayers^{10,67}. It can be shown that $\gamma_b/\gamma_m = 2 \cos \theta_c$, where θ_c is between 0 and 90° (Ch. 5 and ref. 63). From Fig. 2.5 it is evident that for compositions in which θ_c is large, the free energy of bilayer formation is greater, and the oil drop excludes the aqueous droplet to a greater extent. This is to be expected because greater values of θ_c correspond to more strongly adhesive bilayers, as discussed below.

Oil	Lipid	θ_c	ΔA^* ($\mu\text{J m}^{-2}$)
^a Triolein	Monoolein	57.2°	-1,670
^b Silicone oil & hexadecane, 9:1 (v/v)	DPhPC	70°	-1,320
^c Mineral oil	DPhPC	88°	-730
^d Silicone oil & hexadecane, 1:1 (v/v)	DPhPC	47°	-640
^a Squalene	Monoolein	26.5°	-511
^e Hexadecane	Monoolein	5.4°	-22.6
^a Decane	Monoolein	1.97°	-4.5

^a Ref. 25.

^b This work (Ch. 2).

^c Calculated from ref. 67 using $\Delta A^* = 2\gamma_m (1 - \cos \theta)$.

^d This work (Ch. 3).

^e Ref. 63.

Table 2.1 | Energies of bilayer formation. Comparison of the equilibrium contact angle θ_c and free energy of formation of a lipid bilayer ΔA^* for various oils and lipids, including those used to make multisomes.

Discussion

We now compare the free energy of bilayer formation for our system to others, and consider how the energy landscape of a multisome depends on the number of encapsulated droplets.

Intrinsic measure of free energy of bilayer formation. The formation of a bilayer in a multisome deforms the various interfaces in the multisome (Fig. 2.4), and the resulting increase in area incurs a free energy cost that partially negates the gain from bilayer formation (Ch. 5). Because the increase in area depends on the aqueous and oil volumes, and on their ratio, so does the total free energy change of the system upon bilayer formation ΔF (Eq. 2.1). An intrinsic measure that is independent of the multisome geometry would allow the strength of monolayer adhesion in our experimental conditions to be usefully compared to that in other systems. Such a quantity can be obtained by neglecting the energetic cost of monolayer deformation, as follows.

The adhesion of two monolayers of area S to form an equal area of bilayer has an associated free energy change of $\Delta A = S(\gamma_b - 2\gamma_m)$ (ref. 63). Using the relation above between γ_b , γ_m and θ_c , this becomes $\Delta A^* = 2\gamma_m (1 - \cos \theta_c)$, where $\Delta A^* = \Delta A/S$ is the free energy of bilayer formation per unit area. Table 2.1 compares the values of θ_c and ΔA^* for multisomes to those for planar bilayers and droplet interface bilayers, including those in Ch. 3. The values of ΔA^* found here are consistent with the finding discussed in Section 2.2 that the free energy of bilayer formation is generally greater in solvents with larger molecules.

Multiple encapsulated droplets. Whereas the energy landscape for a multisome with a single encapsulated droplet has only one minimum, multiple inner droplets may exist in more than one locally stable configuration. For instance, in multisomes with three inner droplets, the encapsulated droplets were observed to settle in either a linear or triangular arrangement, depending on their positions and velocities at the moment of encapsulation. Further, the droplets may rearrange even after forming bilayers. In multisomes with three inner droplets initially in a linear arrangement, the droplets typically slid along the surface of the oil drop to irreversibly adopt the triangular configuration.

Therefore any desired configuration for a multisome must meet two conditions. First, the arrangement must be metastable. States that are not robust to perturbation might be stabilized

by the presence of auxiliary encapsulated droplets. Second, the arrangement must be accessible from the state of the multisome at the moment of encapsulation. This would require either a highly reproducible means of encapsulation, or some means of driving the inner droplets from one metastable state to another. The use of auxiliary droplets with a reproducible means of encapsulation is demonstrated in Ch. 3.

2.4 Electrical recording

In addition to creating multisomes of defined structure, we aimed to produce functional droplet networks in an aqueous environment. By analogy with networks in bulk oil⁹, the incorporation of membrane pumps, channels and pores into multisome bilayers would allow control over the exchange of material, as well as electrical communication, between the various inner droplets and the external solution. To determine whether the presumed external bilayers of multisomes support the insertion of membrane proteins, we performed electrical measurements across the external bilayer of a multisome with a single inner droplet.

Results and discussion

To perform electrical measurements across a multisome bilayer, we fabricated a glass-insulated Ag/AgCl electrode with an electrically exposed tip (Fig. 2.6a and Ch. 4). Immediately after an aqueous droplet had been transferred into the oil drop, the electrode tip was inserted into the inner droplet. Through micromanipulation of the electrode, the inner droplet was lowered until it adhered to the surface of the oil drop (Fig. 2.6a).

Insertion of multiple protein pores. When wild-type α HL was included in the inner droplet, the ionic current began increasing in a stepwise manner within 1 min of adhesion, as recorded with the inserted electrode at +50 mV relative to the external aqueous solution (Fig. 2.6b). The amplitude of the steps was 18.6 ± 0.8 pA (mean $\pm$ s.d., $n = 16$), which is consistent with the expected current of ~ 18.6 pA for the α HL pore under the given conditions (500 mM KCl, pH 8.0)⁶⁸. The current steps therefore corresponded to consecutive insertions of α HL pores into an external bilayer.

Single-molecule characterization. To confirm that the inserting pore was α HL, the experiment was repeated with a lower concentration of protein. After a few minutes, the current stepped from zero to a steady level, corresponding to the insertion of a single α HL pore into the external bilayer. Immediately afterwards, γ -cyclodextrin (~ 10 μ M) was added to the external aqueous solution, which caused reversible blockades of the current through the pore (Fig. 2.6c) with a blocking amplitude of $63.7 \pm 2.0\%$ (mean $\pm$ s.d., $n = 673$) at -50 mV in 1 M KCl at pH 8.0. A least-squares linear fit to a logarithmic histogram of the dwell times gave a dissociation rate of 4.0 ± 0.6 s⁻¹ (mean $\pm$ standard error of regression) (Fig. 2.6d). A previous study, using an interface bilayer between two aqueous droplets in bulk oil, found a blocking amplitude of $\sim 60\%$ and a dissociation rate of 2.0 s⁻¹ at pH 7.0 (ref. 9). The higher dissociation rate seen here at pH 8.0 is consistent with the finding that the rate of dissociation of β -cyclodextrin from α HL increases with pH (ref. 69). We conclude that α HL pores can insert into the external bilayers of a multisome, and that their behaviour is indistinguishable from that in other lipid bilayer systems.

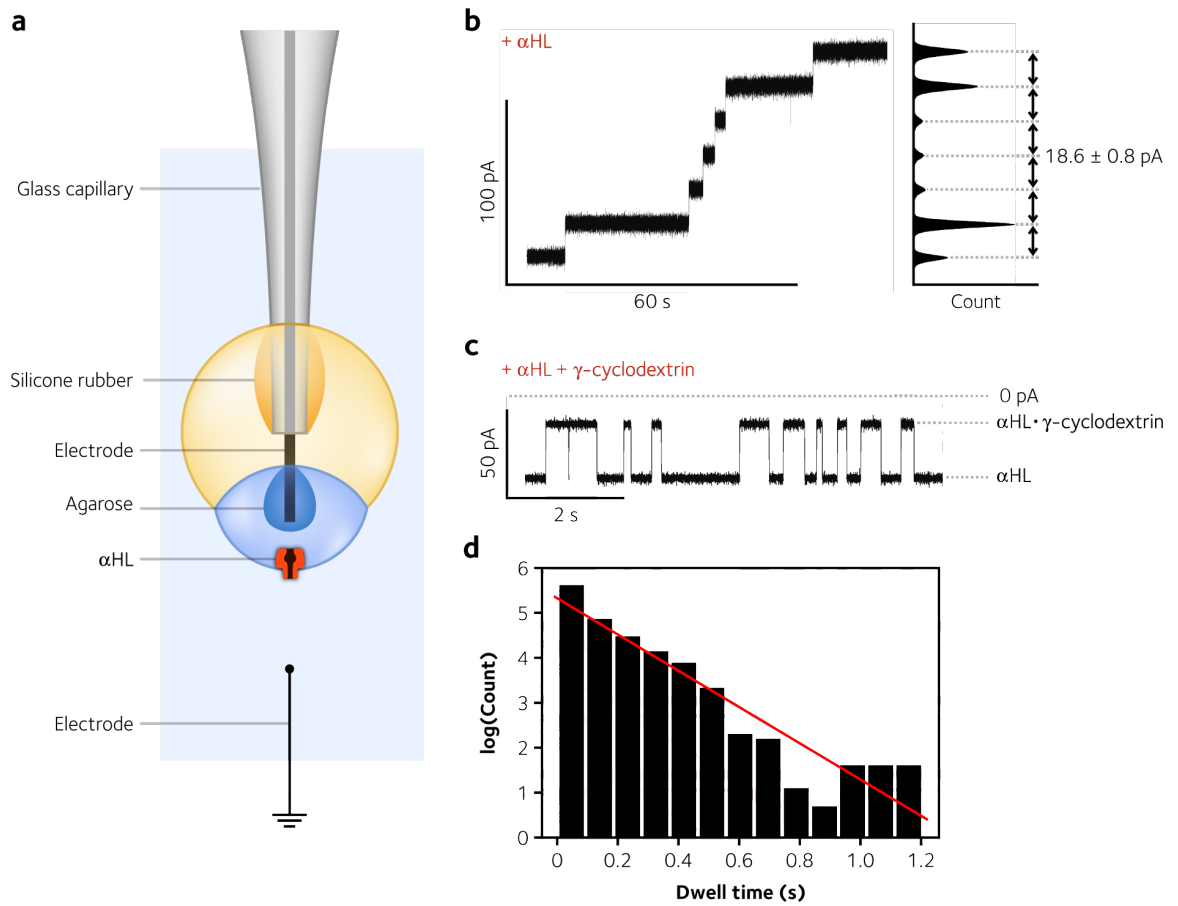


Figure 2.6 | Measurement of ionic currents through α HL pores. (a) Schematic of the measurement of ionic current flowing between an encapsulated droplet and the bulk aqueous solution, through an α HL pore inserted in the bilayer. (b) Stepwise increase in current indicating consecutive insertions of α HL pores into the external bilayer, at +50 mV in 500 mM KCl at pH 8.0. The peaks in the current histogram were separated by 18.6 ± 0.8 pA (mean $\pm$ s.d., $n = 16$), as expected for insertions of individual wild-type α HL pores. (c) Current blockades of a single wild-type α HL pore in the configuration shown in a after adding ~ 10 μ M γ -cyclodextrin to the bulk solution, at -50 mV in 1 M KCl at pH 8.0. The current levels of the unoccupied pore and the pore with γ -cyclodextrin bound are indicated. The γ -cyclodextrin blockades have an amplitude of $63.7 \pm 2.0\%$ (mean $\pm$ s.d., $n = 673$). (d) Logarithmic histogram of the dwell times of the γ -cyclodextrin blockades exemplified in c. The least-squares linear fit shown in red yields a dissociation rate of 4.0 ± 0.6 s^{-1} (mean $\pm$ standard error of regression, $n = 673$).

2.5 Diffusion through pores

We also explored whether the inner droplets of a multisome can use pores to communicate passively with each other and with the external aqueous solution; that is, without the need for an externally applied potential.

Results and discussion

Communication with the environment. We first tested whether an encapsulated droplet could communicate with the external solution. A multisome was made with a single inner droplet that contained α HL pores, and fluo-4 (a Ca^{2+} -sensitive dye) conjugated to a $M_w = 10,000$ dextran to prevent its translocation through α HL. The addition of Ca^{2+} to the external solution caused the initially dark inner droplet to become fluorescent over ~ 1.5 h (Fig. 2.7a), after a delay (see below), whereas a multisome without α HL showed no fluorescence increase ($n = 6$). We concluded that the fluorescence increase in the multisome that contained α HL was caused by the diffusion of Ca^{2+} ions from the external aqueous solution into the inner droplet, through α HL pores in the external bilayer.

We estimated the number of pores in the multisome bilayer by using two aqueous droplets in bulk oil, joined by an interface bilayer. One of the droplets contained α HL at the same concentration as in the multisome experiment. From electrical measurements across this bilayer, several thousand α HL pores are expected to have inserted into the multisome bilayer.

Communication within a multisome. We then tested whether two inner droplets in a multisome could communicate with each other in a similar way, by including dextran-conjugated fluo-4 and α HL in one droplet and Ca^{2+} in the other. The fluorescence of the droplet that contained fluo-4 increased over ~ 1 h (Fig. 2.7b), demonstrating the diffusion of Ca^{2+} ions between the inner droplets through α HL pores in the internal bilayer. Communication by passive diffusion can therefore take place between a multisome and the external solution, and between the inner droplets of a multisome.

Delayed increase in fluorescence. The fluorescence measurements showed a time lag between the start of Ca^{2+} flux and a fluorescence increase. This is due to competitive binding of Ca^{2+} by ethylenediaminetetraacetic acid (EDTA), included in the inner droplets to chelate the small amount of contaminating Ca^{2+} present in the buffer salt, which would otherwise have produced background fluorescence. Ca^{2+} binds to fluo-4 and EDTA at similar rates ($k_{\text{on}} \sim 10^7\text{--}10^8 \text{ M}^{-1} \text{ s}^{-1}$), but dissociates from fluo-4 much more rapidly than from EDTA, with $k_{\text{off}} \sim 400 \text{ s}^{-1}$ and $< 1 \text{ s}^{-1}$, respectively^{70,71}. Therefore on a timescale of minutes the EDTA acts as a sink for almost all the Ca^{2+} in the droplet, and only once the EDTA has been saturated can a significant proportion of the fluo-4 molecules bind Ca^{2+} ions. Calculations of the competitive binding of EDTA and fluo-4 to Ca^{2+} ions (Ch. 5) confirm that the fluorescence profiles in Fig. 2.7 are well explained by the chelation of Ca^{2+} by EDTA.

2.6 Triggered release

We also explored the possibility that the lipid bilayers themselves might be used to functionalize multisomes. By including environment-sensitive lipids in the external bilayers, multisomes were made

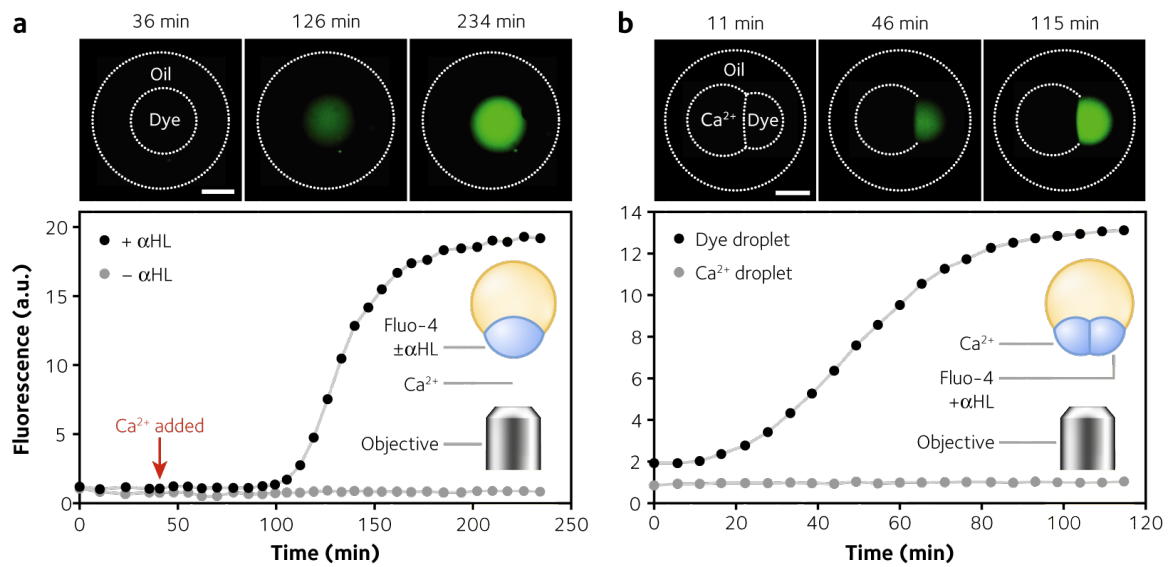


Figure 2.7 | Communication by diffusion through α HL pores. Fluorescence photographs and measurements of multisomes. Oil and inner droplets are outlined in the photographs where they are not visible. Inner droplets containing dextran-conjugated fluo-4 or Ca^{2+} are respectively labelled 'Dye' or ' Ca^{2+} '. (a) Two multisomes with a single inner droplet each, in the same bulk solution; both multisomes contained the dye, and one also contained α HL. The photographs are of the multisome that contained α HL, and the graph includes measurements from both multisomes. Following the addition of Ca^{2+} to the external solution, the droplet that contained α HL increased in fluorescence over ~ 1.5 h, whereas the droplet without protein did not. Scale bar: $300\ \mu\text{m}$. (b) A multisome containing a two-droplet network, in which one droplet contained Ca^{2+} and the other contained the dye and α HL. The dye-containing droplet increased in fluorescence, whereas the Ca^{2+} -containing droplet did not. Scale bar: $300\ \mu\text{m}$.

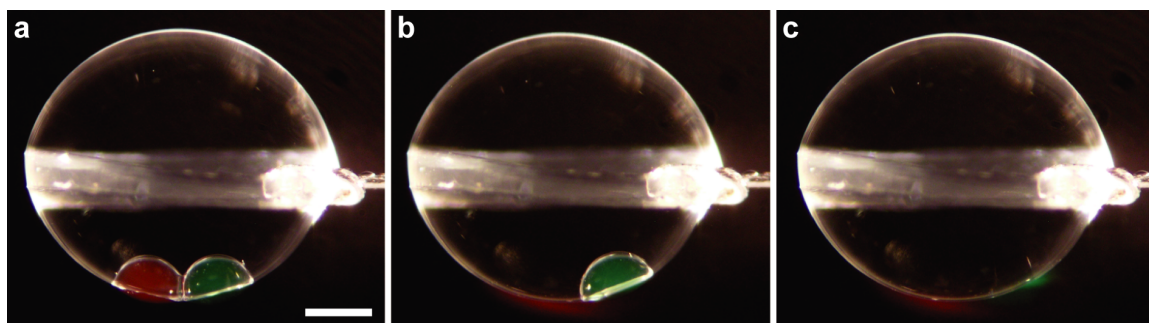


Figure 2.8 | pH-dependent release of encapsulated contents. Photographs of a multisome with two inner droplets, made with a mixture of DOPE and oleic acid. (a) Multisome in pH 8.0 buffer. Exchange of the external buffer with buffer of the same pH had no effect. (b) Bursting of one inner droplet upon lowering the pH of the external buffer to ~ 5.5 . (c) Bursting of the other inner droplet ~ 5 s after the first. Scale bar: 500 μm .

to release their contents in response to a change in the pH of their surrounding solution, or to a change in temperature.

pH sensitivity

Several strategies have been used to engineer liposomes that release their contents upon a change in the pH of their environment⁷². For example, the pH-sensitive polymer poly(2-ethylacrylic acid) associates with and permeabilizes liposome membranes in acidic conditions⁷³. The critical pH for contents release depends on the presence of cholesterol in the membrane⁷⁴.

We rendered multisome bilayers pH-sensitive by another method commonly applied to liposomes. The approach uses a mixture of two lipids⁷²: one, such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), that favours a non-bilayer state, and another, such as oleic acid, that stabilizes the bilayer state at pH values above its pK_a (~ 7.5 when incorporated in a bilayer^{75,76}) but not at lower pH values, when a significant proportion of oleic acid is protonated.

Multisomes made with a mixture of DOPE and oleic acid were stable for at least a day in bulk aqueous solution at pH 8.0 ($n = 7$). Multisomes were allowed to equilibrate for ~ 15 min after formation, and the pH of the solution was then decreased from 8.0 to ~ 5.5 by replacing half the solution with buffer at pH 3.0. This caused the external bilayers to suddenly rupture, allowing the contents of the inner droplets to mix with the external solution (Fig. 2.8). The droplets burst within < 2 min of each other, and usually within < 1 min ($n = 8$). Replacing half the solution with an identical buffer at pH 8.0 instead of pH 3.0 had no effect, which indicates that bilayer rupture was not caused by mechanical disturbance of the solution.

To determine whether the contents of the droplets mixed with each other before diffusing away into the external solution, multisomes with two inner droplets were made, in which one inner droplet contained dextran-conjugated fluo-4, and the other Ca^{2+} (Fig. 2.9a). As shown by fluorescence microscopy, the contents of the two droplets remained separate until the pH of the external solution was lowered (Fig. 2.9b). The droplets subsequently burst, and their contents mixed with each other in the bulk aqueous solution before diffusing away.

Temperature sensitivity

Liposomes made from a lipid with a melting transition have a local maximum of permeability at around the melting temperature, attributable to the boundaries between the solid and fluid phases

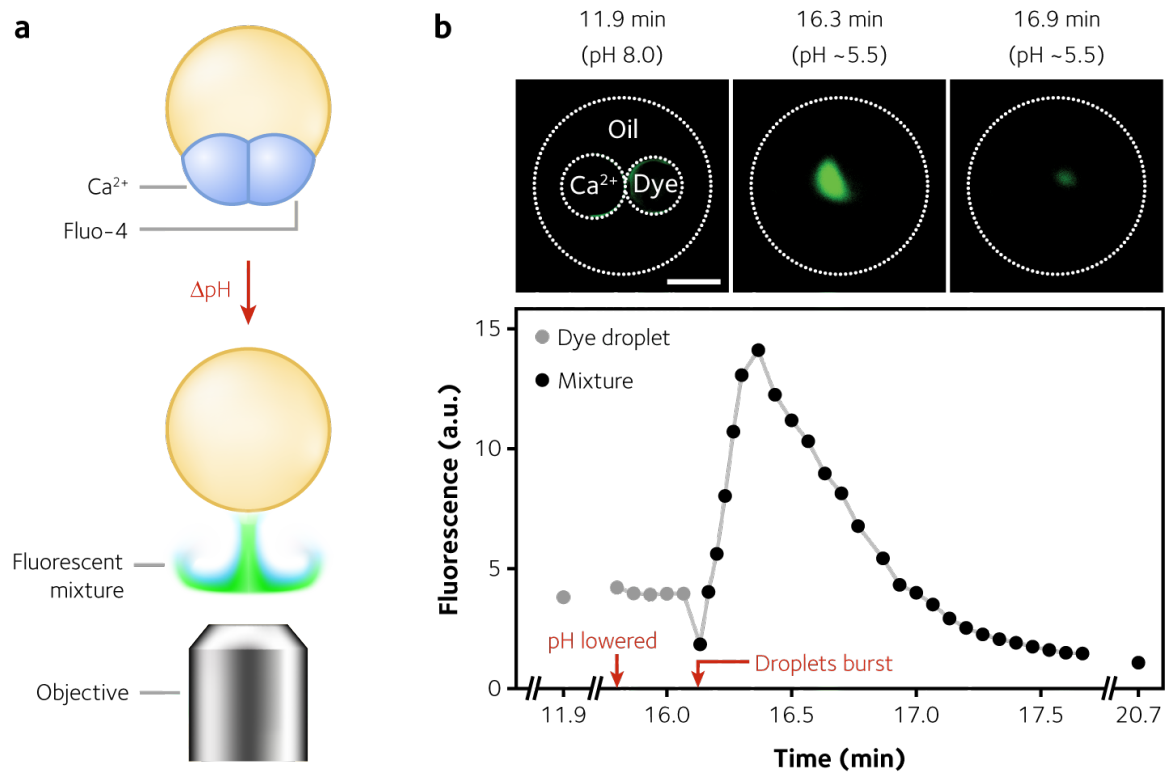


Figure 2.9 | Fluorescence monitoring of pH-dependent release. (a) A multisome is made with a mixture of DOPE and oleic acid, with one inner droplet containing Ca^{2+} and another dextran-conjugated fluo-4. With a decrease in pH, the multisome co-releases its contents into the bulk aqueous solution, where the two solutions mix to produce a signal that is monitored with a fluorescence microscope. (b) Fluorescence photographs and measurements from the experiment in a. On lowering the pH of the external aqueous buffer from 8.0 to ~5.5, the inner droplets burst simultaneously, producing a transient fluorescent cloud. Scale bar: 500 μm .

in the lipid bilayer^{77–79}. We used a similar approach to implement temperature-triggered release from multisomes.

DPPC. A lipid commonly used to make temperature-sensitive liposomes is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), with a melting transition temperature $T_m = 41\text{ }^\circ\text{C}$. However, attempts to form multisomes with DPPC did not yield stable bilayers when performed over a range of lipid concentrations and incubation times.

The rate at which DPPC liposomes spread at a water-air interface to form a monolayer, and the surface tension reached at equilibrium, depends strongly on temperature. Monolayer formation is most rapid and the equilibrium surface tension is lowest above the T_m of the lipid⁸⁰. In our experiments the aqueous droplets and oil drop were incubated at room temperature ($\sim 20\text{ }^\circ\text{C}$), which might have slowed monolayer formation. However, our system is different in that monolayer formation occurs at an oil-water interface, and the lipid is in the oil phase rather than in the form of liposomes in the aqueous phase. Further, it has been previously observed that the production of giant liposomes of pure DPPC is difficult both at room temperature⁸¹ and at $45\text{ }^\circ\text{C}$ (ref. 82). These findings suggest that a large multisome bilayer of pure DPPC might be unstable even if prepared above the T_m of the lipid.

However, multisomes made at room temperature with a 1:1 (mol/mol) mixture of DPPC and DPhPC were stable, with $\sim 90\%$ surviving for at least 12 h ($n = 8$). When multisomes made with this lipid mixture with a single inner droplet were subjected to a temperature gradient, the external bilayers ruptured suddenly at $32.6 \pm 1.6\text{ }^\circ\text{C}$ ($n = 11$), releasing the contents of the inner droplets into the external aqueous solution. The bursting temperature did not show a significant trend as the molar proportion of DPhPC was varied from ~ 15 – 75% , and significantly lower proportions of DPhPC failed to stabilize the bilayers.

That the bursting temperature is considerably lower than the transition temperature of DPPC is likely due to two factors. First, differential scanning calorimetry measurements have shown that the addition of DPhPC to DPPC significantly broadens the melting transition, and decreases the peak transition temperature⁸³. Second, the interfacial tensions of the monolayers in a multisome act to stretch the bilayer with a tension of $\sim 1\text{ mN m}^{-1}$ (Ch. 5). Tension applied to the bilayer of a liposome has been shown to stabilize electrically-induced pores⁸⁴, and similarly the tension in a multisome bilayer might cause mechanical breakdown near the melting transition of the lipid, due to the appearance of packing defects between phase boundaries^{77–79}. The bilayer tension, combined with the early onset of the melting transition caused by the addition of DPhPC, could account for the dramatic disruption of the bilayer observed well below the T_m of pure DPPC.

DSPC. Following the hypothesis that lipids similar to DPPC suffer a similar broadening of the melting transition upon admixture of DPhPC, DPPC was replaced by 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) ($T_m \approx 55\text{ }^\circ\text{C}$) in order to raise the bursting temperature of multisomes to within a range appropriate for clinical mild hyperthermia, with a view toward developing the potential of multisomes as drug delivery vehicles.

Multisomes made with a 3:1 (mol/mol) mixture of DSPC and DPhPC with a single inner droplet were stable at room temperature for at least a day ($n = 11$). When multisomes were held at $37.2 \pm 0.4\text{ }^\circ\text{C}$, 93% survived for at least 30 min ($n = 46$) (Fig. 2.10a). Multisomes made with this lipid mixture were subjected to a temperature ramp of $\sim 1\text{ }^\circ\text{C min}^{-1}$, and the temperature at which each multisome burst was noted (Fig. 2.10b). Excluding the $\sim 3\%$ of multisomes that burst at $\sim 30\text{ }^\circ\text{C}$, the bursting temperature was $43.6 \pm 3.5\text{ }^\circ\text{C}$ (mean $\pm$ s.d., $n = 93$). When multisomes with two inner

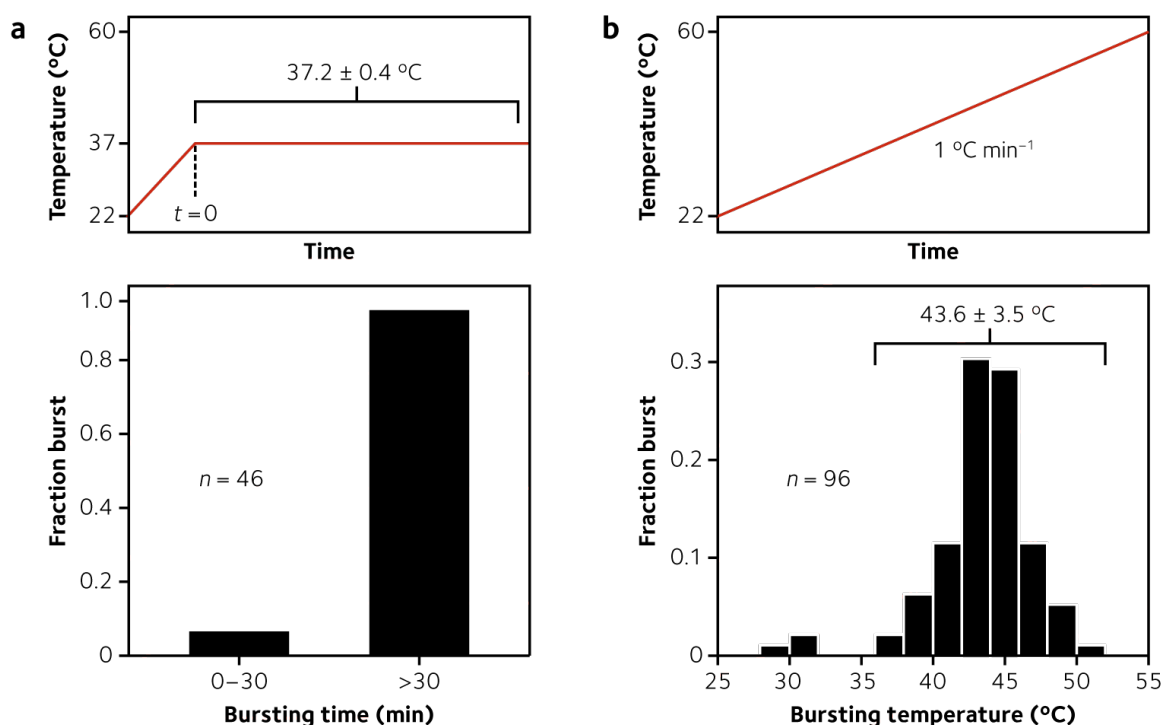


Figure 2.10 | Temperature-dependent release of encapsulated contents. (a) Survival of multisomes held at 37.2 ± 0.4 °C. Top: temperature profile. Bottom: proportion of multisomes that remained intact for at least 30 min. (b) Bursting temperatures of multisomes made with a mixture of DPPC and DSPC with a single inner droplet, subjected to a temperature ramp from room temperature at a rate of ~ 1 °C min^{-1} . Top: temperature profile. Bottom: histogram of bursting temperatures. The bursting temperature was 43.6 ± 3.5 °C (mean $\pm$ s.d., $n = 93$), excluding the three multisomes that burst below 35 °C.

droplets were heated in the same way, in 70% of cases the two inner droplets of each multisome burst within 0.1 °C of each other, at 42.9 ± 3.5 °C (mean $\pm$ s.d., $n = 10$).

The two inner droplets never fused together to form a single, larger inner droplet in either the pH- or temperature-triggered experiments. We conclude that the lipid composition can be chosen to produce multisomes that release their various contents into the aqueous environment upon a specific trigger of pH or temperature. Phase diagrams constructed by differential scanning calorimetry^{85,86} should allow the design of lipid mixtures with a desired melting transition temperature^{79,87} to precisely control the temperature at which multisomes release their contents.

2.7 Future work with multisomes

We envision several directions for the development of multisomes. In basic science, the electrical recording platform developed here might be used to study pore-forming protein complexes that span two lipid bilayers (such as gap junctions³⁶ and nuclear pores⁸⁸) at the single-molecule level by positioning two multisomes with single inner droplets such that their bilayers are apposed. Multisomes might also be developed as vehicles for combinatorial drug delivery, where the inner droplets would contain various drugs that could be released either gradually, by diffusion through pores in external

bilayers, or by pH⁷²- or temperature-triggered⁸⁷ release. In this way, multisomes might deliver multiple drugs with synergistic actions, or prodrugs with their activators⁸⁹, in precise proportions.

Although the manual production technique employed here was appropriate for the study of small numbers of well-defined multisomes, most practical applications of these objects will require reliable and automated means of manufacture. Previous studies have encapsulated aqueous droplets in $\sim 100\text{-}\mu\text{m}$ diameter oil drops (stabilized by surfactants or block copolymers) in bulk aqueous solution, by consecutive shearing⁹⁰ or flow-focusing^{91,92} of aqueous and oil flows. The flow-focusing method has been used to create groups of aqueous droplets joined by bilayers of block copolymers⁹³. These droplet aggregates are structurally similar to multisomes, and the same microfluidic technique should be capable of producing $\sim 100\text{-}\mu\text{m}$ diameter structurally-defined multisomes with functional bilayer interfaces as presented here. The use of lipids, as opposed to alternative interfacial stabilizing agents, allows multisomes to be functionalized based on the richly varied properties of bilayers and membrane proteins.

Droplet networks have been made to transduce a variety of physical and biochemical signals (see Ch. 1). With the ability to function in aqueous environments, multisomes might therefore be developed as a well-defined interface to biological organisms. For example, a droplet network might be used as an intermediary between external electronics and living tissue, or engineered to function autonomously as a synthetic tissue substitute. The following chapter describes the development of droplet networks towards these goals.



CHAPTER 3

3D printed droplet networks

3.1 Introduction

Functional droplet networks have been limited to small groups of droplets, assembled by manual^{1,9,31} or mechanical⁹⁴ manipulation, microfluidic means^{11,95,96} or external fields^{19,97,98}. Larger networks have been constructed by packing droplets into microfluidic containers^{99,100}, but the complexity of the resulting structures is limited by the uncontrolled filling process.

Large, complex droplet networks would constitute a cohesive, macroscopic material composed of distinct aqueous microcompartments that can communicate with each other and the environment through membrane proteins to realize collective functions, and therefore would be functionally analogous to biological tissue (Fig. 3.1). Because neighboring cells in a tissue are separated by two membranes, they must communicate through specialized membrane proteins such as gap junctions³⁶ to produce the emergent properties that distinguish a tissue from a collection of independently functioning cells. By contrast, in a droplet network a single lipid bilayer separates adjacent aqueous compartments, so that cooperative properties may emerge in a simpler environment¹⁰¹.

Here, we develop an automated system for printing three-dimensional (3D) networks in software-defined mm-scale geometries from tens of thousands of heterologous droplets $\sim 50\ \mu\text{m}$ in diameter. The structures are self-supporting, and can be printed in bulk oil or within capsules that reside in aqueous solution. We demonstrate that objects can be printed with membrane proteins in specific bilayers. The networks can also be programmed with osmolarity gradients to fold after printing into various defined geometries.

3.2 Development of the printing system

Our strategy was to eject aqueous droplets within a bath of lipid-containing oil that was mounted on a motorized stage, in order to build up a droplet network in horizontal layers. Although layer-by-layer deposition is commonly employed in 3D printing, the construction of droplet networks raises challenges that preclude the use of a commercially available printing system. In this section we first enumerate these challenges, and then describe how they are addressed by our printing apparatus and process. Finally, we demonstrate the use of the 3D printer for the precise construction of large and complex droplet networks.

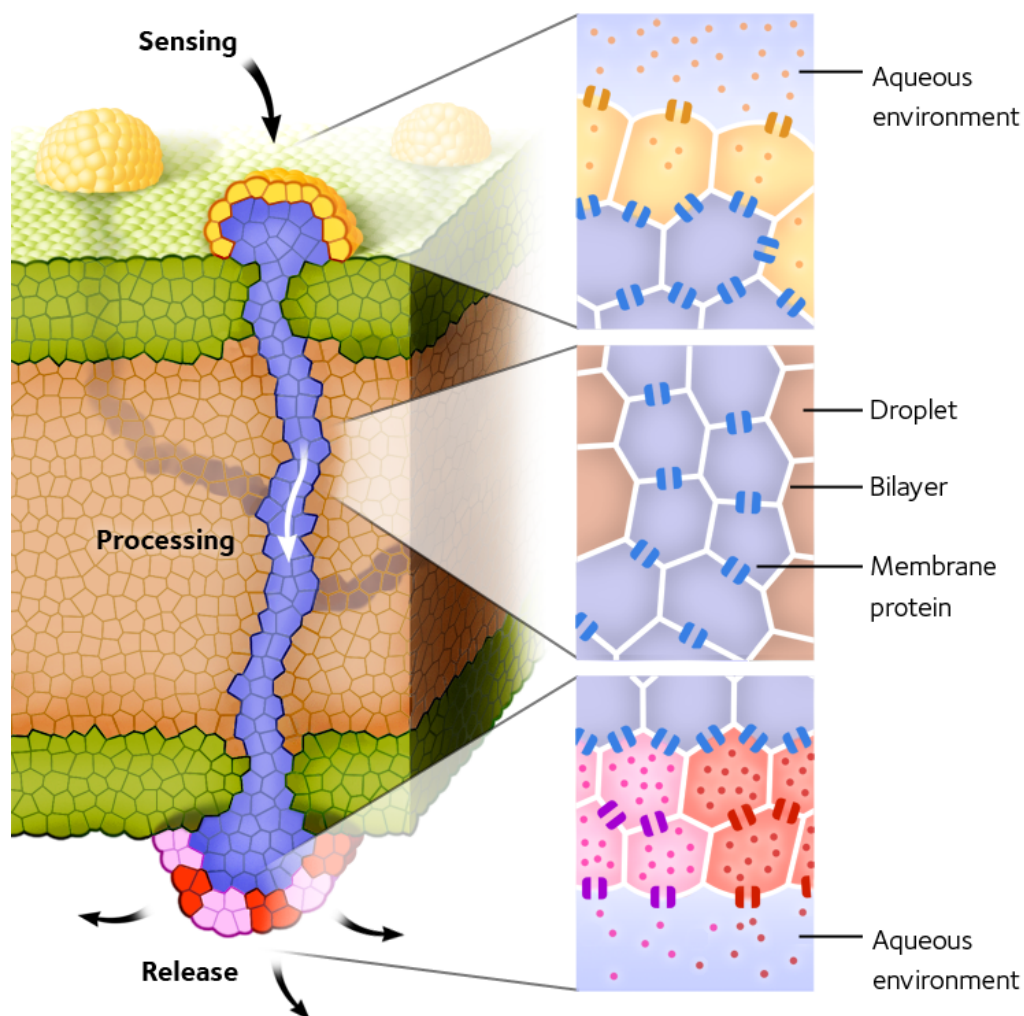


Figure 3.1 | Illustration of a printed droplet network. Single lipid bilayers join adjacent aqueous droplets, and separate peripheral droplets from the aqueous environment. The droplets communicate with each other and the environment through membrane proteins in the bilayers (depicted in insets). Neighboring compartments can communicate directly through membrane proteins that span a single bilayer, in contrast with living tissues.

Printing challenges

The need for a special printing process is due principally to two properties of droplet networks. First, each droplet must acquire a lipid monolayer, or incubate, before coming into contact with another droplet. The required incubation period limits the rate at which printing can take place. Second, the droplets must be printed in oil to allow bilayer formation, whereas most two- or three-dimensional printing technologies operate in air. The dynamic viscosity of the oil mixture used was ~ 10 times greater than that of water. This had several distinct consequences, as outlined below.

Sinking rate. The high viscosity of the oil slowed the descent of each aqueous droplet from its point of ejection towards its intended position in the network. Under our experimental conditions, the Reynolds number for a sinking droplet of diameter $\sim 50 \mu\text{m}$ is of the order of 10^{-4} . Stokes' law gives the terminal velocity of the droplet as $\sim 20 \mu\text{m s}^{-1}$, which agrees with experimental observations.

Given this slow sinking rate, it is desirable to minimize the distance the droplet must fall by ejecting the droplet as close as possible to the growing network during printing. The droplet generator (see below) produces from its nozzle a short-lived protrusion of the aqueous phase into the oil phase $\sim 50\text{--}150 \mu\text{m}$ long, which breaks up within tens of ms to create a single droplet. The aqueous protrusion was deformed by obstacles placed $\lesssim 150 \mu\text{m}$ from the nozzle, which caused droplet formation to become unreliable or abolished it entirely. Each droplet was therefore ejected $\gtrsim 200 \mu\text{m}$ above its final position, and consequently required several seconds to sink from its point of ejection into its intended position in the network.

Oil thinning. Once a droplet came into contact with a growing network, it did not immediately adhere and form a bilayer. This is likely due to the finite time required for the viscous oil layer between the falling droplet and the network to thin under the weight of the falling droplet. Droplets typically formed bilayers with the network $\sim 1\text{--}3$ s after first coming into contact with the network.

Displacement. Droplets not yet incorporated into the network or adhered to a printing substrate were displaced from their intended positions by viscous drag produced by the motion of the nozzles in the oil, and by the ejection of further droplets. The geometries of commercial print-heads, typically designed to operate in air, would create prohibitively great viscous drag.

The above effects make a commercial print-head unsuitable to print droplet networks both rapidly and precisely. Further, the use of a commercial print-head to produce millimetre-scale droplet networks would require quantities of membrane proteins in aqueous solution, and of lipids in oil, that are orders of magnitude greater than the amounts that comprise the final printed network. This problem, albeit economic rather than technical, would preclude repeated experimentation with pure components.

Solutions to printing challenges

To solve the above problems, we developed a custom droplet generator and printing algorithm that allowed droplet networks to be printed relatively rapidly and precisely (see also Ch. 4). We begin with a brief description of the system, and then specify how it addresses the printing challenges.

The droplet generator employs a piezoelectric element for actuation and a finely tapered glass capillary as a nozzle (Fig. 3.2). The device was loaded with aqueous buffer, and the nozzle tip immersed in a solution of lipids in oil. A voltage pulse applied to the piezoelectric element caused it to expand and contract in rapid succession, which produced a pressure wave that propagated through

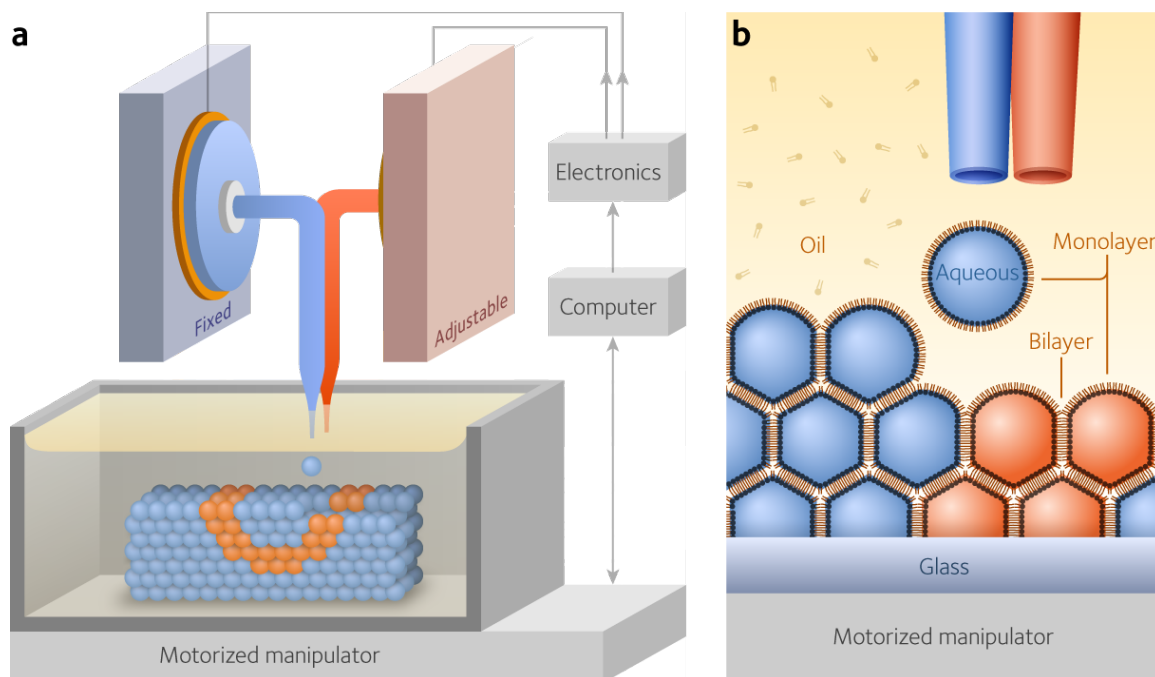


Figure 3.2 | Printing setup. (a) Schematic of the printing process. Two droplet generators eject droplets of different aqueous solutions into a well filled with a solution of lipids in oil. The well is mounted on a motorized micromanipulator controlled by a computer. The same computer controls the ejection of droplets from the two generators through custom electronics, and synchronizes droplet production with the motion of the manipulator. The droplet generator labelled *Adjustable* is mounted on a manual micromanipulator, while the other is fixed. (b) Schematic of a droplet network being printed. Aqueous droplets ejected into the oil acquire a lipid monolayer as they fall through the oil, and then form bilayers with droplets in the growing network.

the nozzle and caused droplet ejection at the nozzle tip. The diameter of droplets ejected into the oil in this way could be tuned to between $\sim 10 \mu\text{m}$ and $200 \mu\text{m}$ by varying the amplitude and duration of the voltage pulse and choosing an appropriate nozzle diameter (Fig. 3.3).

The diameter of ejected droplets did not depend in a simple way on the width or amplitude of the voltage pulse. The observed relationship is likely determined by the resonant behaviour of the piezoelectric element, and by the complex propagation of the pressure wave through the chamber and nozzle of the droplet generator. The resonant frequency of the element (6.3 kHz when mechanically uncoupled¹⁰²) is in turn expected to depend sensitively on the manner in which the element is mounted onto the droplet generator. However, a given pulse width and amplitude produced droplets of a consistent size for a given nozzle (Fig. 3.3). It was therefore not necessary to predict the droplets produced by a given pulse shape, but instead only to determine experimentally the pulse parameters that produced single droplets of the desired diameter.

To print networks of heterologous droplets, two droplet generators were used with their nozzles placed side by side, and each layer was printed in two sequential passes, one for each type of droplet. A 3D network is defined by the user as a series of horizontal cross-sections one droplet thick, and the computer accordingly synchronizes the motion of the oil bath with the ejection of droplets from the two nozzles to construct the desired network automatically.

The droplet generator and printing algorithm were designed to address the printing challenges in the following ways:

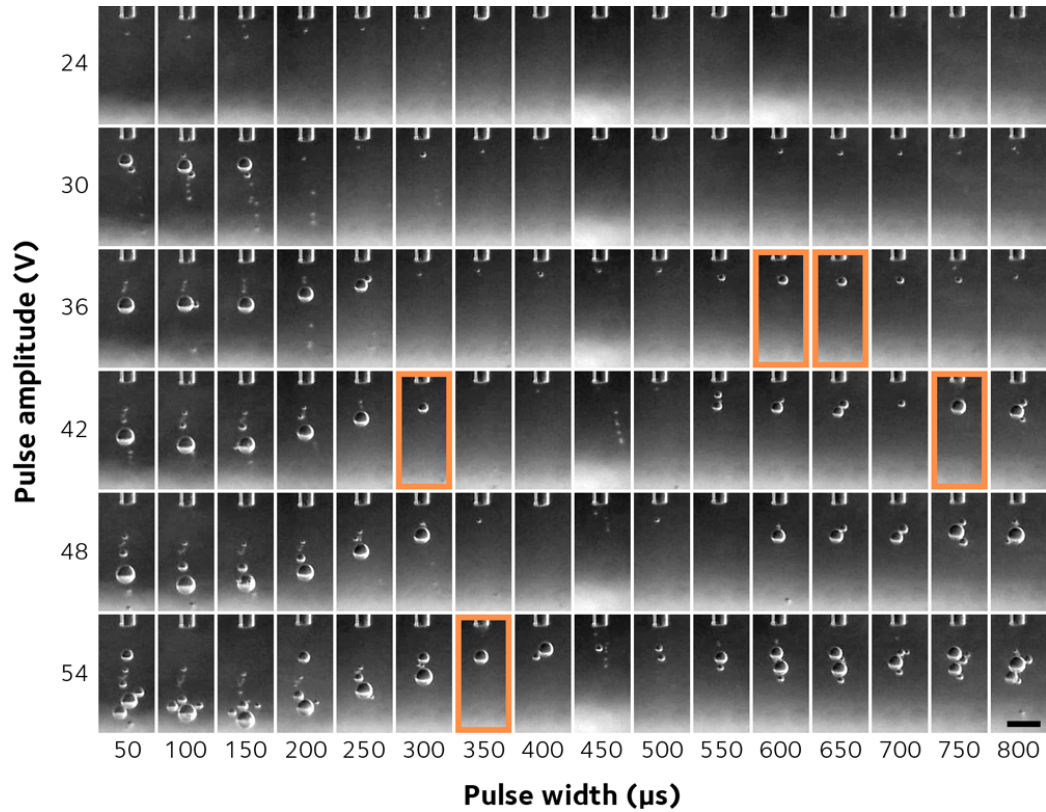


Figure 3.3 | Droplet ejection as a function of pulse width and voltage. Photographs taken immediately after droplet ejection using the apparatus shown in Fig. 3.2b, imaged horizontally through one eyepiece of a stereomicroscope. Photographs were taken for various widths and amplitudes of the voltage pulse applied to a droplet generator. To determine the reproducibility of the ejected droplets, each combination of pulse width and amplitude was applied five times with an interval of a few seconds between pulses. After every combination was tested, the entire procedure was repeated. Droplet production was consistent for each combination of pulse width and amplitude ($n = 10$) for a given nozzle, but varied between nozzles. The highlighted photographs indicate conditions for this particular nozzle that produced single droplets of a suitable size for printing droplet networks. Scale bar: 200 μm .

Optimized nozzle geometry. The viscous drag produced by the motion of the nozzle in the oil was minimized by using thin-walled nozzles with a relatively small outer diameter of $\sim 100\ \mu\text{m}$. The nozzles were formed from tapered glass capillaries as described in Ch. 4. When using capillaries shaped to have a similar inner diameter but an outer diameter of 1.5 mm, the motion of the nozzle displaced the droplets by a significantly greater distance. The nozzle geometry used also minimized the volume of lipid in oil solution required for printing, and allowed the device to operate with as little as $5\ \mu\text{l}$ of aqueous solution. The filling procedure for small aqueous volumes, and the design parameters of the nozzle that affected droplet generation, are detailed in Ch. 4.

Row delay. Initially, a delay was introduced after the ejection of each droplet to allow it to incorporate into the network without being displaced from its intended position. Although this improved the printing accuracy, the required delay of $\sim 3\text{--}10\ \text{s}$ after each droplet made the printing of large networks prohibitively slow. The use of a similar delay after printing each row of droplets, instead of after ejecting each droplet, allowed a significant reduction in printing time without a significant cost to print quality. Although the droplets in each row were then displaced by motion of the nozzle and subsequent ejections in that row, every droplet suffered approximately the same displacement, so that droplets were placed in the correct relative positions within their row. Because adjacent rows were printed in opposite directions, to ensure alignment between rows the printing algorithm was made to offset the positions at which droplets in alternate rows were ejected (Ch. 4).

Increased sinking rate. To reduce the row delay necessary to prevent droplet displacement, we increased the rate at which droplets sank through the oil in two ways. First, we used a salt concentration of 1 M KCl in the aqueous phase, which increased the density of the droplets relative to the oil solution. Second, we used droplets $\gtrsim 30\ \mu\text{m}$ in diameter, because larger droplets suffer proportionately less viscous drag for their weight.

Decreased incubation time. For a given concentration of lipid in the oil, smaller droplets were found to require less time to acquire a lipid monolayer. The incubation time in our experiments was on the order of one second, compared to several minutes in previous studies with larger droplets⁹. This is likely because adsorption of lipid onto the droplet surface creates a lipid-depleted region around the droplet, and for smaller droplets this depleted volume is more accessible to replenishment by diffusion from the lipid bath¹⁰³. The droplet diameter is therefore a compromise between rapid sinking (which occurs with larger droplets) and a short incubation time (which requires smaller droplets). In our experiments we found droplet diameters in the range $\sim 30\text{--}60\ \mu\text{m}$ to be a good compromise.

A secondary effect that might accelerate the incubation of small droplets preferentially is the dissolution of water into the oil. Although no data was available for the solubility of water in silicone oil, the solubility of water in hexadecane is a mole fraction of $\sim 7 \times 10^{-4}$ (ref. 104). Assuming this solubility for the oil mixture used, the typical oil volume of $\sim 750\ \mu\text{l}$ could dissolve $\sim 30\ \text{nl}$ of water. Isolated droplets of volume $\sim 65\ \text{pL}$ dissolved entirely within a few minutes. As a droplet shrinks, the lipids already adsorbed on its surface are concentrated, which contributes to the formation of a well-packed monolayer. Over a given time interval, smaller droplets experience a greater relative change in area, and therefore also a greater relative increase in lipid surface density. However, because dissolution is negligible over the required incubation period of $\sim 1\ \text{s}$, this effect is not likely to be a significant determinant of the incubation time in our experiments.

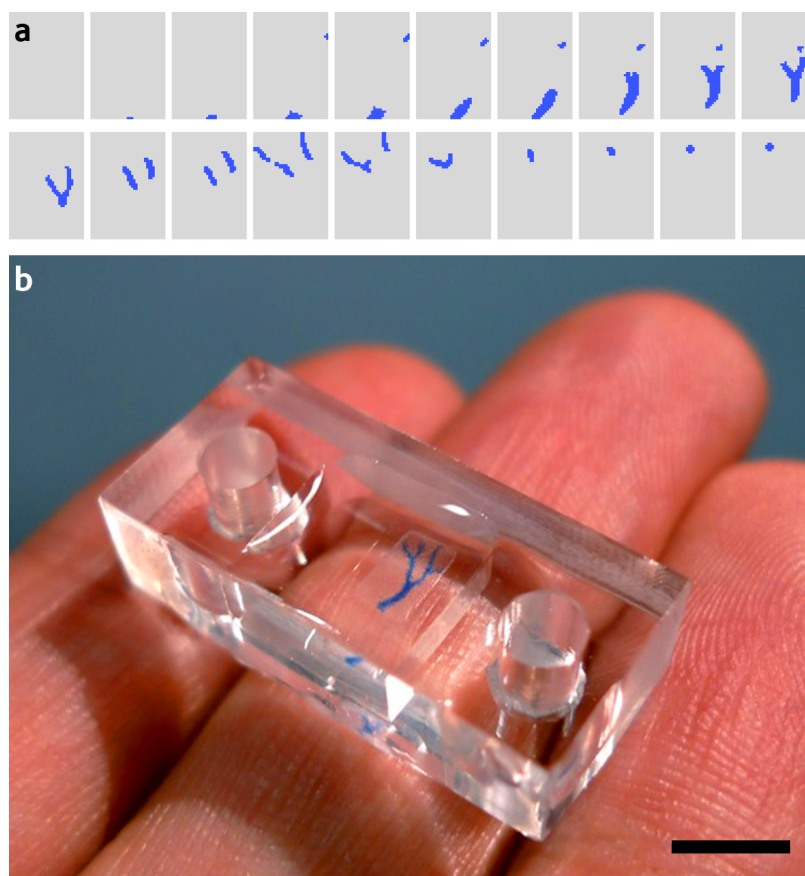


Figure 3.4 | A printed droplet network. (a) Images that define the desired horizontal cross-sections of a three-dimensional droplet network. The design comprises 20 layers of 50×35 droplets each. (b) Network printed according to the design in a. Scale bar: 5 mm.

Printed networks of volume $\sim 1 \mu\text{l}$ also dehydrated, although over several days rather than minutes due to their smaller surface area relative to their volume. That the water in an entire network dissolved beyond the solubility limit in hexadecane may be due to a greater solubility of water in silicone oil, or to evaporation of water from the oil into the air.

Results and discussion

With the above optimizations, the printing system was used to construct precisely defined networks several millimetres in size, comprised of up to $\sim 35,000$ heterologous droplets of diameter $\sim 50 \mu\text{m}$ ejected at a rate of $\sim 1 \text{ s}^{-1}$ (Figs. 3.4, 3.5). Although this printing rate is adequate for the production of networks on this scale, we anticipate that the printing time could be decreased further, as follows.

Lower viscosity. One strategy would be the use of an oil mixture with lower viscosity, to increase the sinking rate of droplets and reduce their displacement from their intended positions. The consequent reduction in sinking time will require that the oil also encourage the rapid formation of a lipid monolayer.

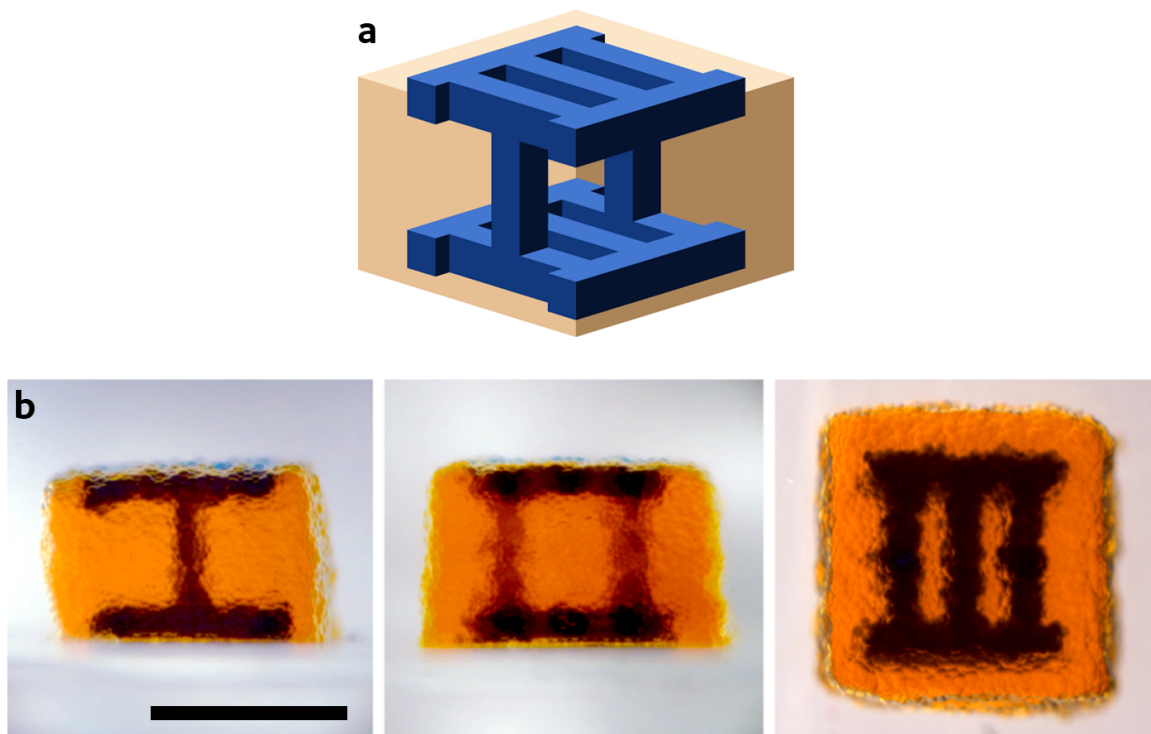


Figure 3.5 | Printed droplet networks. (a) Schematic of a three-dimensional design, which consists of 28 layers of 24×24 droplets each. (b) Three orthogonal views of a single network printed according to the design in a. Scale bar: 1 mm.

Multiple nozzles. Another strategy is the simultaneous use of multiple nozzles, which may reduce the printing time by a factor equal to the number of nozzles. This strategy is commonly employed by commercial ink-jet printers, which employ thousands of nozzles in parallel¹⁰⁵. However, the motion of a large number of nozzles through the oil would greatly increase the viscous drag exerted on previously ejected droplets. In our experiments we ameliorated this problem by allowing each row of droplets to be displaced by the same amount, and introducing a delay between the printing of consecutive rows. With multiple nozzles in parallel, in each horizontal pass the print-head could print multiple rows, so that the required delay need not be proportional to the number of nozzles.

Printing outside oil. Finally, we have found that aqueous droplets can be dropped into the oil phase from above the oil-air interface. Commercial print-heads might therefore be operated in air to print into an oil phase, which would avoid some of the problems that arise from the high viscosity of the oil. The time required for droplets to sink in the oil could be minimized by maintaining the oil level at a constant, small height above the network throughout the printing process.

An aqueous droplet placed carefully at the interface between air and a dense oil can form a partially-submerged lens rather than sink through the oil. To avoid the formation of surface-bound lenses, this strategy would require the droplets to enter the oil at high speed.

3.3 Mechanical properties

Printed droplet networks are cohesive and self-supporting (Fig. 3.4, 3.5), and retain their shape under gentle perturbations. The mechanical properties of droplet networks might therefore be meaningfully expressed in the same way as for a bulk material. In Ch. 5 we derive some mechanical properties of droplet networks, and their dependence on the droplet size and the strength of droplet adhesion. Here we present the main results of the analyses.

The mechanical properties were derived in detail for a pair of droplets joined by a bilayer (Fig. 3.6a), and for a linear chain of droplets of arbitrary length (Fig. 3.6b). We first outline the key assumptions of our model. We then present the mechanical behaviour of the droplet pair and linear chain near and far from equilibrium, and estimate the conditions for a droplet network to be self-supporting. Finally, we outline how the results for these simple systems might extend to fully three-dimensional networks.

Assumptions

The principal assumptions used to calculate the mechanical properties of droplet networks are as follows:

Constant interfacial tensions. The model assumes that the interfacial tensions of the lipid monolayers and bilayers in a droplet network remain constant. Two conditions must be satisfied for this assumption to hold. First, the monolayers must be allowed to recruit the necessary lipids from the oil bath to maintain an equilibrium interfacial tension during deformation of the network. Second, the bilayers must be close to mechanical equilibrium with the monolayers during deformation of the network. In our experiments the droplets required ~ 1 s to incubate, and pairs of droplets relaxed on a similar timescale during bilayer formation. Perturbations with a timescale greater than a few seconds can therefore be expected to take place at constant monolayer and bilayer tensions. Under perturbations that change the monolayer-coated area rapidly compared to the time required for the monolayers to reach an equilibrium tension, the interfacial tensions and therefore the mechanical properties of the network will likely depend on the rate of deformation. Therefore although the network is expected to behave as a viscoelastic material in general, here we consider the limit of slow deformations.

Spherical cap geometries. We assume that the droplets are composed of spherical caps. This follows from the assumption that for each monolayer or bilayer on a droplet, the interface adopts the geometry that minimizes its interfacial free energy. The droplets are assumed to be identically sized, so there exists no Laplace pressure between adjacent droplets and the bilayers are therefore planar. The monolayers must enclose a given volume with the minimal area, which together with the constraint of planar bilayers requires the droplet to adopt the geometry of a spherical cap, in agreement with observations.

Changing contact angle under deformation. Finally, we assume that the contact angle between a pair of droplets joined by a bilayer changes as the droplets are pulled apart or pushed together. This follows from the assumption that the interfacial tension is identical at all points on the monolayer-coated surface of a given droplet.

Mechanical behaviour near equilibrium

We express the mechanical behaviour of a droplet network near its equilibrium state in two ways: in terms of effective springs that join connected droplets, and in terms of the Young modulus of the network.

Effective spring constants. Under gentle perturbation from equilibrium the free energy of bilayer formation is approximately quadratic in the extension of the droplet network (Ch. 5), so that droplets behave as point masses joined by Hookean springs. It should be noted that the bilayers and monolayers are not assumed to behave as elastic surfaces, but rather the droplet network as a whole behaves as an elastic material. Deformation of the network changes the monolayer and bilayer surface areas from their equilibrium values, and the associated increase in free energy produces a restoring force that is approximately quadratic in extension for small perturbations (Ch. 5). The effective spring constants for the droplet pair and linear chain, k_{pair} and k_{chain} respectively, were calculated to be:

$$k_{\text{pair}} = \gamma_m \pi (2 - \cos \theta_{\text{eq}});$$

$$k_{\text{chain}} = \frac{\gamma_m \pi}{4N} (3 \sec \theta_{\text{eq}} + 2 \cos \theta_{\text{eq}} - \cos^3 \theta_{\text{eq}}),$$

where γ_m is the monolayer interfacial tension, θ_{eq} is the equilibrium contact angle between a pair of droplets joined by a bilayer, and N is the number of droplets in the linear chain. Note that k_{chain} is the effective spring constant of the entire chain, so that chains composed of more droplets are less stiff. For N identical Hookean springs of stiffness k_0 connected in series, the equivalent spring constant of the chain is equal to k_0/N . Therefore each unit of the linear chain can be attributed an effective spring constant of Nk_{chain} , which can be compared directly with k_{pair} (Fig. 3.6c).

In both cases the spring constant increases monotonically as the equilibrium contact angle increases in the range $0 \leq \theta_{\text{eq}} \leq 90^\circ$; that is, droplets that are more strongly adhesive are effectively connected by springs of greater stiffness. Under our experimental conditions, θ_{eq} was measured as $47 \pm 4^\circ$ ($n = 7$), and $\gamma_m \approx 1 \text{ mN m}^{-1}$ (ref. 67), so that $k_{\text{pair}} = 4.1 \text{ mN m}^{-1}$ and $Nk_{\text{chain}} = 4.3 \text{ mN m}^{-1}$. Therefore each bilayer in a linear chain of droplets behaves like the bilayer in an isolated droplet pair, but with $\sim 5\%$ greater stiffness.

For droplets that are more strongly adhesive, Fig. 3.6c shows that each unit in the chain rapidly becomes much more stiff than a single droplet pair. In the extreme case of $\theta_{\text{eq}} \approx 90^\circ$ the droplets in the chain become flattened into large, thin discs with a drastically increased cross-sectional area. The extension of the chain by a given length therefore constitutes a progressively more severe deformation from the equilibrium geometry; this causes the energy required for that deformation, and therefore the effective stiffness k_{chain} , to diverge at $\theta_{\text{eq}} = 90^\circ$. By contrast, at this extreme contact angle each droplet in the isolated pair forms a hemisphere of an overall spherical pair, and this geometry places an upper bound on the energy of deformation and therefore also on k_{pair} .

Young's modulus. The Young modulus of a material is defined as the ratio of the applied stress to the resulting strain in the same direction. Like the effective spring constant it is a measure of resistance to deformation, but the Young modulus also takes into account the area over which the external force is applied. The Young moduli of the droplet pair and chain, E_{pair} and E_{chain}

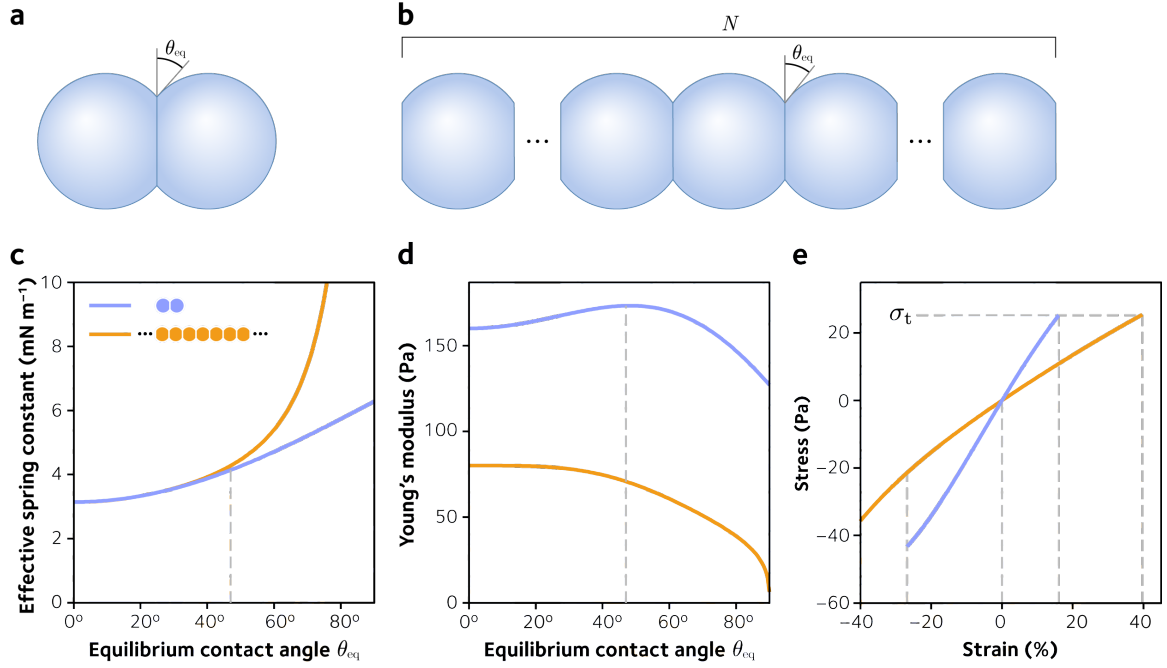


Figure 3.6 | Mechanical properties of printed networks. (a) A droplet pair joined by a bilayer with an equilibrium contact angle θ_{eq} . (b) A linear chain of N droplets joined by bilayers with an equilibrium contact angle θ_{eq} . (c) The effective spring constant between a single pair of droplets, k_{pair} (blue line), and between each pair of droplets in a linear chain, Nk_{chain} (orange line), as a function of equilibrium contact angle θ_{eq} . The quantity Nk_{chain} represents the effective spring constant of each bilayer in the chain, and is independent of chain length. The curves were calculated assuming the monolayer interfacial tension $\gamma_m = 1 \text{ mN m}^{-1}$. (d) Young's modulus as a function of equilibrium contact angle θ_{eq} , for a single pair of droplets (blue line) and a linear chain of droplets (orange line). Neglecting end effects, the latter is independent of the number of droplets in the chain. The dashed line marks the equilibrium contact angle of 47° used in this work. The curves were calculated assuming the initial droplet radius $R_0 = 25 \text{ }\mu\text{m}$ and the same monolayer interfacial tension as in c. (e) Stress-strain relationship for a droplet pair (blue line) and linear chain of droplets (orange line). The vertical dashed lines indicate, from minimum to maximum strain, the compressive strain that would cause the droplet pair to adopt a spherical geometry; zero strain; the ultimate tensile strain for a droplet pair of 16%; and the ultimate tensile strain for a droplet chain of 40%. The horizontal dashed line indicates the tensile stress for both systems of 25 Pa.

respectively, were calculated to be (Ch. 5):

$$E_{\text{pair}} = \frac{2^{1/3} \gamma_m}{R_0} (2 - \cos \theta_{\text{eq}})^{4/3} (1 + \cos \theta_{\text{eq}})^{5/3};$$

$$E_{\text{chain}} = \frac{\gamma_m}{4R_0} (3 \cos \theta_{\text{eq}} + 2 \cos^3 \theta_{\text{eq}} - \cos^5 \theta_{\text{eq}}) (12 \sec^2 \theta_{\text{eq}} - 4)^{1/3},$$

where R_0 is the radius of each droplet before bilayer formation. Note that whereas $k_{\text{chain}} \propto 1/N$, the tensile modulus is independent of chain length. In our experiments $R_0 = 25 \mu\text{m}$, and using the same values for γ_m and θ_{eq} as above we obtain Young moduli of $E_{\text{pair}} = 170 \text{ Pa}$ and $E_{\text{chain}} = 71 \text{ Pa}$. Interestingly, this range overlaps with the elastic moduli of brain, fat and other soft tissues¹⁰⁶. The Young moduli would be greater for networks with smaller droplets (smaller R_0), or in conditions with greater monolayer and bilayer interfacial tensions (greater γ_m at constant θ_{eq}).

The Young modulus of the droplet pair is maximal at an intermediate value of θ_{eq} (Fig. 3.6d). Setting $dE_{\text{pair}}/d\theta_{\text{eq}} = 0$, one readily finds this to be $\theta_{\text{eq}} = \arccos(2/3) \approx 48^\circ$, which coincidentally is approximately equal to the value of θ_{eq} in our experiments. For the droplet chain, E_{chain} decreases monotonically with increasing θ_{eq} ; that is, the chain is less rigid if composed of droplets that are more strongly adhesive. The derivation in Ch. 5 reveals that although increasing θ_{eq} increases the stiffness of the chain, there is a concomitant shortening of the equilibrium length and increase in the cross-sectional area of the droplet chain, which overall produces a decrease in E_{chain} .

That only the tensile modulus of the droplet chain approaches zero at large values of θ_{eq} can be understood by again considering the case of $\theta_{\text{eq}} \approx 90^\circ$. The droplets in the linear chain are flattened as described above; the applied force is therefore distributed over an increased cross-sectional area, so that the ratio of stress to strain approaches zero. By contrast, the spherical geometry of the droplet pair at this contact angle places an upper bound on the cross-sectional area.

Mechanical behaviour far from equilibrium

The expressions derived for the free energy of bilayer formation also allow us to determine the behaviour of the droplet pair and chain far from equilibrium. The mechanical response to extension or compression of a droplet network can be described by its stress-strain relationship. This is derived for a droplet pair and chain (Ch. 5), and depicted for our experimental conditions (Fig. 3.6e). As expected from the result for Young's modulus (Fig. 3.6d), a droplet chain is calculated to require less stress than a droplet pair to reach a given strain.

A further measure of the mechanical strength of droplet networks is the greatest stress that can be applied without causing droplets to separate irreversibly, that is, the ultimate tensile strength, σ_t . The result is identical for a pair of droplets and a linear chain (Ch. 5):

$$\sigma_t = \frac{2\gamma_m}{R_0} (1 - \cos \theta_{\text{eq}}).$$

With our values of γ_m , R_0 and θ_{eq} , we obtain a tensile strength of $\sigma_t = 25 \text{ Pa}$. Because the tensile strength is the same for a droplet pair and chain, and the chain achieves a greater strain for a given stress (Fig. 3.6e), the strain at which the droplets in a chain separate is 40%, compared to 16% for a droplet pair. The tensile strength increases monotonically with θ_{eq} in the range $0 \leq \theta_{\text{eq}} \leq 90^\circ$, so networks composed of more strongly adhesive droplets can withstand greater stress before the droplets separate entirely.

Conditions for self-support

Because the density of the aqueous droplets is greater than that of the surrounding oil, the upper layers of a network exert a pressure on the lower layers. In a network with enough layers, this pressure might be enough to force apart the droplets on the lower layers, so that the network cannot support its own weight. Having derived the mechanical behaviour of a droplet pair allows us to estimate the requirements for a network to be self-supporting.

We consider a vertical column of droplets within a network, the weight of which acts to separate two droplets on the bottom layer. To make a conservative estimate, we will consider the network to no longer be self-supporting if these bottom droplets are forced apart. We also assume that the entire weight of the column is supported by only these two droplets, and neglect the interaction of the droplet pair with other droplets in the bottom layer that could prevent their separation. With these assumptions, we find the following condition for the network to be self-supporting (Ch. 5):

$$N \leq \frac{3\gamma_m \left(8 - [32(8 - \cos 3\theta_{eq} + 9 \cos \theta_{eq})]^{1/3}\right)}{8gR_0^2(\rho_{drop} - \rho_{env})}, \quad (3.1)$$

where N is the number of droplets in the vertical column, ρ_{drop} is the density of the droplets, ρ_{env} is the density of the surrounding oil, and g is the acceleration due to gravity. Using the same values as above for R_0 , θ_{eq} and γ_m , and assuming $\rho_{drop} = 1 \text{ g cm}^{-3}$ for the aqueous droplets and $\rho_{env} = 0.9 \text{ g cm}^{-3}$ for our oil mixture, we arrive at a conservative upper limit of $N = 4,300$ layers in a self-supporting droplet network, which corresponds to a network thickness of $\sim 20 \text{ cm}$. Given the assumptions in the calculation, we expect the upper limit under our experimental conditions to be significantly greater. The above expression suggests that the layer limit should be higher still for networks printed with smaller droplets (smaller R_0), in more adhesive conditions (greater θ_{eq}), or in bulk aqueous solution (smaller $\rho_{drop} - \rho_{env}$).

Mechanical properties of three-dimensional networks

It was not analytically tractable to derive the mechanical properties of a fully three-dimensional lattice of droplets in the same way as for a droplet pair and chain. For small perturbations about equilibrium, the droplets in a 3D network could again be modelled as connected by springs, and scaling arguments (Ch. 5) suggest that some properties of the Young modulus would be similar to those of the simpler systems.

However, there are several ways in which a 3D network can be expected to exhibit different mechanical properties from the simpler systems. First, each droplet in a typical 3D network has more neighbours than in a pair or chain. The changes in monolayer and bilayer areas under a perturbation, and therefore the values of k and E , will depend on the number of neighbours and their arrangement. Second, under large deformations some droplets may detach, form new bilayers or both, so that the tensile strength of the network would not be given by the simple arguments used here. Further, in addition to tensile stress a 3D network can be subjected to bending, shear and torsion. The response of 3D networks to arbitrary perturbations might be explored in detail using numerical calculations or a simplified mechanical model (Ch. 5), and validated with experimental studies.

Experimental verification

The theoretical predictions presented here for large droplet networks could be tested as for other bulk materials. The behaviour of smaller networks, such as a pair or linear chain of droplets, might be investigated as follows. For a pair of droplets joined by a bilayer, each droplet could be held at

the end of a micropipette by a controlled suction¹⁰⁷. If the droplet does not wet the pipette, the minimum suction pressure required to draw the droplet into the pipette is given by the difference in the Laplace pressures of the droplet surfaces outside and within the pipette: $P_{\text{crit}} = 2\gamma (r_{\text{in}}^{-1} - r_{\text{out}}^{-1})$, where γ is the interfacial tension, and r_{in} and r_{out} are the radii of the inner and outer portions of the droplet, respectively^{108,109}. By maintaining the pressure below P_{crit} the droplets may be held without distortion. Measurement of the force exerted by the droplets as a function of their extension and compression, for example for comparison with Fig. 3.6c, might be accomplished by using one of the micropipettes as a calibrated cantilever. This approach has been used to measure the force required to stretch a single water droplet in oil between two microcapillaries, and thereby deduce the interfacial tension of the droplet surface¹¹⁰.

3.4 Printing in aqueous solution

In Ch. 2, droplet networks were stabilized in bulk aqueous solution by encapsulation within small drops of oil to form multisomes, with prospective applications in synthetic biology and medicine¹. Whereas previously the multisomes were created manually and therefore were limited in complexity, here we demonstrate the printing of complex encapsulated networks.

Printing process

Encapsulated networks were printed inside an oil drop suspended in aqueous solution by a wire frame with a hydrophobic coating (Fig. 3.7 and see Ch. 4). We adapted the procedure for printing in bulk oil to print encapsulated networks in a controlled manner, with the following modifications.

Immersion of printing nozzles. In order to enter the oil drop, the printing nozzles first had to pass through the bulk aqueous phase. To prevent leakage of the nozzle contents into the bulk aqueous solution during this transit, a plug of the same oil mixture used for the oil drop was sucked into the tip of each nozzle, by applying suction at the inlet of each droplet generator with a micropipette. Once the nozzle tips had been placed inside the oil drop in aqueous solution, the oil plugs were expelled by applying positive pressure at each inlet in the same way.

Removal of excess oil. Because aqueous droplets in the encapsulated network pack closely and have a greater density than the oil, most of the oil volume is displaced above the network during printing. Once printing is complete, excess oil can be removed by suction through one of the printing nozzles.

User-directed printing. The printing pattern used for bulk oil, in which droplets were printed in sequential parallel rows, was found to be inadequate for printing inside an oil drop in bulk aqueous solution. This was due to the curved lower surface of the oil drop, and to the time-dependent geometry of the oil drop.

In contrast to the droplets printed in bulk oil on a horizontal substrate, droplets ejected inside the recipient oil drop in bulk aqueous solution rolled down the curved lower surface of the oil drop over a few seconds. Because the timescale of rolling was comparable to the rate at which droplets were printed, the droplets in an encapsulated network printed row-by-row were not in the desired arrangement.

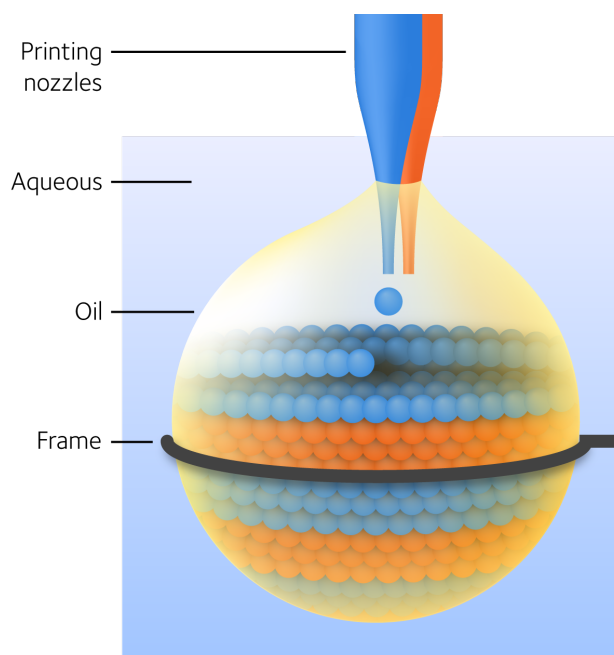


Figure 3.7 | Schematic of printing in bulk aqueous solution. The nozzles eject aqueous droplets into a drop of oil that is suspended in bulk aqueous solution by a wire frame. The frame is attached to the bulk container, which is translated by a motorized micromanipulator. Excess oil can be removed after printing by suction through a printing nozzle.

The printing algorithm might be readily adapted for an oil drop with a given static geometry. For example, the aqueous droplets could be prevented from rolling away from their intended positions by printing each layer in an outwardly growing spiral pattern. However, the geometry of the oil drop changed during printing for two reasons. First, because the volume of oil is conserved, the ejection of an aqueous droplet increases the total volume enclosed by the surface of the oil drop. Second, the printing nozzles are wetted by the oil drop, so that the oil drop is distorted in a manner that depends on the positions of the nozzles inside it.

Two broad strategies might be employed by a printing algorithm to account for the curved and time-dependent geometry of the oil drop. In one approach, the printing pattern is set before printing according to a known relationship between the position at which each droplet is ejected and the position of that droplet in the final printed network. However, the final position of a given droplet is likely to depend on the order in which other droplets in the network are printed, so that such a relationship would be difficult to specify for complex networks.

In the other approach, employed here, the printing pattern is determined during printing in real time using feedback on the positions of already-ejected droplets and the geometry of the oil drop. The automated acquisition and algorithmic interpretation of this feedback is beyond the scope of this work. However, a person who observes the printing process through a stereomicroscope can readily acquire this information, and control the printing process in real time through a user interface to the printer. To this end, a graphical user interface was written that allowed real-time control of both droplet generators and of the manipulator (Ch. 4).

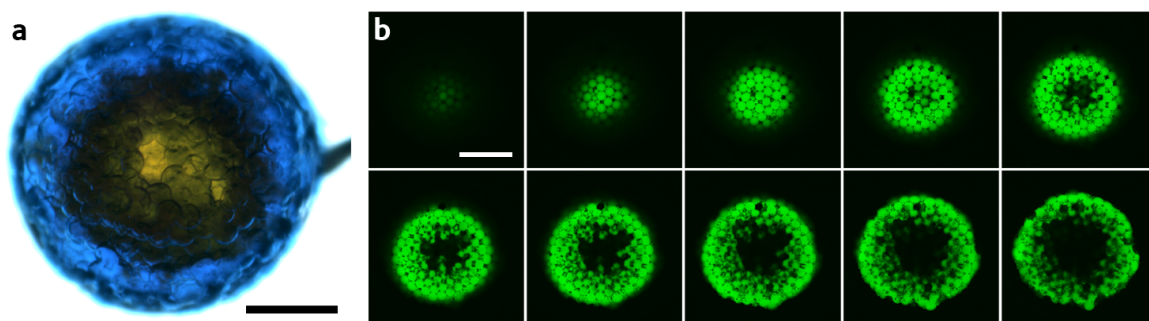


Figure 3.8 | Confocal microscopy of an encapsulated printed network. (a) Photograph of a network printed in aqueous solution, viewed from above. A core of orange droplets is surrounded by a shell of blue droplets, which contain the fluorescent dye pyranine. Scale bar: 400 μm . (b) Horizontal sections of the network in **a** obtained by confocal microscopy, showing the fluorescent shell of droplets around the non-fluorescent core. The sections span approximately the bottom 150 μm of the network. Scale bar: 400 μm .

Results and discussion

The above techniques allowed encapsulated printed networks to be printed in a controlled way (Figs. 3.8, 3.9). The encapsulated networks were stable for at least several weeks. We expect that the ability to print complex, stable droplet networks in aqueous solution will serve to expand the functions previously demonstrated with simple encapsulated networks, including communication with the aqueous surroundings through membrane pores, and pH- or temperature-triggered release of contents¹. We now discuss the factors that determine the geometries of encapsulated networks.

Wire frame. The volume of oil initially available for printing is limited by the geometry of the wire frame used to support the oil drop in aqueous solution. When a wire loop was loaded with an oil volume $\gtrsim 50\%$ of the volume of a sphere bounded by the loop, the initially lenticular oil drop deformed over a few minutes until it broke up to form a drop that floated upwards in the aqueous phase, while the residual oil remained attached to the loop. In Ch. 5 we show that buoyancy is expected to dominate over interfacial tension forces in this case. The gradual change in geometry of the oil drop was presumably due to a progressive decrease in interfacial tension caused by the adsorption of lipid at its surface.

Nozzles. The maximum suspended volume of oil also depended on the geometry of the nozzles. When the two nozzles were inserted into the oil drop approximately parallel to each other and separated by $\lesssim 200 \mu\text{m}$, most of the oil rose spontaneously in the space between the nozzles. Larger frames may therefore be best designed as fine meshes to prevent the oil drop from breakup instability and from rising between the nozzles by capillary action.

Volume and surface area. The geometry of an encapsulated network is not fully determined by the wire frame. Because the volume of oil remains constant during printing, the encapsulated network may have a greater volume than the oil. Further, an encapsulated network does not necessarily minimize its external area (Fig. 3.9). Non-minimal surface areas are kinetically stable, because the rearrangement of the network to attain the minimal bounding area would require the unfavourable separation of connected droplets. Further, the interfacial tension of an internal bilayer (between two encapsulated droplets) is at least approximately equal to that of an external bilayer (between

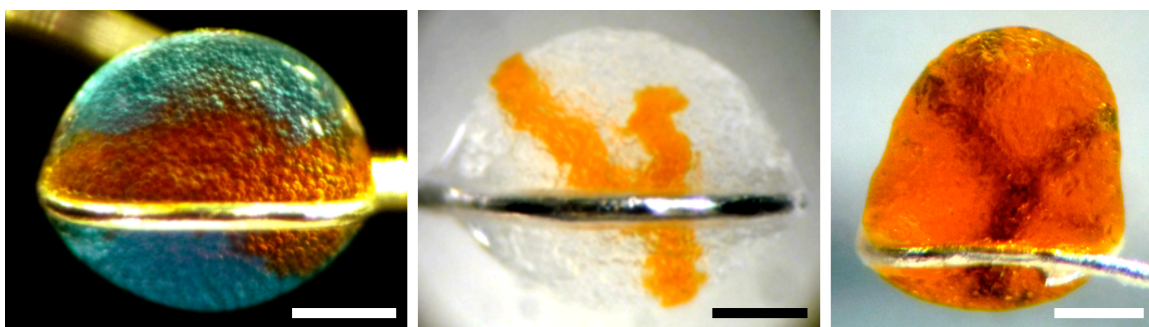


Figure 3.9 | Droplet networks printed in bulk aqueous solution. Micrographs of three networks printed in bulk aqueous solution. Scale bars: 400 μm .

an encapsulated droplet and the bulk aqueous solution)¹, so an encapsulated network would not decrease its surface energy significantly by minimizing its bounding area.

3.5 Conductive droplet pathway

We next established whether membrane proteins could be included in specific bilayers, and so allow droplets in the printed material to communicate in a controlled way.

Results

As a proof of principle, we printed a network in which only the droplets along a defined pathway contained αHL pores, in order to create an ionically conductive route across an otherwise insulating network (Fig. 3.10a,b). To probe the network electrically in a non-destructive way, a drop of buffer of diameter $\sim 500\ \mu\text{m}$ containing αHL was manually pipetted onto each of two Ag/AgCl electrodes with agarose-coated ends. The drops were then brought into contact with different parts of the network, so that they formed bilayers with the droplets on the network surface (Fig. 3.10a).

When the two large drops were placed on either end of the αHL -containing pathway (Fig. 3.10b), we measured a stepwise increase in ionic current under an applied potential (Fig. 3.10c, and see below). After one of the drops was separated and brought back into contact with the network away from the αHL -containing pathway (Fig. 3.10d), only transient currents were observed (Fig. 3.10e, and see below). When this drop was separated from the network again and replaced in its original position, a stepwise increase in current was again observed (data not shown). Droplet networks in which no droplets contained αHL showed negligible current flow (Fig. 3.11a), whereas the current measured across droplet networks in which every droplet contained αHL was similar to that measured across the droplet pathway (Fig. 3.11b).

Discussion

The relatively large magnitude of the current measured across the droplet pathway (Fig. 3.10c) compared to that measured away from the pathway (Fig. 3.10e) indicates that only the pathway allows the uninterrupted flow of ionic current. The electrical recordings do not, however, immediately suggest explanations for the stepwise increase in current measured along the pathway, or for the transient spikes of current away from the pathway.

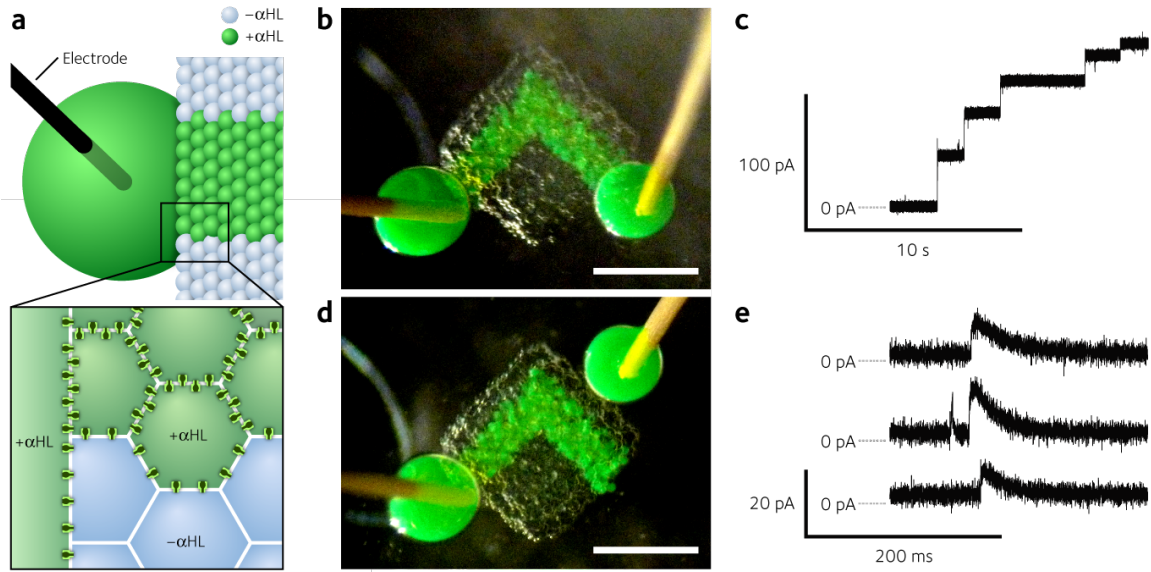


Figure 3.10 | Electrically conductive pathway. (a) Schematic of part of a network printed with a pathway that allows the flow of ionic current. Only the green droplets and the large drop contain α HL pores. The large drop is impaled with an Ag/AgCl electrode. The magnified section illustrates the α HL pores in the bilayers around the α HL-containing droplets. (b) Photograph of a printed network with electrode-impaled drops placed on either end of the conductive pathway. The green droplets contain α HL, while the other droplets contain no protein. Scale bar: 500 μ m. (c) Stepwise increase in the ionic current as measured in the configuration in b, at 50 mV in 1 M KCl at pH 8.0. (d) Photograph of the network in b, after separating one of the large drops and rejoining it onto the network away from the conductive pathway. Scale bar: 500 μ m. (e) Selected portions of a single recording as measured in the configuration in d at 50 mV, showing transient increases in ionic current.

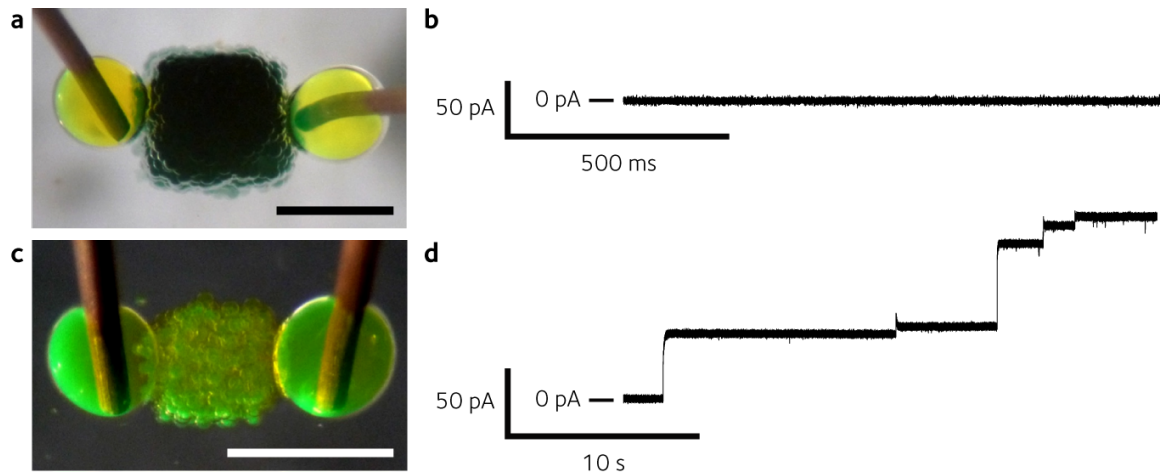


Figure 3.11 | Electrical measurements of droplet networks with and without α HL. (a) Photograph of a network in which none of the droplets contained α HL. The electrode-impaled drops contained α HL. Scale bar: 500 μ m. (b) Typical portion of the current measured at 50 mV in the configuration shown in a. No steps or transient spikes of current were measured from this network. (c) Photograph of a network in which all of the droplets contained α HL. The network droplets and the electrode-impaled drops were of the same solution as the electrode-impaled drops in a. Scale bar: 500 μ m. (d) Portion of the current measured at 150 mV in the configuration shown in c, immediately after the large drops were placed onto the network. Similar current steps were measured after twice removing and replacing the large drops.

To interpret these aspects of the measured currents, we performed computational simulations of the electrical behaviour of the droplet network in Fig. 3.10. As detailed below, the simulations suggest that most of the bilayers along the droplet pathway in the network contained several α HL pores at the time of recording, and that the other bilayers in the network contained none, so that the pathway presented an already established conductive route through the otherwise insulating network.

Electrical model. A previous study analyzed the electrical behaviour of three droplets joined by bilayers in a linear configuration¹¹¹, but the procedure is not readily applied to larger systems. We therefore formulated a model that allows the straightforward simulation of the electrical behaviour of arbitrarily complex droplet networks (Ch. 5). In the model, the bilayer that joins connected droplets is modelled as a capacitor, and any pores in the bilayer are represented by resistors in parallel with the capacitor. In this case, only part of the network in Fig. 3.10 was simulated to decrease the computation time (Fig. 3.12a,c); the simulation results are not expected to change significantly with the inclusion of the rest of the network. The simulations used the following parameter values, as an approximation of the conditions of the experiments in Fig. 3.10: an applied potential of 50 mV, bilayer diameters of 45 μ m, a bilayer specific capacitance of 650 nF cm⁻² (ref. 9), a bilayer conductance of 1 pS, and a pore conductance of 1 nS (ref. 9). By comparing the experimentally measured and simulated currents, we aimed to deduce the likely configurations of α HL pores in the network.

Currents measured on the pathway. The currents measured along the pathway, such as in Fig. 3.10c, were consistent with the simulations (Fig. 3.12) only if the following conditions were met:

1. Most of the bilayers in the conductive pathway contained at least several α HL pores, so that the pathway had negligible resistance compared to a single pore. This is reasonable, given the relatively high concentration of α HL used and the relatively long time available for pores to insert into bilayers in the pathway.
2. Many of the bilayers between the pathway and one of the electrode-impaled drops (drop *a* in Fig. 3.12a) contained at least one α HL pore, while no pores were present in any of the bilayers that joined the pathway to the other electrode-impaled drop (drop *b* in Fig. 3.12a). This is plausible, given that one of the large drops was placed onto the network approximately tens of seconds before the other.
3. The bilayers formed by the second electrode-impaled drop to be connected to the pathway (drop *b* in Fig. 3.12a) initially contained no α HL pores, and the first current step corresponded to the insertion of a pore in any bilayer between that drop and the pathway. Each subsequent step was due to the insertion of additional pores in these bilayers. This condition is reasonable, because the experimental electrical recording began immediately after the second electrode-impaled drop was joined to the pathway.

Under these conditions, the simulated current steps closely matched the measured currents in both the step amplitudes and relaxation timescale (Fig. 3.12b). Further, as shown in Ch. 5 the above assumptions can be used to calculate the resistance of a single α HL pore from the measurements in Fig. 3.10c, which yields a resistance of 1.0 ± 0.3 G Ω ($n = 4$), in good agreement with the known value of 1 G Ω (ref. 9). Similar calculations (Ch. 5) give the resistance of the pathway as 0.23 ± 0.05 G Ω .

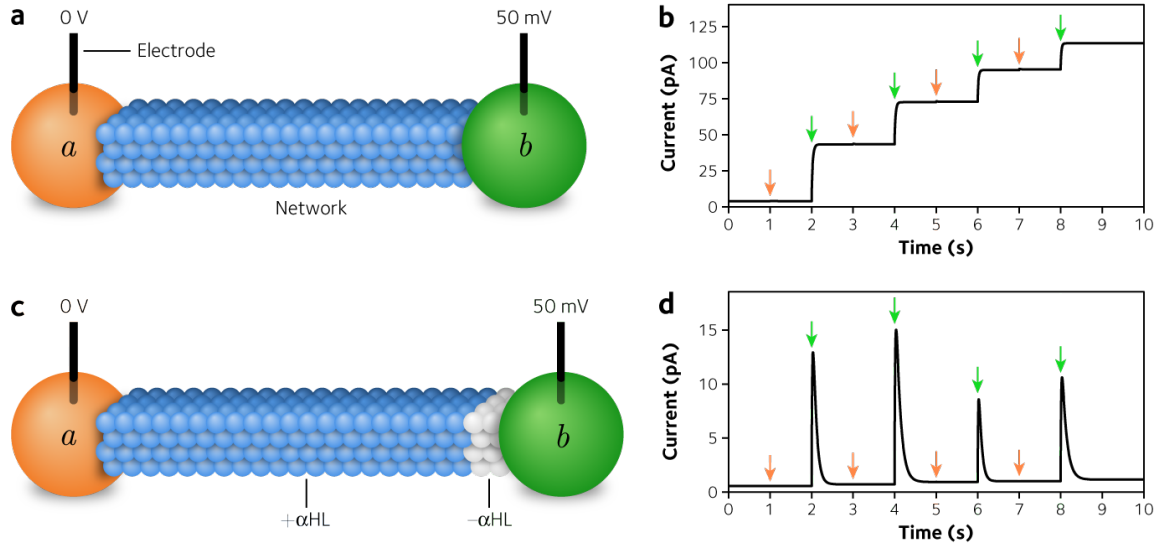


Figure 3.12 | Electrical simulations of a conductive droplet pathway. (a) Schematic of the system simulated as a model of the electrical recording conditions in Fig. 3.10b. The network consists of 4 rows, 20 columns and 4 layers of droplets in a face-centred cubic arrangement, and represents only the conductive pathway part of the network in Fig. 3.10. Two large drops are positioned at either end of the network, and form bilayers with the network droplets at the end of each row. (b) Simulated current between the electrodes in the system depicted in (a). Five α HL pores are assumed to be initially present in each bilayer within the network. Each of the bilayers between the network and drop *a* is assumed to initially contain one pore, while those between the network and drop *b* are assumed to contain no pores. The insertion of a single α HL pore into a bilayer between the network and drop *a* is simulated to occur at 1 s, 3 s, 5 s and 7 s (orange arrows), and into a bilayer between the network and drop *b* at 2 s, 4 s, 6 s and 8 s (green arrows). Each pore inserts into a different bilayer. Note that to decrease the computation time, the time interval of the simulation was made shorter than that in Fig. 3.10c. (c) Schematic of the system simulated as a model of the electrical recording conditions in Fig. 3.10d. The network is identical to that in *a*, except for the addition of two columns of droplets that do not contain α HL. (d) Simulated current between the electrodes in *c*. The simulation was performed with the same conditions as described in *b*, except that no pores were initially present in any of the bilayers formed by the rightmost column of droplets in the network. The arrows signify pore insertions as described in *b*.

Currents measured away from the pathway. Similarly, the transient current peaks measured with one drop on the pathway and one off the pathway (Fig. 3.10d) were consistent with the simulations if, in addition to the above conditions, a thin layer of droplets was added that did not allow the flow of current between one electrode-impaled drop and the rest of the network. These insulating droplets represent the droplets in the network in Fig. 3.10d that did not contain α HL. The large drop chosen to be adjacent to this insulating layer was drop *b* in Fig. 3.12c, which represents the drop that was removed from the network and replaced away from the pathway in Fig. 3.10d.

Under the above conditions, both the amplitudes and relaxation time-dependence of the current peaks in the simulation (Fig. 3.12d) were consistent with the transient currents measured experimentally (Fig. 3.10e). The model can be validated further by using an approximate model of the network in which each transient current spike is attributed to the charging of a single bilayer formed by one of the droplets without α HL (Ch. 5). As shown in Ch. 5, this model can be used with the measurements in Fig. 3.10e to calculate the capacitance of a single bilayer in the network as 28 ± 6 pF ($n = 3$). By comparison, the observed bilayer diameter of ~ 45 μ m and the previously measured specific capacitance of droplet bilayers of 650 nF cm $^{-2}$ (ref. 9) predict a bilayer capacitance of ~ 10 pF. The discrepancy between the two calculated values is likely due to oversimplification of the model (Ch. 5), and to inaccuracy in the measurement of the bilayer diameter.

Rate of α HL insertion. The simulation results suggest that the current steps in Fig. 3.10c, and the current spikes in Fig. 3.10e, corresponded to insertions of α HL into bilayers between the network and the large drops impaled by electrodes, rather than into bilayers between droplets in the network. This conclusion is also in agreement with the experimental observation that the rate of α HL insertions into droplet interface bilayers decreases with time.

In droplets that contained α HL in the concentration used in the electrical recording experiments, the rate of pore insertion decreased from ~ 0.5 s $^{-1}$ a few minutes after the droplets were formed to a negligible rate after a few hours. The attrition of the rate of pore insertion is likely to have two contributions. First, the droplets were made by diluting a concentrated stock solution of α HL heptamers solubilized with SDS. The dilution is likely to encourage the dissociation of SDS from the protein, which would destabilize the heptamers. Second, whereas the stock and diluted solutions of α HL were refrigerated at -80 $^{\circ}$ C and 0 $^{\circ}$ C, respectively, the experimental system was at room temperature, which would also increase the rate of heptamer degradation.

The droplets on the conductive pathway were formed from a freshly diluted solution that was at room temperature during printing for ~ 2 h before electrical recording, whereas the large drops impaled on electrodes were formed only ~ 15 min before electrical recording. Any pores that inserted in bilayers during electrical recording are therefore more likely to have been in the large drops than in the printed droplets.

Summary. The electrical model and the observed time dependence of α HL pore insertions both indicate that only the droplets along the defined pathway in Fig. 3.10 allowed the passage of ionic current, and that the conductance of the pathway was approximately constant during the recordings. The stepwise increase in current in Fig. 3.10e was most likely caused by pore insertions into the bilayers between the large drops and the pathway droplets. The current spikes in Fig. 3.10e correspond to pore insertions in the bilayers between the drop placed away from the pathway and insulating droplets in the network, which transmit transient capacitive currents but do not permit a steady resistive current. Based on these findings, we maintain that droplet networks can be printed with

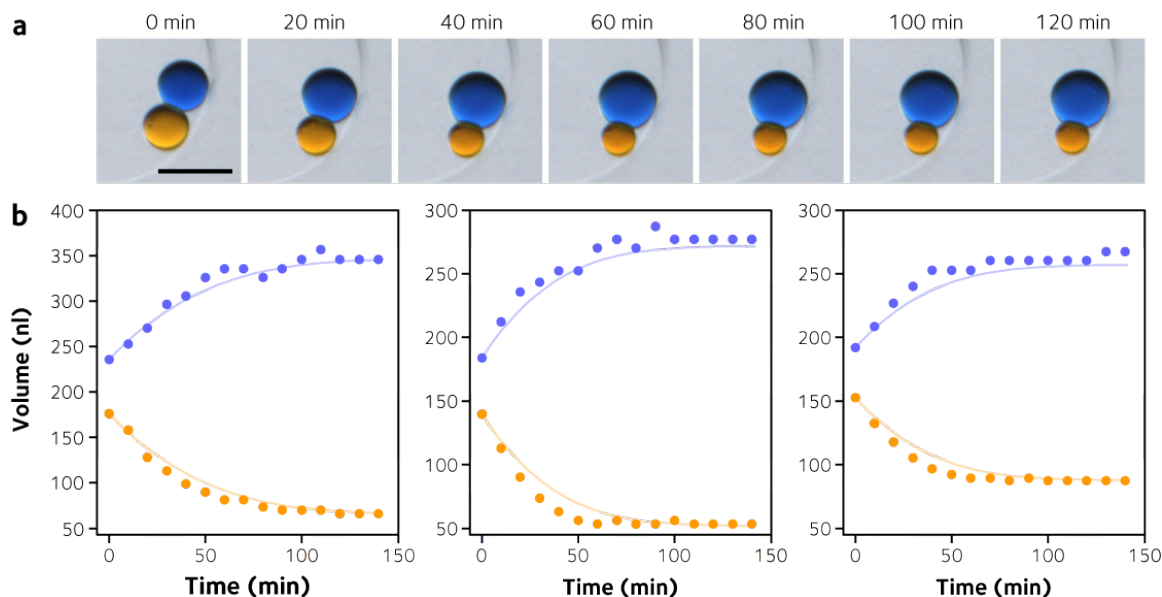


Figure 3.13 | Measurement of water permeability of droplet interface bilayers. (a) Photographs of a single pair of droplets with different salt concentrations, taken every 20 min after the droplets were joined by a bilayer. The blue and orange droplets initially contained 1 M KCl and 250 mM KCl, respectively. Scale bar: 1 mm. (b) Volumes of three pairs of droplets as exemplified in a. The droplet and bilayer diameters were measured from photographs, and the droplet volumes calculated with the assumption of spherical cap geometries. The circles show the measured volumes of the blue and orange droplets, and were used to calculate the permeability coefficient P as described in Ch. 5. The lines show the droplet volumes calculated using Eq. 5.52 with the average measured value of $P = 27 \mu\text{m s}^{-1}$, and demonstrate that the data are well explained by the model with the average value of P .

protein pores in specific bilayers, and can be designed to tolerate variation in the number of proteins that insert in each bilayer.

3.6 Self-folding droplet networks

Finally, we explored the capabilities of printed networks in which the droplets communicate by water transport. Water permeates readily through lipid bilayers even in the absence of protein channels or pores, with a permeability coefficient of $27 \pm 5 \mu\text{m s}^{-1}$ (mean $\pm$ s.d., $n = 6$) under our experimental conditions (Fig. 3.13), consistent with other permeability measurements of droplet interface bilayers^{67,112} and other lipid bilayer systems^{113,114}, which are typically in the range ~ 0 – $70 \mu\text{m s}^{-1}$. Consequently, two droplets of higher and lower osmolarity joined by a bilayer will respectively swell and shrink until their osmolarities are equal (Fig. 3.14a). By extension, water transfer within a network composed of droplets of different osmolarities will cause spontaneous deformation of the network as long as adhesion between droplets is maintained (Fig. 3.14b).

More permeable lipid bilayers are known to be more compressible in the plane of the bilayer¹¹⁴. Using this known relationship together with the above permeability measurement, we estimate that the droplet bilayers have a surface compressibility of $\sim 5 \times 10^{-6} \text{ N cm}^{-1}$, which is similar to that of bilayers made from the lipid 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

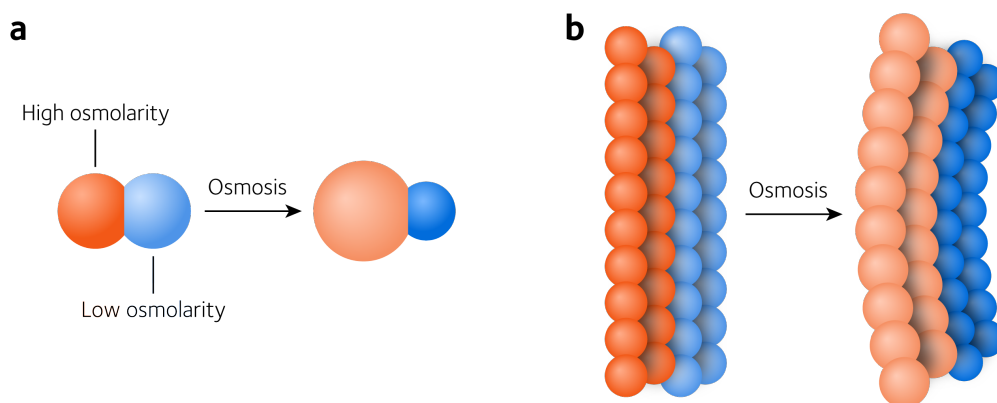


Figure 3.14 | Schematic of osmosis in droplet networks. (a) Schematic of two droplets of different osmolarities joined by a lipid bilayer. The flow of water through the bilayer causes the droplets to swell or shrink. (b) Schematic of a droplet network that comprises two strips of droplets of different osmolarities. The transfer of water between the droplets induces an overall deformation of the network.

Development of folding networks

To print droplet networks that folded in a predictable way, it was necessary to alter the printing substrate; control the relative timescales of printing and folding; prevent mechanical failure of the networks during folding; and predict the final geometry of a given folding network. We discuss each of these points below.

Printing substrate. Static networks in bulk oil were printed on a glass surface, which is partially wetted by the aqueous droplets and therefore fixes their positions during printing. Conversely, droplet networks were printed on a poly(methyl methacrylate) surface, which was not wetted by droplets and therefore allowed the printed network to deform freely. Consequently, the substrate needed to be close to horizontal to prevent the network from drifting laterally under gravity during printing. The printing nozzles were also positioned $\sim 150 \mu\text{m}$ higher than when printing static networks, to prevent the ejection of droplets from displacing already-printed droplets.

Timescales of printing and folding. If a network folds to a significant degree before printing has completed, the later droplets may incorporate into the network at incorrect positions. Folding during printing should therefore be minimized by decreasing the printing time, and increasing the folding timescale. The printing time was minimized as described earlier in this chapter. The folding timescale was increased by controlling the salt concentrations and the droplet size, as follows.

The rate of water transfer between two droplets is proportional to the *difference* in their osmolarities, while the volume of water transferred, and therefore the equilibrium geometry of the folded network, is partially determined by the *ratio* of osmolarities. We therefore slowed the folding process to a timescale of hours by reducing the difference in the initial salt concentrations between the two droplet types to $\sim 100 \text{ mM}$, while maintaining a high ratio of initial salt concentrations of ~ 10 .

Folding was slowed further by using larger droplets. Consider a pair of droplets joined by a bilayer, both with initial volume V but each with a different initial osmolarity. The initial rate of water transfer is proportional to the bilayer area, A . Because $A \propto V^{2/3}$, water is transported more rapidly

between larger droplets. However, the rate of water transfer as a proportion of the droplet volume is $\propto A/V \propto V^{-1/3}$. Therefore relative to their initial volume, larger droplets change in volume more slowly, and by extension a network composed of larger droplets will fold more slowly.

Buckling. A length mismatch can develop between connected regions of a droplet network as a result of the swelling and shrinking of droplets with different osmolarities. Networks that are thin in one dimension can resolve this mismatch without a significant energetic cost by buckling into that dimension. This is analogous to the buckling instability in tissues that grow at inhomogeneous rates, such as certain leaves^{115,116} and flower petals¹¹⁷, as well as in some synthetic systems^{118,119}. We found that networks designed to fold in the horizontal plane were less prone to buckle out of that plane if printed with additional horizontal layers. This is presumably because bending a thicker network out of the horizontal plane would involve the unfavourable exposure of more monolayer area or total surface area.

Fracture. In networks that are thick along the axis about which folding takes place (for example, the axis perpendicular to the page in Fig. 3.14b), the length mismatch between connected regions discussed above can produce stresses that detach neighbouring shrinking droplets from each other (Fig. 3.15). This prevents further folding around the fracture zone, because the swelling and shrinking portions of a droplet network must be connected to each other and within themselves to induce an overall deformation. We found that fracture could be prevented by making the shrinking region of the network thicker perpendicular to the interface between the swelling and shrinking regions (for example, the horizontal axis on the page in Fig. 3.15a, and the axis perpendicular to the page in Fig. 3.15c). This extra thickness presumably distributes the stresses induced by folding among a greater number of bilayers, so that the forces on each connected pair of shrinking droplets are no longer sufficient to separate the droplets.

Model. To aid in the design of folding networks, we formulated a simple computational model that allowed us to qualitatively predict the folding behaviour of a given droplet network (Ch. 5). The model consists of two components that separately account for the mechanical behaviour of the network and for the transfer of water between droplets. The mechanical component of the model treats each droplet as a point mass, and each pair of droplets joined by a bilayer is modelled as being connected by a stiff spring of a natural length that depends on the sizes of the two connected droplets. As in Section 3.3, the spring does not represent the elasticity of the lipid bilayer, but rather the restoring force produced by the free energy cost of deforming a droplet pair from its equilibrium geometry. The osmotic component models the transfer of water between connected droplets at a rate proportional to the area of the connecting bilayer and to the difference in their osmolarities.

Results and discussion

We used the above guidelines for the various aspects of the printing process, together with the model of folding networks, to design droplet networks that folded in a controlled way.

Folding strip. In one experiment, we printed a network that consisted of two strips of droplets of different salt concentrations, connected along their lengths (Fig. 3.16a). The network folded spontaneously in the horizontal plane over ~ 3 h, until droplets at opposing ends of the network formed bilayers in a closed ring. The evolution of the network geometry is in good qualitative agreement with that of a simulated folding network with similar initial conditions (Fig. 3.16b).

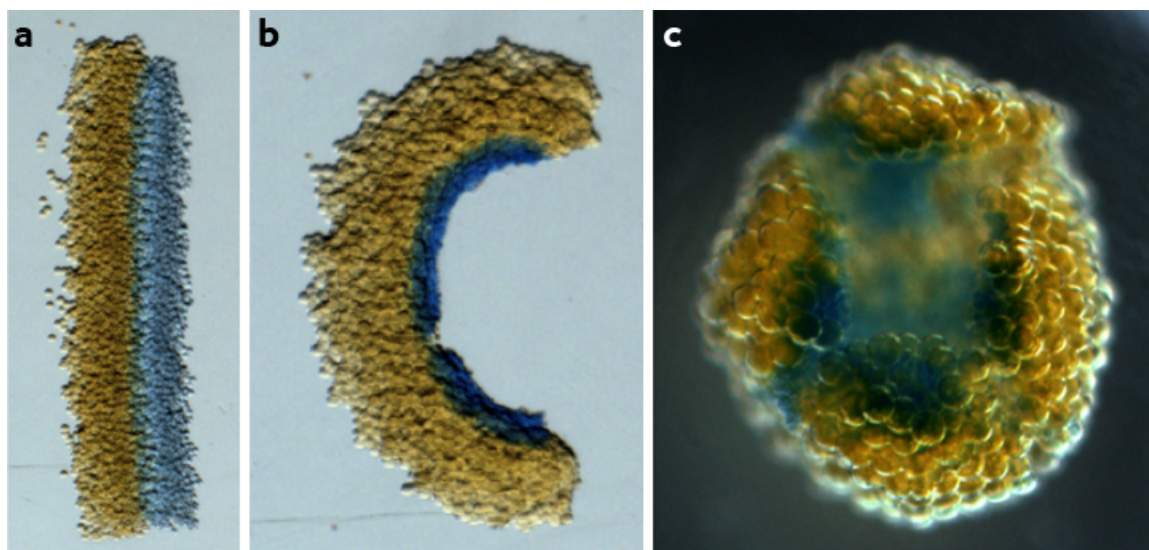


Figure 3.15 | Fracture of folding networks. (a) A network designed to fold into an arc. The orange and blue droplets initially contain 250 mM KCl and 16 mM KCl, respectively. (b) The network in (a) after the completion of folding. The point of fracture in the region of blue droplets is evident. (c) An originally horizontal cross-shaped network designed to fold out of the horizontal plane to form a cup. The initially upper (blue) and lower (orange) layers originally contained 8 mM and 80 mM KCl, respectively. The blue layer fractured near the base of the upper arm. Note that the fractured arm folded to a lesser extent than the others.

Folding petals. We also programmed a network to fold spontaneously out of the horizontal plane to attain a geometry that would be difficult to print directly. We printed a flower-shaped network with four petals, in which the lower layers had higher an osmolarity than the upper layers. The permeation of water from the upper into the lower layers induced a curvature that raised the petals and folded them inwards (Fig. 3.17a). The geometry of the folded network approximated that of a hollow sphere, with the originally upper layer contained within a shell formed by the originally lower layer (Fig. 3.17b). The behaviour of this network was also well matched by a similar simulated network (Fig. 3.17c).

In Ch. 5 we calculate the energy available from the osmosis-driven flow of water in a given droplet network. The osmotic energy density of the flower-shaped network is estimated to be $\sim 4.3 \text{ kJ m}^{-3}$, which corresponds to an osmotic energy of the order of $1 \text{ } \mu\text{J}$. This is comparable to the total energy of bilayer formation in the network, and several orders of magnitude greater than the energy required to lift the droplets against gravity.

Lipid recruitment. In a folding droplet network, lipid molecules presumably adsorb from the oil phase onto the growing surface of each swelling droplet. Because droplets in a bulk solution of lipid in oil require only $\sim 1 \text{ s}$ to incubate and folding takes place over several hours, swelling droplets at the surface of a folding network are expected to acquire lipids at a sufficient rate to maintain a constant surface density of lipids. However, droplets within the network must recruit lipid from surrounding pockets of oil, which may be replenished by percolation from the bulk oil solution or isolated entirely.

We estimate that the swelling droplets in the flower-shaped network would require the adsorption of all the lipid contained in a volume of oil $\sim 20 \text{ } \mu\text{m}$ to a side, which is comparable to the dimensions of the oil pockets in Fig. 3.8b. The time scale for diffusion of the lipid through any oil pathways through the network is of the order of several minutes (Ch. 5). These estimates suggest that the

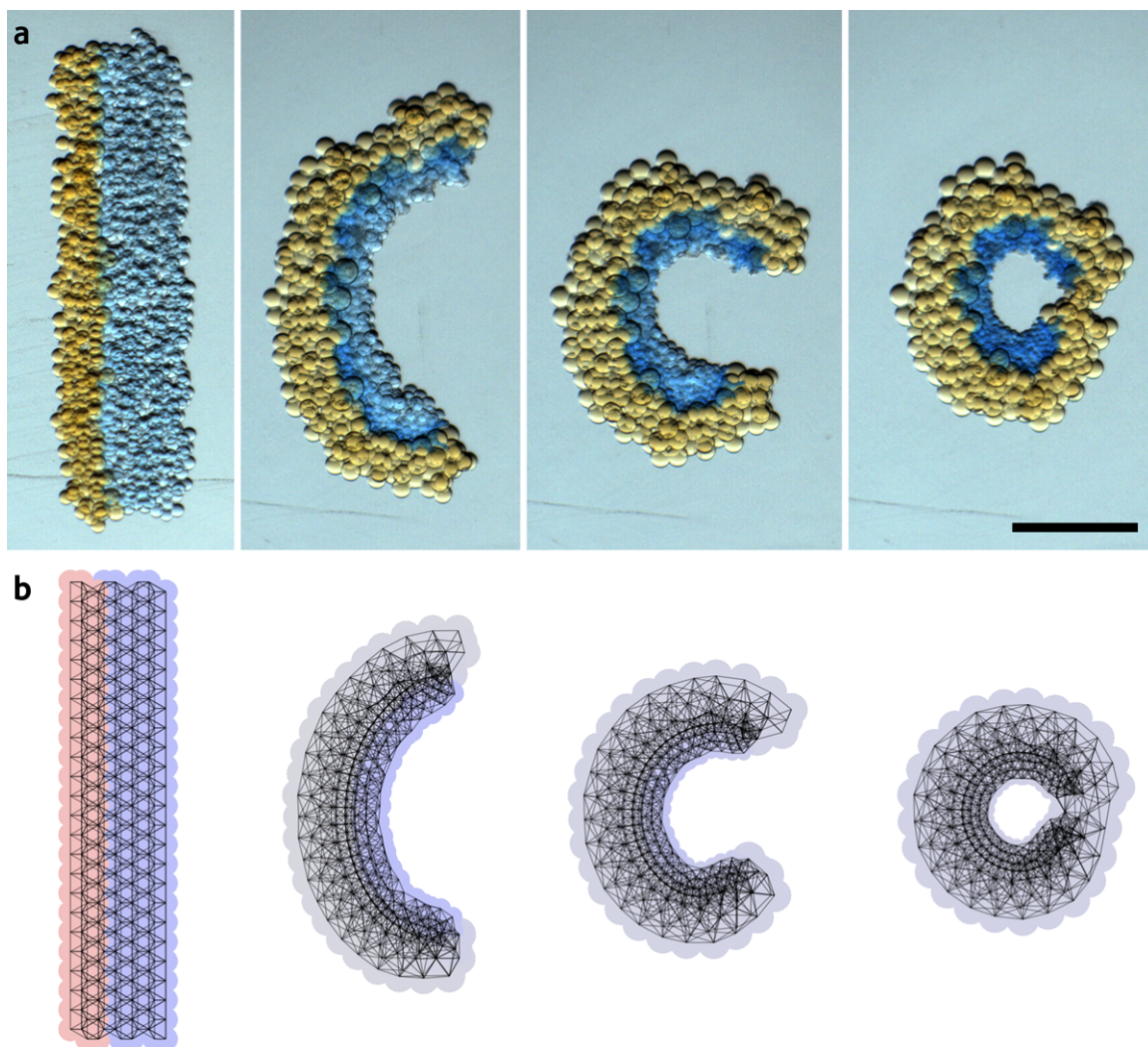


Figure 3.16 | A droplet network self-folding to form a ring. (a) Photographs of a rectangular network folding into a circle over ~ 3 h. The orange and blue droplets initially contain 250 mM KCl and 16 mM KCl, respectively. Note the increased horizontal thickness of the lower-osmolarity region compared to the network in Fig. 3.15a,b. Scale bar: 250 μm . (b) Frames from a simulation of a folding network with a similar geometry to the network in a. Blue and red represent the lowest and highest initial osmolarities, respectively, and grey represents the average of the two. The bilayer between each connected pair of droplets is represented by a line that joins the centres of the two droplets. The simulation parameters are given in Table 5.1.

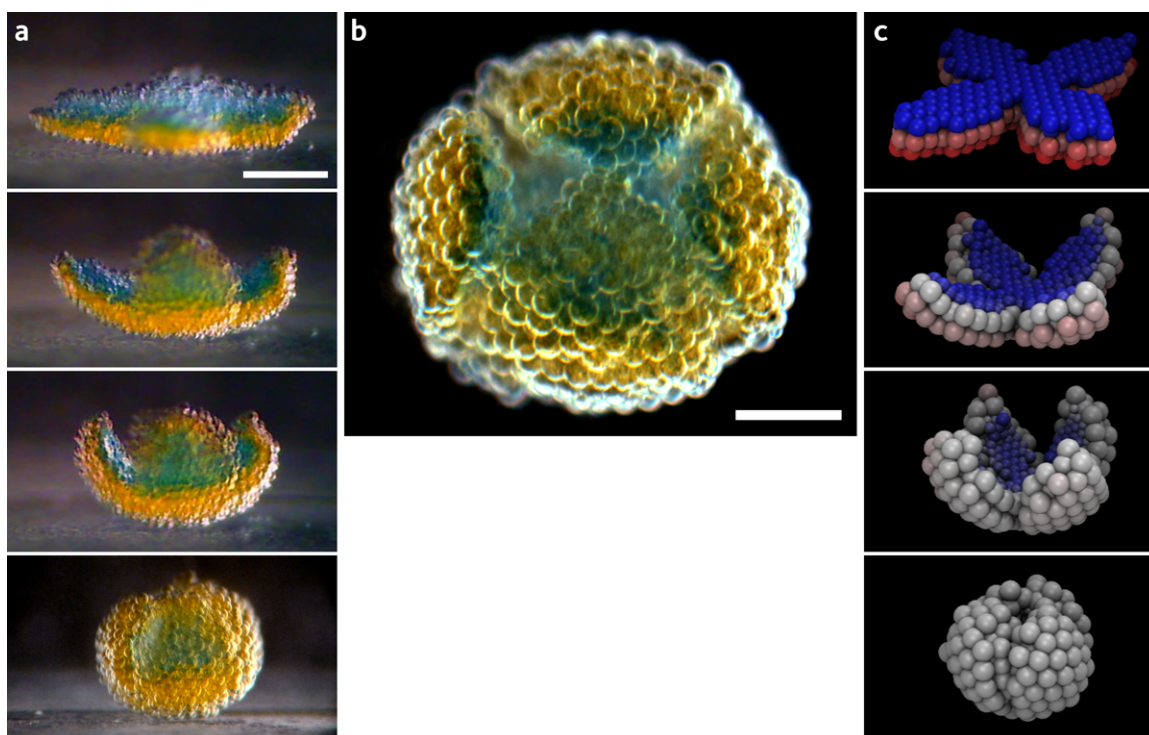


Figure 3.17 | A droplet network self-folding to form a hollow sphere. (a) Photographs of a flower-shaped network folding spontaneously into a hollow sphere. The orange and blue droplets initially contain 80 mM KCl and 8 mM KCl, respectively. The photographs cover a period of 8 h. Scale bar: 400 μm . (b) The network in a in its final configuration, photographed from above. Scale bar: 200 μm . (c) Frames from a simulation of a folding network with a similar initial geometry to the network shown in a. Blue and red represent the lowest and highest initial osmolarities, respectively, and white represents the average of the two. The simulation parameters are given in Table 5.1.

swelling droplets in the networks presented here were able to maintain a constant surface density of lipids by adsorption from surrounding interstitial oil, whether these were isolated or connected to the bulk oil phase. However, it might be necessary to use a higher lipid concentration for larger folding networks; for networks in which some droplets experience a greater increase in volume; or for networks composed of more strongly adhesive droplets, which would reduce the volume of interstitial oil.

Comparison with other folding materials. Other materials exist that deform through non-uniform volume changes. For instance, the function of the bimetallic strip relies on a discontinuous thermal expansion coefficient. Also, hydrogel systems have been spatially patterned to suffer inhomogeneous changes in volume upon a uniform change in solvent concentration or temperature^{120, 121}. Droplet networks are distinct from these materials in three principal ways. First, they are readily printed in designed three-dimensional geometries. Second, droplet networks are composed of discrete compartments that can be selectively functionalized, for example with membrane proteins. Third, rather than being driven by an external stimulus, droplet networks fold as a result of water transfer driven by osmolarity gradients entirely within the network. The latter characteristics make the folding of droplet networks closely analogous to the nastic movements exhibited by certain plants^{122, 123}. Deformation at constant volume is also exhibited by muscular hydrostats, which are actuated by muscle contraction rather than osmotic flow¹²⁴. It would be interesting to see whether the osmotic shape change might be made reversible, for instance by using osmolytes that are responsive to heating or illumination.

3.7 Future work with printed networks

Printed droplet networks represent a platform for the construction of structured, macroscopic materials that can employ lipid bilayers and membrane proteins to transduce physical and chemical signals in a designed way. In particular, this material could serve synthetic biology as a simple, well-defined system for the reproduction of multicellular behaviors unattainable with a single-compartment chassis; for instance, folding droplet networks might be used for the study of processes driven by differential cell growth.

An exciting and challenging prospect is the integration of printed droplet networks and living systems, such as biological tissues. One significant challenge for an encapsulated droplet network intended to be interfaced with an organism is biocompatibility. This might be achieved by modification of the external bilayers of the network, similarly to biocompatible liposomes for use in drug delivery¹²⁵. In some cases, it could be desirable to remove the residual oil in an encapsulated network. This might be accomplished by the use of an oil mixture that contains a volatile component, which could dissolve into the bulk aqueous phase after printing⁹³.

Further, for a droplet network to contribute function to a living tissue, it must transduce physical and chemical signals in a manner compatible with its biological environment. Although the droplet network in Fig. 3.10 is functionally analogous to a nerve axon in that it enables rapid communication along a defined path, the two systems have different mechanisms of signal propagation. Droplet networks have been made that function under external chemical¹, electrical³¹ or optical⁹ stimuli, and more complex networks with functional membrane channels or pores could exhibit more sophisticated behaviours³¹. For instance, a printed network might employ voltage-gated ion channels to transmit

an action potential, aquaporins or ion channels for finer control of folding, or light-sensitive channels or pumps such as bacteriorhodopsin to replicate the function of the retina⁹.



CHAPTER 4

Experimental details

This chapter provides information on materials and methods for the experiments described in Chs. 2 and 3. This includes *(i)* the various aqueous and oil solutions used; *(ii)* the manual production of multisomes; *(iii)* the procedures for electrical recording and fluorescence microscopy; *(iv)* the procedure for the temperature-sensitivity measurements; *(v)* the droplet generator and printing containers; *(vi)* the driving electronics for the droplet generator; *(vii)* the graphical user interface to the droplet printer; and *(viii)* the printing algorithm.

4.1 Aqueous solutions

Except where indicated otherwise, the buffer in all experiments was 25 mM Tris·HCl, 1 M KCl, 100 μ M EDTA, pH 8.0. This pH was chosen to match that of the α HL solution described below, to ensure that the pH of the droplets was independent of their α HL concentrations.

Diffusion through pores. The buffer used in the experiments of Ch. 2 on diffusion through pores in multisome bilayers (Fig. 2.7) was 25 mM Tris·HCl, 500 mM KCl, pH 8.0. The potassium salt of fluo-4 conjugated to a $M_w = 10,000$ dextran (Invitrogen) was dissolved in pure water, and included in droplets of buffer at a final dye concentration of 25 μ M. Dye-containing droplets also contained 50 μ M EDTA disodium salt. Ca^{2+} -containing droplets consisted of buffer with 100 mM CaCl_2 .

pH-sensitive multisomes. The buffer used in the pH-sensitivity experiments of Ch. 2 (Fig. 2.9) was 10 mM Tris·HCl, 10 mM succinic acid, 50 mM KCl, pH 8.0 or 3.0. Dye-containing droplets also contained 25 μ M dextran-conjugated fluo-4 and 50 μ M EDTA disodium salt. Ca^{2+} -containing droplets consisted of buffer with 10 mM CaCl_2 .

Self-folding droplet networks. In the folding experiments of Ch. 3, a solution of 25 mM Tris·HCl, 1 M KCl, 100 μ M EDTA, pH 8.0 was diluted to obtain the salt concentrations given in the captions to Figs. 3.15, 3.16 and 3.17.

Visible dyes

The dyes used in the multisome experiments of Ch. 2 were fluorescein and sulphorhodamine 101 (both from Invitrogen). The dyes used in the 3D printing experiments of Ch. 3 were xylene cyanol FF, orange G and pyranine (all from Sigma-Aldrich). The dye concentrations used in each case are listed in Table 4.1.

Figure	Droplets	Dyes
Fig. 2.3	Red droplets	25 μ M sulphorhodamine 101
	Green droplets	25 μ M fluorescein
Fig. 2.8	Red droplets	25 μ M sulphorhodamine 101
	Green droplets	25 μ M fluorescein
Fig. 3.4b	Blue droplets	1 mM xylene cyanol FF
Fig. 3.5b	Blue droplets	1 mM xylene cyanol FF
	Orange droplets	10 mM orange G
Fig. 3.8	Blue droplets	900 μ M xylene cyanol FF and 100 μ M pyranine
	Orange droplets	10 mM orange G
Fig. 3.10b,d	Large green drops	10 mM pyranine
	Green droplets	10 mM pyranine
	Other droplets	50 μ M xylene cyanol FF
Fig. 3.11	Large green drops	10 mM pyranine
	Green droplets	10 mM pyranine
	Blue droplets	1 mM xylene cyanol FF
Fig. 3.13	Blue droplets	1 mM xylene cyanol FF
	Orange droplets	2.5 mM orange G
Fig. 3.15a,b	Blue droplets	320 μ M xylene cyanol FF
	Orange droplets	2.5 mM orange G
Fig. 3.15c	Blue droplets	160 μ M xylene cyanol FF
	Orange droplets	800 μ M orange G
Fig. 3.16	Blue droplets	320 μ M xylene cyanol FF
	Orange droplets	2.5 mM orange G
Fig. 3.17a,b	Blue droplets	160 μ M xylene cyanol FF
	Orange droplets	800 μ M orange G

Table 4.1 | Dye concentrations.

Protein

The α HL used in the electrical recording experiments of Ch. 2 was prepared by E. Mikhailova. Wild-type α HL monomers were prepared by *in vitro* transcription/translation, and heptamerized by incubation with rabbit red blood cell membranes. The heptamers were then purified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)¹²⁶ to a final concentration of $\sim 1 \mu\text{g ml}^{-1}$ (ref. 127). For the electrical recording experiments of Ch. 2, the protein concentration in the encapsulated droplets was 10 to 100 times lower.

The α HL used in the fluorescence experiments of Ch. 2 and the electrical recording experiments of Ch. 3 was prepared by Q. Li. The procedure used has been described previously¹²⁸. Briefly, a culture was grown from a single colony of the Wood 46 strain of *Staphylococcus aureus*. Spontaneously oligomerized heptamers of α HL were purified by cation exchange chromatography and gel electrophoresis, and stored at $\sim 2 \text{ mg ml}^{-1}$ in 20 mM sodium phosphate buffer with 150 mM NaCl and 0.3% (w/v) SDS at pH 8.0. This protein solution was added to the aqueous droplets at a 50-fold dilution for the fluorescence experiments of Ch. 2, and at a 100-fold dilution for the electrical recording experiments of Ch. 3.

4.2 Lipids and oils

The structures and selected properties of the lipids and oils used in this work are given in Table 4.2. Lipids were purchased from Avanti Polar Lipids and dissolved in pentane (DPhPC, DOPE) or chloroform (DPPC, DSPC) at 10 mg ml^{-1} . Portions of these stock solutions were evaporated by using a nitrogen stream followed by at least 30 min under vacuum. The residues were re-solubilized in various oil mixtures.

In all the experiments of Ch. 2 except for those on pH and temperature sensitivity, the lipid used was DPhPC at $0.1\text{--}0.2 \text{ mg ml}^{-1}$, and the oil was a 9:1 (v/v) mixture of silicone oil (Silicone Oil AR 20) and hexadecane (both from Sigma-Aldrich). The pH sensitivity experiments used a 2:1 (mol/mol) mixture of DOPE and oleic acid (Sigma-Aldrich) at 10 mg ml^{-1} total concentration, in a 19:1 (v/v) mixture of silicone oil and hexadecane. The temperature sensitivity experiments with DPPC used a 1:1 (mol/mol) mixture of DPPC and DPhPC at 0.5 mg ml^{-1} total concentration, in a 9:1 (v/v) mixture of silicone oil and hexadecane. The temperature sensitivity experiments with DSPC used a 3:1 (mol/mol) mixture of DSPC and DPhPC at 1 mg ml^{-1} total concentration in the same oil mixture.

All the experiments of Ch. 3 used DPhPC at $0.2\text{--}0.5 \text{ mg ml}^{-1}$ in a 1:1 (v/v) mixture of silicone oil and hexadecane.

4.3 Production of multisomes

This section describes how the multisomes in Ch. 2 were suspended in bulk aqueous solution and produced by manual pipetting.

Oil	Structure	Density (g cm ⁻³)	Melting point (°C)
Hexadecane		0.77 (note 1)	18 (note 1)
Silicone oil	Poly(phenylmethylsiloxane) Structure unknown	1.01 (note 1)	Unknown
1-bromododecane		1.04 (note 1)	-10 (note 1)

Lipid	Structure	Property
DPhPC		No melting transition under conditions of this work (note 2)
DOPE		Forms bilayers when mixed with oleic acid below pK _a (note 3)
Oleic acid		pK _a ~ 7.5 when in a bilayer (note 4)
DPPC		Melting transition temperature = 41 °C (note 5)
DSPC		Melting transition temperature ~ 55 °C (note 6)

Table 4.2 | Structures and properties of lipids and oils. Structures and selected properties of the lipids and oils used in this work.

Note 1: ref. 129.

Note 2: ref. 83.

Note 3: ref. 72.

Note 4: refs. 75, 76.

Note 5: ref. 130.

Note 6: refs. 131, 132.

Suspension in the bulk aqueous phase. Multisomes were suspended within the bulk aqueous buffer from loops of silver wire or plastic of diameter ~ 0.8 – 1.5 mm to fix their positions while avoiding any interaction with the walls of the container that might disrupt the structure. Silver loops were made by wrapping $100\text{-}\mu\text{m}$ diameter silver wire around a cylindrical template. Plastic loops consisted of cross-sections cut from pipette tips. Each loop was then attached to a silver wire fixed to an inner wall of a polystyrene cuvette. Multisomes could be dislodged from silver loops by mechanical disturbance, whereas plastic loops held multisomes very reliably because of their strong adhesion to the oil drop.

Production of multisomes. A solution of lipids in oil was dispensed onto a loop submerged in buffer to make a suspended oil drop with a volume of ~ 0.2 – $2\text{ }\mu\text{l}$. Micromachined poly(methyl methacrylate) (PMMA) wells were filled with the same oil solution, and aqueous droplets with a volume of ~ 0.5 – 70 nl were made in these wells using a $2\text{ }\mu\text{l}$ pipette with an electrophoresis gel-loading tip. The tip was filled with ~ 200 nl of the aqueous solution, and then immersed in the oil solution. Just enough of the solution was expelled to expose a small pendant droplet, and this droplet was separated from the pipette tip by pressing the tip against the bottom of the well. After incubation, a pipette was used to transfer one or more of the aqueous droplets into the oil drop located in the bulk aqueous solution.

Incubation time. In experiments that used only DPhPC, the incubation time was ~ 10 min; for the DOPE/oleic acid mixture, ~ 25 min; and for the DSPC/DPhPC mixture, ~ 10 min. Multisomes with several inner droplets could be formed either by transferring an intact network of aqueous droplets joined by bilayers, or by transferring unconnected droplets, which then formed bilayers *in situ*.

4.4 Electrical recording

This section describes equipment and procedures for electrical measurements of multisomes and printed droplet networks.

Plain electrodes. Ag/AgCl electrodes were prepared by treating 25- or $100\text{-}\mu\text{m}$ -diameter silver wire (Scientific Wire Company and Sigma Aldrich, respectively) with 25% sodium hypochlorite solution for at least 30 min, and then coating the end of each electrode with agarose gel. The gel coating allowed the electrode to penetrate an aqueous droplet in oil.

Glass-sheathed electrodes. Electrodes were sheathed with glass to record ionic currents across the external bilayer of a multisome (Fig. 2.6a). The glass insulated the electrode from the bulk aqueous solution, and its hydrophilicity prevented the oil drop from spreading along its surface. Glass-sheathed electrodes were made by threading $25\text{-}\mu\text{m}$ diameter silver wire through a glass capillary (Drummond) with internal and external diameters of $142\text{ }\mu\text{m}$ and $559\text{ }\mu\text{m}$, respectively. The capillary was then pulled (PC-10, Narishige) with the wire inside it, such that both the capillary and wire separated into two pieces. The wire inside one of the pulled capillary pieces was soldered to an electrode pin at the larger opening of the capillary. Tweezers were used to trim $\sim 50\text{ }\mu\text{m}$ of glass from the pulled end of the capillary, exposing the end of the wire. This end was then treated with sodium hypochlorite solution as described above. A region near the pulled end of the capillary was coated with silicone rubber (3140 RTV Coating, Dow Corning) to prevent current leakage between the inner droplet and

the external aqueous solution (Fig. 2.6a). Finally, the exposed tip of the electrode was coated with agarose gel.

Recording. Currents were measured using Ag/AgCl electrodes with a patch-clamp amplifier (Axopatch 200B, Axon Instruments) and 16-bit digitizer (1322A, Molecular Devices). In the multisome experiments of Ch. 2, data were acquired at 10 kHz with a 2 kHz low-pass Bessel filter, and for analysis were further filtered with a 400 Hz low-pass Bessel filter. Comparisons of conductance values with those from previous studies^{9,68} were made for the same voltage polarities relative to pore orientation. Currents measured through multisome bilayers, and through droplet interface bilayers in bulk oil, showed occasional bursts of current leakage when pure silicone oil was used. These leaks were suppressed by mixing the oil with a small fraction of hexadecane. In the 3D printing experiments of Ch. 3, signals were processed with a 5 kHz low-pass Bessel filter and acquired at 20 kHz.

4.5 Fluorescence microscopy

The fluorescence microscopy in Ch. 2 was performed with a Nikon Eclipse TE2000-S inverted microscope with a Nikon CFI DL $\times 10$ objective, using a mercury arc lamp for illumination and the appropriate filter cube. Photographs were taken with a Hamamatsu C9100 EMCCD camera, with an exposure of 400 ms and gain of 179. Fluorescence intensities were measured using the IMAGEJ software package¹³³. Fluorescence photographs were pseudo-coloured green, and their brightness levels adjusted equally within each figure panel. The confocal microscopy in Ch. 3 was performed with an Olympus FluoView 1000 laser scanning confocal microscope with a UPLSAPO $\times 10$ objective.

4.6 Temperature sensitivity

For the temperature-sensitivity experiments of Ch. 2, a container made to hold 12 multisomes on plastic loops was placed on a heating block, and the temperature of the bulk aqueous solution was measured with a thermocouple. The bulk solution was continuously stirred to ensure temperature homogeneity. The heating block thermostat was controlled by using feedback from the temperature measurements to achieve the ramped and constant temperature regimes illustrated in Fig. 2.10.

4.7 Droplet generator

This section describes the droplet generator developed for the 3D printing experiments of Ch. 3. First we detail the components of the device, and then describe the procedures for filling the device with aqueous solution.

Description of the device. Each droplet generator consisted of a micromachined PMMA chamber, a piezoelectric transducer (7BB-20-6L0, Murata), or *piezo*, affixed to the chamber with epoxy adhesive, and a printing nozzle (Fig. 4.1). The nozzle for each droplet generator was formed from a glass capillary (Drummond) with external and internal diameters of 1.4 mm and 1.0 mm, respectively.

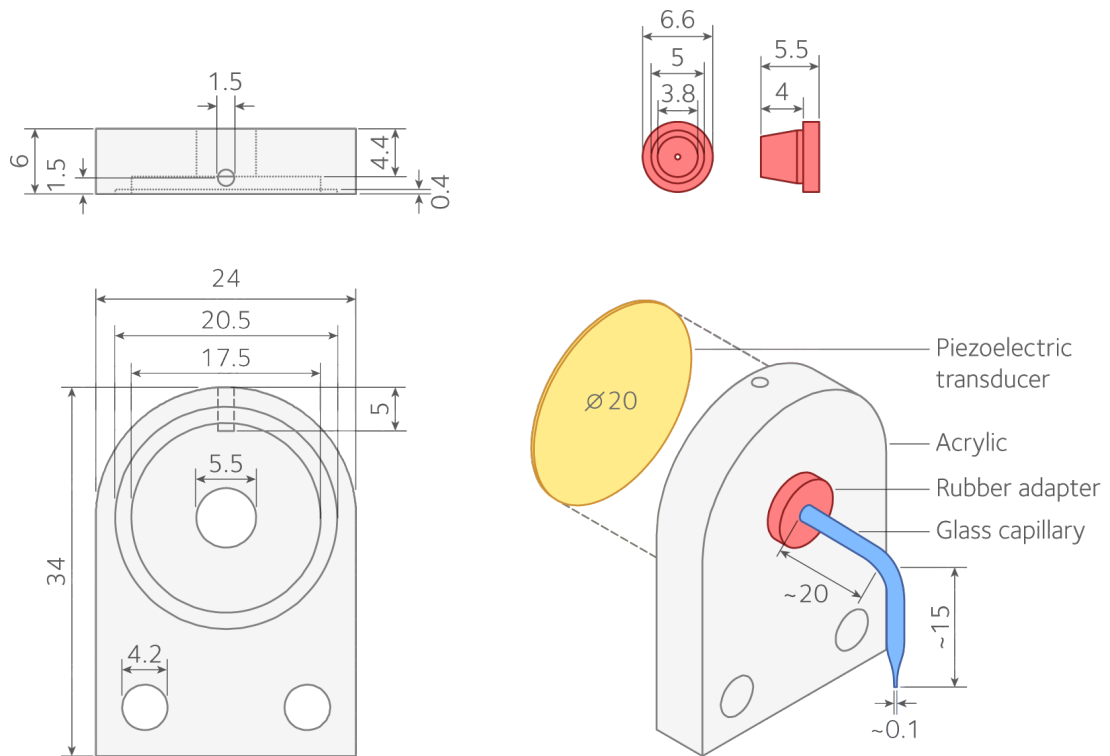


Figure 4.1 | Diagram of a droplet generator. The device consists of a piezoelectric transducer affixed onto a micromachined poly(methyl methacrylate) chamber. A rubber septum is fitted opposite the transducer, and into the septum is inserted a pulled and bent glass capillary. The two lower holes were used to fix one device in place, and to mount the other onto a manual micromanipulator. All dimensions are in mm.

The capillary was pulled (PC-10, Narishige), and its pulled end trimmed by gently passing another pulled capillary tip against it¹³⁴ to give a flat-ended tip of diameter between $\sim 60\ \mu\text{m}$ and $120\ \mu\text{m}$. The capillary was then bent by 90° over a flame, $\sim 15\ \text{mm}$ from the pulled end. Finally, the capillary was trimmed $\sim 35\ \text{mm}$ from the pulled end (Fig. 4.1). The capillary was fitted onto the chamber using a silicone rubber adapter (Drummond).

Filling the device. The chamber was filled with $\sim 400\ \mu\text{l}$ of aqueous solution through the inlet on the top of the device by using a micropipette with a gel-loading tip. The nozzle spontaneously filled with this solution through capillary action.

The volume of aqueous solution required for printing could be reduced to $\sim 5\ \mu\text{l}$, which minimized the wastage of solution in the experiments that employed αHL . To do this, the droplet generator was first filled with water. The nozzle of the generator was then immersed in a well filled with hexadecane, and suction was applied at the inlet of the generator by using a micropipette so that $\sim 5\ \mu\text{l}$ of hexadecane was drawn into the nozzle. The nozzle was then immersed into another well that contained the aqueous sample, and suction applied to load a similar volume of the aqueous solution. The hexadecane formed a plug within the nozzle that prevented the aqueous sample in the nozzle tip from mixing with the larger volume of water. For significantly smaller loadings of the aqueous sample, the size of ejected droplets was found to vary with the volume of aqueous sample remaining in the nozzle.

4.8 Droplet production

Here we describe various factors, other than the amplitude and duration of the voltage pulse, that significantly affected droplet production by the droplet generators.

Fluid levels. Droplet ejection depended strongly on the height difference h between the level of the aqueous phase in the chamber of the droplet generator and the level of the oil solution in the well (Fig. 4.2), which created hydrostatic pressure on the aqueous-oil interface in the nozzle. In general, a greater h yielded larger droplets. Evaporation of the aqueous solution from the chamber gradually changed the aqueous level, so that the size of ejected droplets remained consistent for only $\sim 6\ \text{h}$. Evaporation might be prevented by adding a thin layer of oil on top of the aqueous phase in the chamber.

Aqueous volume. Droplet ejection also depended on the volume V of aqueous solution in the droplet generator chamber, independently of its height above the oil level (Fig. 4.2). In general, the size of ejected droplets was more readily controlled when the aqueous chamber was completely full than when it was filled incompletely. This is likely because upon the application of a voltage pulse to the piezo, a less-filled chamber couples less of the vibration from the transducer to the fluid in the nozzle, which limits the size of the ejected droplets.

Lipid concentration. The amplitude of the voltage pulse required to eject droplets of a given size was lower for higher concentrations of lipid in the oil. This is to be expected: the adsorption of lipids at the aqueous-oil interface in the nozzle decreases the tension of that interface, thereby lowering the energy required to deform the interface to the extent required for droplet formation.

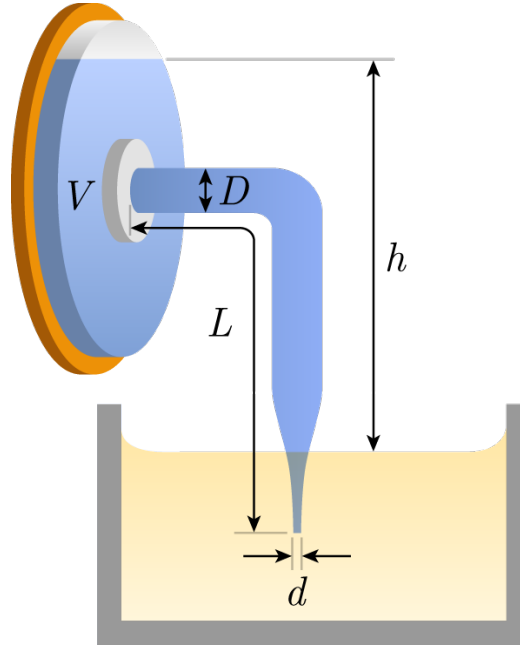


Figure 4.2 | Schematic of a droplet generator. The droplet generator chamber is filled with a volume V of aqueous solution. The nozzle of length L has an internal diameter D at its base, and a tip diameter d . The level of the aqueous solution in the chamber is a distance h above the level of the oil solution in the well.

Nozzle geometry. Droplets could not be produced reliably in the available range of voltages if the nozzle length L was too great, its internal diameter D was too small, or its tip diameter d was too great or too small (Fig. 4.2). The dimensions given in Fig. 4.1 allowed reliable droplet production within the available voltage range. Because the nozzles were produced manually from glass capillaries, under otherwise almost identical conditions the two generators generally produced droplets of different sizes. However, the two generators could be made to eject identical droplets by using different voltage pulses for each.

4.9 Printing containers

Different containers were used to print static networks in bulk oil, self-folding networks, and networks in bulk aqueous solution.

Printing in bulk oil. The container for networks printed in bulk oil was a well micromachined from PMMA with a glass observation window on one side. The oil-filled volume was typically $\sim 15 \times 10$ mm horizontally and ~ 5 mm deep. Spontaneously folding networks were printed directly on the bottom surface of the well, while all other networks in bulk oil were printed on a piece of glass coverslip placed in the well.

Printing in bulk aqueous solution. The container for networks printed in bulk aqueous solution was a polystyrene cuvette, with a glass coverslip bottom in the case of the network imaged by confocal microscopy. Printing in bulk aqueous solution took place inside a drop of oil that was suspended on a

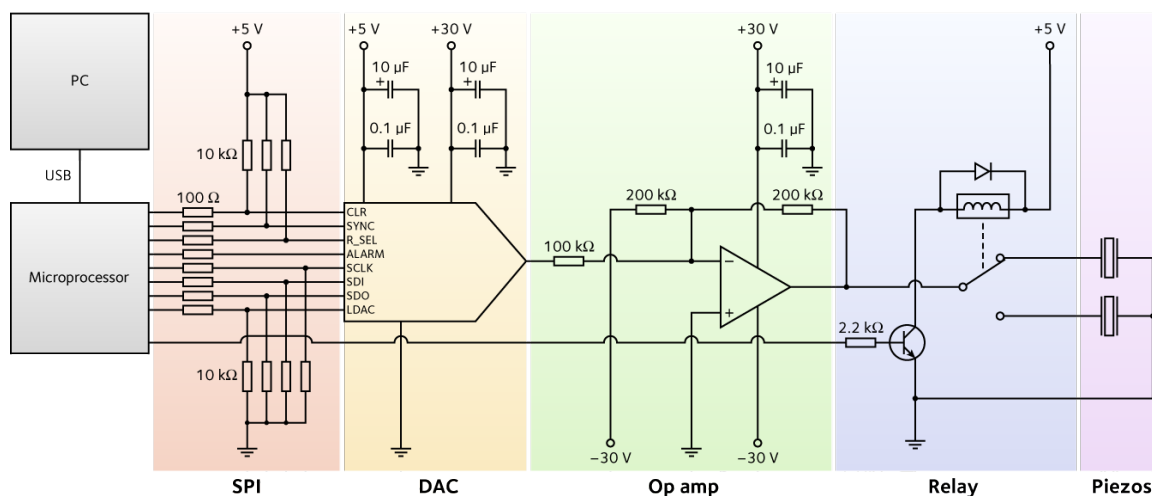


Figure 4.3 | Schematic of driving electronics for droplet generators. The electronics allow a computer to apply a voltage in the range -30 V to $+30$ V, with 12-bit resolution, to one of two piezoelectric transducers. Abbreviations: PC, personal computer; USB, universal serial bus; SPI, serial peripheral interface; DAC, digital-to-analog converter; op amp, operational amplifier.

wire frame, which was made as follows. PMMA shavings were dissolved in chloroform at 100 mg ml^{-1} . One end of a $100\text{-}\mu\text{m}$ diameter silver wire (Sigma-Aldrich) was dipped in this solution up to five times, so that it acquired a thin hydrophobic coating. The coated end of the wire was then wrapped around a cylindrical template using tweezers to form a loop, such that the loop was the only length of the wire coated with polymer. The uncoated end was attached to the polystyrene container using epoxy adhesive.

4.10 Electronics for droplet generators

The electronic circuit built to drive the droplet generators is shown schematically in Fig. 4.3. The circuit interprets instructions from a computer to produce a square voltage pulse of specified duration and amplitude, and applies this voltage to the piezo in either of two droplet generators. This section describes how the circuit generates and applies the voltage pulse.

Piezo selection. First, the computer sends a serial message that represents the desired piezo to a microcontroller board (Arduino Uno, SmartProjects). The microcontroller interprets this message, and activates a relay (NRP-04, NCR) through a transistor such that the desired piezo will receive the voltage output of the circuit. Once a piezo is selected in this way, it is held at the maximum negative voltage of -30 V. The piezo terminal that is connected to the voltage output was chosen such that a negative voltage produces compression of the piezo.

Voltage output. The computer then sends a serial message to the microcontroller that represents the instruction to generate a voltage pulse. The duration and amplitude of the pulse can be either specified in the serial message or previously programmed into the microcontroller. The microcontroller interprets this message, and writes a value that encodes the amplitude of the voltage pulse to the digital-to-analog converter (DAC) (AD5504, Analog Devices) through a serial peripheral interface

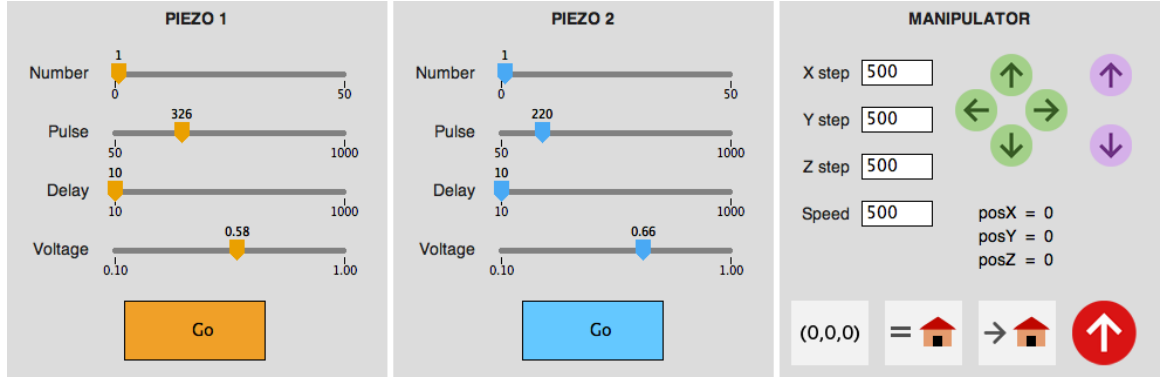


Figure 4.4 | Graphical user interface to the 3D printer. The left-hand and centre panels allow, for each of the two generators, variation of the duration (in μs) and amplitude (as a proportion of 60 V) of the voltage pulse, the triggering of droplet ejection, variation of the number of droplets to be ejected per trigger, and variation of the delay (in ms) between multiple droplets made per trigger. The right-hand panel allows interactive control of the manipulator, and variation of the manipulator step size and speed.

(SPI) bus. The DAC outputs a corresponding voltage between 0 V and +30 V with 12-bit resolution. The DAC output enters an operational amplifier (OPA551, Texas Instruments) circuit that acts as a current buffer, and also offsets and increases the output voltage range to -30 V to +30 V. The output of this circuit is applied to the piezo previously selected by the relay. After the specified duration of the voltage pulse, the microcontroller again sets the piezo to the maximum negative voltage. Voltage pulses typically had a duration of 100–800 μs and a peak-to-peak amplitude of 20–60 V.

4.11 Graphical user interface

A graphical user interface that enables real-time control of the droplet printer was written in the PROCESSING programming language (Fig. 4.4). The interface affords precise control of the amplitude and duration of the voltage pulse applied to each droplet generator. Droplet ejection can be triggered on demand, with a user-defined number of droplets and time delay between multiple droplets. These features allow the user to quickly determine, for each generator, the conditions required for the production of droplets of a specific size. The interface also gives the user direct control of the motorized micromanipulator (PatchStar, Scientifica), which was used to determine the correct spacing between droplets of a given size, as well as the relative displacement of the two nozzles. The latter was compensated for in the printing software to prevent a systematic displacement between the two types of droplets. The interface can be controlled through the computer keyboard or mouse.

4.12 Printing algorithm

Droplet networks were printed according to an algorithm executed by the computer that controlled the two droplet generators and the motorized micromanipulator. First we describe the basic pattern in which networks are printed, and then discuss modifications to this pattern that respectively

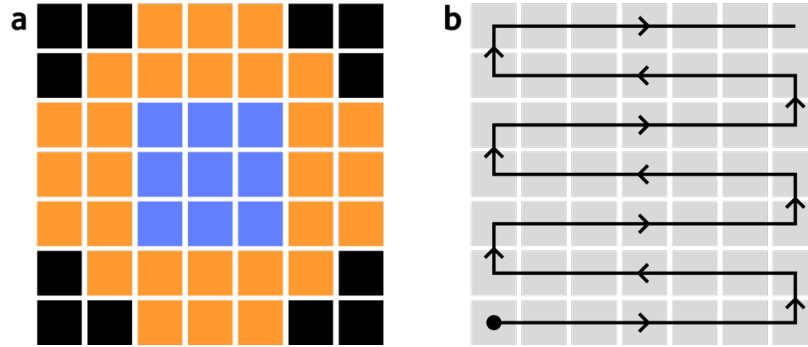


Figure 4.5 | Printing pattern. (a) An example of a map of 49 pixels, including A (orange), B (blue) and empty (black) pixels. (b) Path taken by the printing nozzles across the map in each of the two passes, in the simplest variation of the printing algorithm.

improve the accuracy, speed and reliability of the printer. Finally, we describe the implementation of the printing pattern as a computer algorithm, and provide typical values for the various printing parameters.

Basic printing pattern

The printing pattern was defined in terms of *layers*, *passes* and *goals* as below.

Layers. The algorithm builds droplet networks by printing one horizontal layer at a time. The network to be printed is defined by a series of images, or *maps*, each of which represents one or more of the layers in the network (Fig. 3.4a). The number of layers represented by each map is specified by the user. Each pixel in a map may have one of three colours. Depending on its colour, each pixel represents a droplet from one of the two droplet generators, or the absence of a droplet; these are respectively referred to here as *A*, *B*, and *empty* pixels.

Passes. Each layer is printed in two passes: in the first pass only the *A* droplets of the layer are printed, and in the second pass only the *B* droplets. Each pass is printed one row at a time, with each row parallel to the horizontal dimension x .

Goals. The path that the printing nozzles are instructed to follow in a given pass is defined by an ordered set of coordinates, or goals, each of which represents a location at which a droplet is to be ejected. For each layer, a goal is set for each pixel in the corresponding map. The goal order begins at the minimum x and y , increases left to right along x in even-numbered rows, increases along y , and increases right to left in odd-numbered rows (Fig. 4.5).

Improving printing accuracy

When the rectangular pattern in Fig. 4.5b was used to print networks designed to be cuboidal, the resulting networks often had sloped walls and a convex upper surface (Fig. 4.6a,b). Observation of the printing process revealed that these deformations arose as a consequence of the finite time required for two droplets to form a bilayer after coming into contact (see Ch. 3). Droplets ejected at the outermost edges of the network therefore tended to roll down the outer walls for some distance before incorporating into the network. Although droplets in the first few layers sometimes rolled

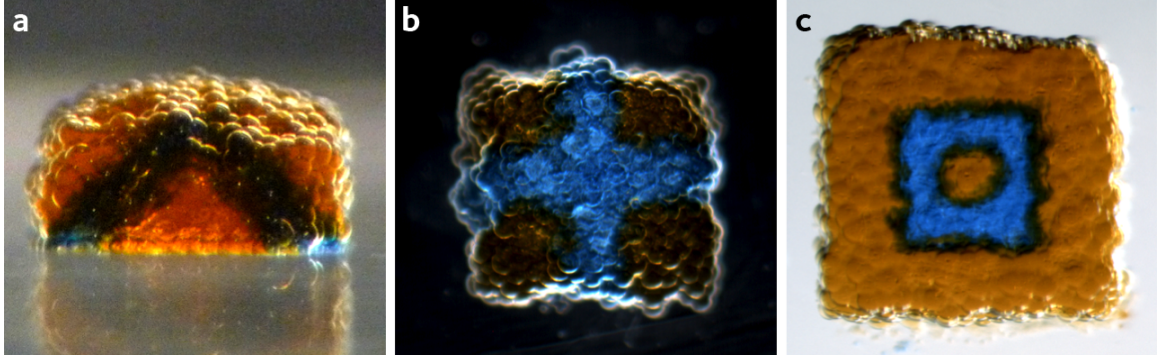


Figure 4.6 | Printing distortions. All the networks in this figure were printed with the simple printing pattern of Fig. 4.5b. (a) Horizontal view of a network designed to be cuboidal, but resulting in a convex top face. (b) View from above of a network designed to be cuboidal, but resulting with inwardly sloped walls. (c) View from above of a network designed to be cuboidal and faithfully printed by the simple printing pattern.

towards the centre of the network instead of away from it, this increased the probability that droplets subsequently printed at the same position would roll outwards. The tendency of droplets to roll down the outer walls caused a depletion of droplets in the outermost parts of upper layers of the network, and an excess around the outermost parts of lower layers. In a few cases, however, networks printed in the same way did not exhibit the same distortion (Fig. 4.6c). In these cases, the motion of the nozzles through the oil dragged droplets towards the centre of the network before the droplets rolled down the sides.

We found that the printing pattern could be modified slightly to considerably and reliably improve the accuracy with which networks were printed, so that droplets could be placed within approximately one droplet diameter of their intended positions in the network (Fig. 3.5b). The modification consisted of (i) the addition of an outer margin of one or two pixels to the network maps, and (ii) the modification of the printing algorithm so that in addition to the normal ejection of the droplets in each row, a droplet was ejected an additional n times at each of the marginal goals, where $n \sim 2$ or 3 depending on the network printed. Although the first marginal droplets in each layer typically rolled down the outer walls of the network for a few layers, the following marginal droplets in that layer tended to incorporate into the network on top of these. In this way, the printing algorithm forms a barrier of marginal droplets that grows at the pace of the network, and prevents the internal droplets from rolling out of their intended boundary.

Reducing printing time

In a given pass, in general not all goals represent droplets of the colour corresponding to that pass; for example, in a layer that contains both A and B droplets, the A pass would include goals for B droplets. This can cause the manipulator to spend a significant time in travelling to goals at which no droplets are produced. This could be solved by simply skipping (that is, omitting from the printing path) all the goals that do not require droplets in the current pass. However, the motion of the nozzles through the oil can cause the displacement of droplets that have been recently produced (see Ch. 3). Therefore, skipping every goal that does not correspond to the current pass would cause some droplets in the network to be displaced differently to others, depending on their positions along the printing path.

Parameter	Typical value
Spacing between droplets in x and y	50 μm
x -offset for alternate rows	25 μm
x - and y -offsets for alternate layers	25 μm
Manipulator speed during printing	200 $\mu\text{m s}^{-1}$
Delay after ejecting each droplet	200 ms
Delay after printing each row	2 s
Goal skipping threshold	4
Interface reset period	10

Table 4.3 | Printing parameters. x and y are the two horizontal dimensions, and rows of droplets are printed along x . In each layer, droplets in alternate rows can be printed with a displacement along x to promote the formation of a regular close-packed arrangement. Similarly, displacements in x and y can be set for droplets in alternate layers.

The algorithm prevents this problem by allowing the manipulator to skip to the next goal that requires a droplet in the current pass only if no droplets have been ejected at any of the previous n goals, where n is the *goal skipping threshold*. The goal skipping threshold is chosen to allow recently-ejected droplets enough time to incorporate into the network, and so be unaffected by the motion of the printing nozzles.

Preventing leakage

If the droplet generator chamber was too high above the level of oil solution in the well, the hydrostatic pressure from the aqueous solution caused the printing nozzle to spontaneously leak its contents into the oil. With the chamber ~ 1 cm above the oil and a typical nozzle with tip diameter $\sim 80 \mu\text{m}$, this leakage took place in two stages: an initial phase in which the aqueous volume gradually grew out of the nozzle to form an approximately hemispherical protrusion over several minutes, followed by a rapid phase in which the contents of the aqueous chamber emptied into the oil over a few seconds.

During the slower initial phase of leakage, the ejection of a droplet returned the aqueous-oil interface at the nozzle to its original planar geometry. The droplet generator that was used in given pass was therefore prevented from leaking its contents, because it ejected droplets at the relatively high rate of $\sim 1 \text{ s}^{-1}$. However, because each pass took up to several minutes, the generator not used in a given pass could reach the rapid phase of leakage and empty its contents.

The application of certain voltage waveforms to a droplet generator returned the aqueous-oil interface at its nozzle to a planar geometry without causing the ejection of a droplet. The voltage waveform we used to reset the interface typically consisted of three square pulses, each of duration 40 μs and amplitude ~ 12 V, with a 20 ms interval between them. In each pass, this waveform was applied every n goals to the droplet generator not used in that pass, where n is the *interface reset period*.

Computer algorithm

The printing pattern with the above modifications was implemented as a computer algorithm written in the PROCESSING programming language. The algorithm (Fig. 4.7) begins with an *initialization* phase that is executed once, followed by a *printing* phase that persists until printing has completed.

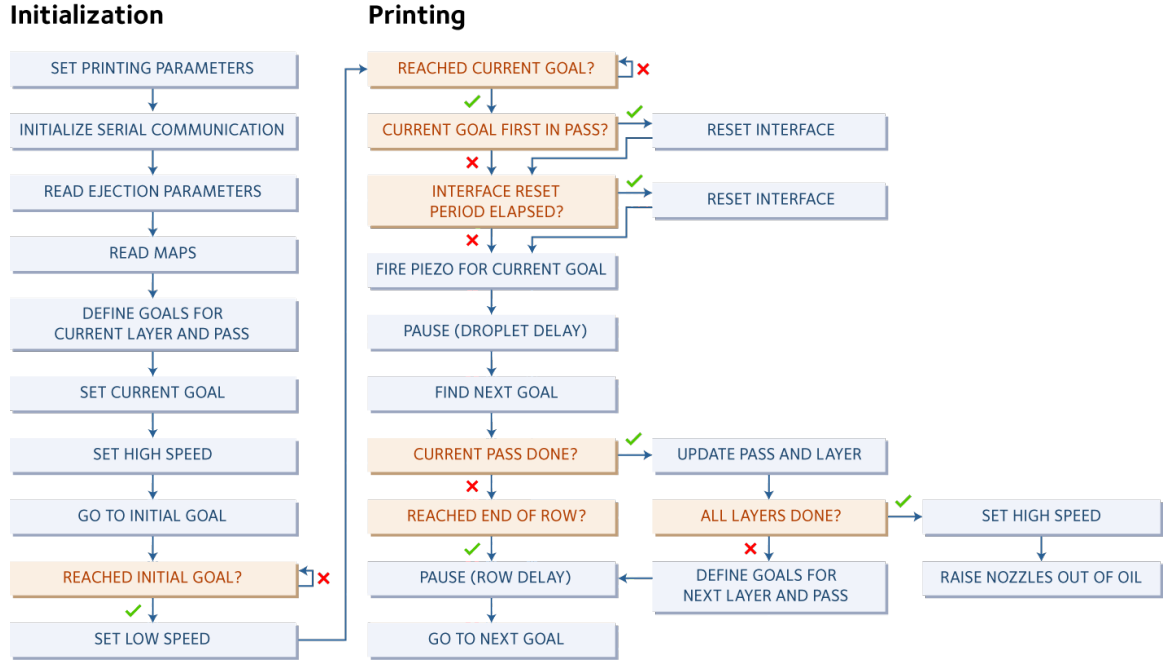


Figure 4.7 | Outline of the printing algorithm. For each condition (coloured orange), the instruction marked by a cross is executed if the condition is false, and the instruction marked by a check is executed if the condition is true. The delays after making each droplet or row are explained in Ch. 3.

Initialization. In the initialization phase, the user first inputs the parameters in Table 4.3. The program then loads into memory all the maps that define the network to be printed. A path for the printing nozzles, encoded as a series of goals according to the pattern detailed above, is established for the first pass of the first layer. Finally, the manipulator is instructed to move to the first goal.

Printing. In the printing phase, the computer synchronizes the motion of the oil bath with the ejection of droplets from the two generators. When each goal is reached, a droplet is ejected only if the goal type corresponds to the current pass. After allowing a delay after ejection, the next goal is determined according to the basic pattern of Fig. 4.5, together with the strategies described above to improve accuracy and reduce printing time. If the current goal represents the end of a row, another delay is introduced. The delays after each droplet and row are explained in Ch. 3. Finally, if the interface reset period has elapsed upon reaching the current goal, the oil-aqueous interface of the idle generator in the current pass is reset as described above.



CHAPTER 5

Mathematical derivations

This chapter presents mathematical derivations for various results in Chs. 2 and 3. For Ch. 2, we calculate the free energy landscape of a multisome with a single inner droplet. The calculations for Ch. 3 are in five sections: (i) the derivation of some mechanical properties of printed droplet networks; (ii) the derivation of equations used to simulate the electrical behaviour of droplet networks; (iii) the calculation of bilayer permeability from experimental measurements; (iv) the formulation and computational implementation of a model of self-folding networks; and (v) the calculation of the free energy available from osmosis in folding networks. Auxiliary calculations are provided in the Appendix.

5.1 Energy landscape of a multisome

In this section we derive the free energy landscape of a multisome with a single inner droplet (Fig. 2.4). Following the definition of the system, an expression is derived for the free energy of bilayer formation for a multisome from its initial state into an arbitrary geometry. Physical constraints dictate the range of attainable geometries. Finally, the free energy landscape of bilayer formation is calculated by evaluating the free energy throughout the attainable range of geometries.

Definitions and assumptions

Let the oil drop, without the aqueous droplet inside, have radius R_1 , and the aqueous droplet have radius R_2 . We assume that once the bilayer has formed, each of the two monolayers and the bilayer minimizes its own surface area so that the oil drop and aqueous droplet can be constructed from spherical caps (Fig. 5.1). This assumption is validated by photographs of multisomes with single inner droplets, such as Fig. 2.3a. The geometry of the spherical caps is defined by the contact angles θ_i and radii of curvature r_i , where $i \in \{1, 2, 3\}$. A similar parameterization has been used for vesicles containing phase-separated aqueous solutions¹³⁵, which are physically distinct from multisomes but mathematically equivalent under certain conditions.

We assume that the multisome has two types of interface: lipid monolayers, with interfacial tension γ_m ; and a lipid bilayer, with interfacial tension γ_b . As shown in the Appendix, buoyancy effects are expected to be negligible compared to the effects of interfacial tension. Both monolayer and bilayer interfacial tensions were assumed to be independent of curvature at the very low curvatures investigated here (radius of curvature $>50 \mu\text{m}$, corresponding to curvature $<0.02 \mu\text{m}^{-1}$).

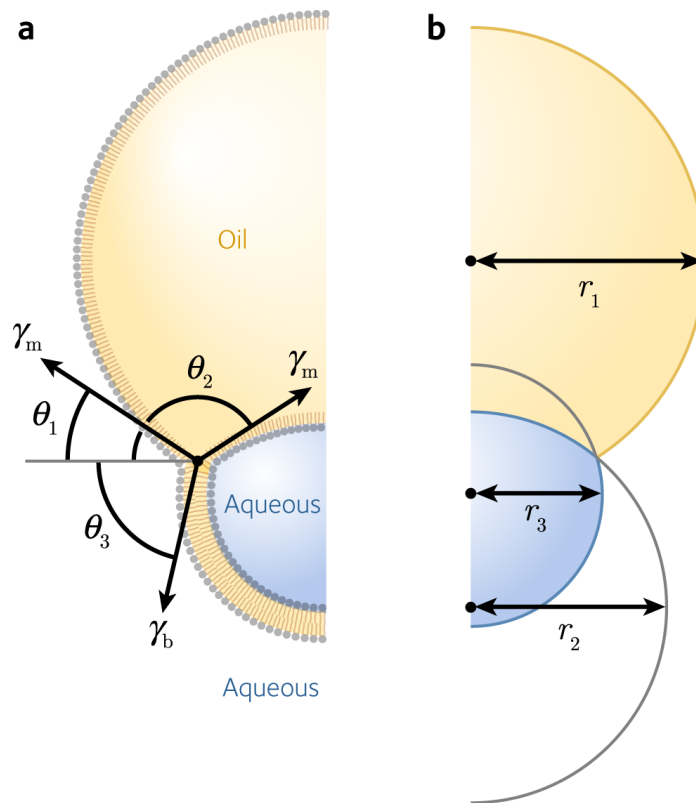


Figure 5.1 | Geometrical definitions. (a) Contact angles θ_i , defined relative to the plane that contains the circular intersection of the two monolayers and the bilayer. (b) Radii of curvature r_i of the spherical caps that define the two monolayers (r_1 and r_2) and the bilayer (r_3).

Free energy of bilayer formation

When the aqueous droplet contacts the edge of the oil drop to form a bilayer, there is a free energy change composed of (i) a favourable contribution from the joining of two monolayers to form a bilayer, and (ii) an unfavourable contribution from distortion of the monolayer-covered surfaces. The unfavourable contribution exists because the initial spherical geometries of the oil drop and aqueous droplet minimize their surface areas per unit volume, and their volumes remain constant during the formation of the bilayer; it follows that any distortion from this geometry will incur an increase in surface area. The net change in free energy during bilayer formation is given by the difference in the free energies of the multisome in its initial and final states.

Initial state. We define the initial state to be prior to the formation of a bilayer. The aqueous droplet is then contained entirely within the oil drop, and both the aqueous droplet and oil drop have a spherical surface coated with a lipid monolayer. Recalling that interfacial tension is interfacial energy per unit area, the interfacial energy of this system is given by the tension of each interface multiplied by its area. The free energy of the initial state is then:

$$F_{\text{initial}} = \gamma_m (A_1 + A_2), \quad (5.1)$$

where $A_i = 4\pi R_i^2$ with $i \in \{1, 2\}$ are the initial areas of the oil drop and aqueous droplet, respectively.

Final state. In the final state of the system, a portion of the aqueous droplet has adhered to the surface of the oil drop to form a bilayer. As before, the free energy of this state is given by the interfacial tension of each surface multiplied by its area. The free energy of the final state is therefore:

$$F_{\text{final}} = \gamma_m (a_1 + a_2) + \gamma_b a_3, \quad (5.2)$$

where a_i with $i \in \{1, 2, 3\}$ are the areas of the spherical caps with radii of curvature r_i defined in Fig. 5.1. The area of each spherical cap can be expressed (see Appendix) in terms of its radius of curvature and associated contact angle: $a_i = 2\pi r_i^2 (1 + \cos \theta_i)$.

Free energy change. The free energy of formation of a multisome bilayer is given by the difference in free energy between the final (Eq. 5.2) and initial (Eq. 5.1) states:

$$\Delta F = \gamma_m (a_1 + a_2) + \gamma_b a_3 - \gamma_m (A_1 + A_2).$$

Substituting our expressions for the A_i and a_i and rearranging, we obtain:

$$\Delta F = 2\pi\gamma_m \left[r_1^2 (1 + \cos \theta_1) + r_2^2 (1 + \cos \theta_2) + \frac{\gamma_b}{\gamma_m} r_3^2 (1 + \cos \theta_3) - 2 (R_1^2 + R_2^2) \right].$$

The ratio γ_b/γ_m is related (see Appendix) to the equilibrium contact angle of a droplet interface bilayer in bulk oil, θ_{eq} , by: $\gamma_b/\gamma_m = 2 \cos \theta_{\text{eq}}$. The above expression then becomes:

$$\Delta F = 2\pi\gamma_m \left[r_1^2 (1 + \cos \theta_1) + r_2^2 (1 + \cos \theta_2) + 2r_3^2 \cos \theta_{\text{eq}} (1 + \cos \theta_3) - 2 (R_1^2 + R_2^2) \right]. \quad (5.3)$$

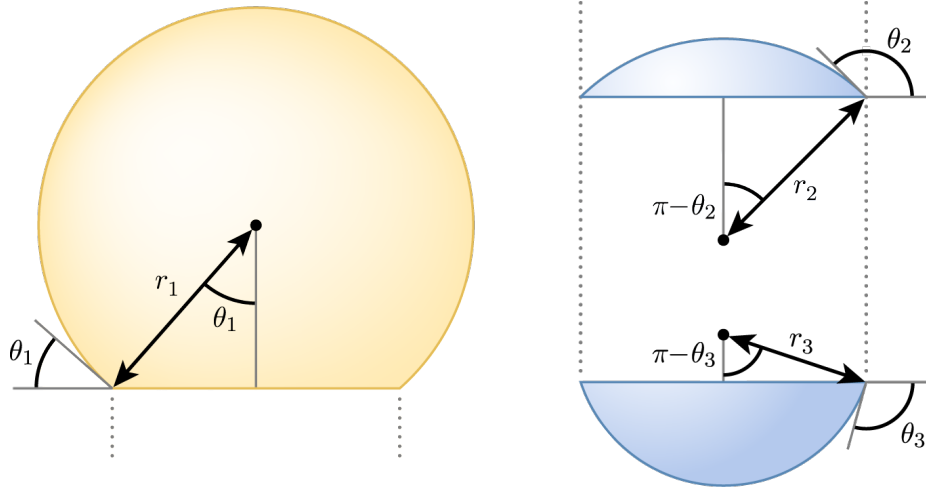


Figure 5.2 | Boundary matching. The three spherical caps that comprise the multisome must coincide at their edges, as indicated by the dotted lines.

Constraints

Equation 5.3 gives the free energy of bilayer formation ΔF for a multisome with a given initial geometry (R_1, R_2) and final geometry $(r_1, r_2, r_3, \theta_1, \theta_2, \theta_3)$. However, not all final geometries are accessible to a given multisome. For a given initial geometry, some combinations of the r_i and θ_i , where $i \in \{1, 2, 3\}$, would describe a multisome with different oil or aqueous volumes to the initial state, or one composed of spherical caps that do not coincide at their edges. The r_i and θ_i are therefore not free variables, and it is necessary to impose constraints on their values. Each constraining equation can be visualized as defining some subset of the $(r_1, r_2, r_3, \theta_1, \theta_2, \theta_3)$ space, so that the intersection of all such subsets defines the region in the parameter space accessible to a given multisome.

Boundary matching. We first impose the constraint that the surfaces of the three spherical caps must coincide at their edges. The circular openings of the spherical caps must therefore all share the same diameter (Fig. 5.2). The following conditions are straightforwardly derived with reference to this figure:

$$\begin{aligned} r_1 \sin \theta_1 &= r_2 \sin \theta_2; \\ r_2 \sin \theta_2 &= r_3 \sin \theta_3. \end{aligned} \tag{5.4}$$

Volume conservation. The second constraint we impose is the conservation of volume of the oil drop and of the aqueous droplet. We denote the oil volume by $V_{\text{oil}} = 4\pi R_1^3/3$, the volume of the aqueous droplet by $V_{\text{aq}} = 4\pi R_2^3/3$, and the volumes of the three spherical caps that comprise the encapsulated droplet after bilayer formation by V_1 , V_2 and V_3 (Fig. 5.3). The conservation of oil volume implies that $V_{\text{oil}} = V_1 - V_2$, so that

$$\frac{4\pi}{3} R_1^3 = V_1 - V_2. \tag{5.5}$$

Similarly, conservation of volume for the aqueous droplet implies that $V_{\text{aq}} = V_2 + V_3$, so that

$$\frac{4\pi}{3} R_2^3 = V_2 + V_3. \tag{5.6}$$

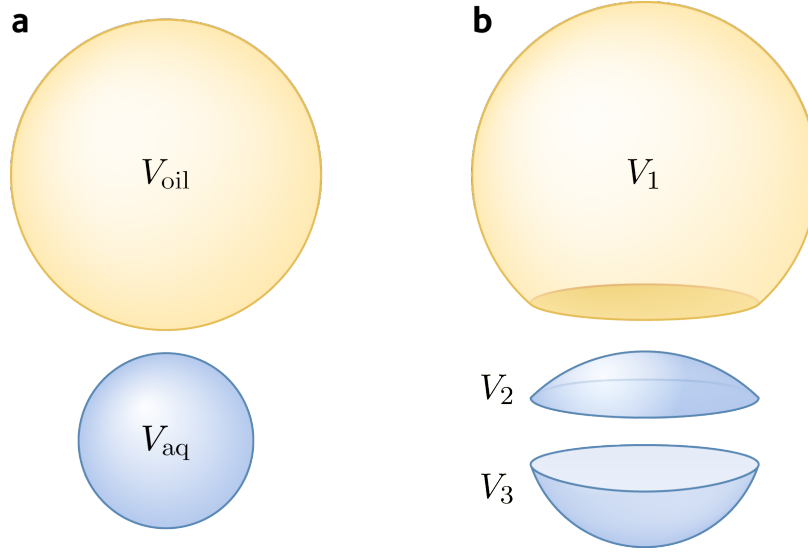


Figure 5.3 | Volume conservation. (a) The oil drop and aqueous droplet prior to the formation of a bilayer, with volumes V_{oil} and V_{aq} , respectively. The aqueous droplet is illustrated outside the oil drop to emphasize that V_{oil} is the volume of the oil phase only. (b) Exploded geometry of the multisome after the formation of a bilayer. V_1 , V_2 and V_3 are the volumes of the spherical caps that represent, respectively, the monolayer-coated surface of the oil drop, the monolayer-coated surface of the aqueous droplet, and the bilayer-coated surface of the aqueous droplet.

The volume V_i of each spherical cap can be expressed (see Appendix) in terms of its radius of curvature r_i and associated contact angle θ_i as:

$$V_i = \frac{\pi r_i^3}{12} \alpha(\theta_i),$$

where for brevity we define the function

$$\alpha(\theta) = 8 - \cos 3\theta + 9 \cos \theta. \quad (5.7)$$

Substituting this expression for V_1 , V_2 and V_3 in Eqs. 5.5 and 5.6, we obtain two constraints that express the conservation of the oil and aqueous volumes:

$$\begin{aligned} 16R_1^3 &= r_1^3 \alpha(\theta_1) - r_2^3 \alpha(\theta_2); \\ 16R_2^3 &= r_2^3 \alpha(\theta_2) + r_3^3 \alpha(\theta_3). \end{aligned} \quad (5.8)$$

Imposing the constraints

We now impose the four constraints represented by Eqs. 5.4 and 5.8 on the expression for the free energy of bilayer formation, Eq. 5.3. The four independent constraining equations involve six variables (the r_i and θ_i ; R_1 and R_2 are given constants), so together involve only two free variables. The choice of variables to be left free is arbitrary; it was found that keeping θ_2 and θ_3 free facilitated the numerical solution of the equations and the visualization of the results. We therefore proceed to eliminate r_1, r_2, r_3 and θ_1 by combining the constraining equations.

First we eliminate r_2 and r_3 by rearranging Eqs. 5.4 to give:

$$r_2 = r_1 \frac{\sin \theta_1}{\sin \theta_2}; \quad r_3 = r_2 \frac{\sin \theta_2}{\sin \theta_3}.$$

Substituting the first of these into the second, we obtain r_2 and r_3 in terms of r_1 and the θ_i :

$$r_2 = r_1 \frac{\sin \theta_1}{\sin \theta_2}; \quad r_3 = r_1 \frac{\sin \theta_1}{\sin \theta_3}. \quad (5.9)$$

This leaves r_1 and θ_1 to be eliminated. Substituting Eqs. 5.9 into the volume conservation equations, Eqs. 5.8, yields:

$$\begin{aligned} 16R_1^3 &= r_1^3 \left[\alpha(\theta_1) - \left(\frac{\sin \theta_1}{\sin \theta_2} \right)^3 \alpha(\theta_2) \right]; \\ 16R_2^3 &= r_1^3 \left[\left(\frac{\sin \theta_1}{\sin \theta_2} \right)^3 \alpha(\theta_2) + \left(\frac{\sin \theta_1}{\sin \theta_3} \right)^3 \alpha(\theta_3) \right]. \end{aligned} \quad (5.10)$$

The first of these can be rearranged to give r_1 in terms of θ_1 and θ_2 :

$$r_1^3 = 16R_1^3 \left[\alpha(\theta_1) - \left(\frac{\sin \theta_1}{\sin \theta_2} \right)^3 \alpha(\theta_2) \right]^{-1}. \quad (5.11)$$

Finally, we obtain an expression for θ_1 by substituting Eq. 5.11 into the last unused constraining equation, Eq. 5.10, to obtain:

$$\alpha(\theta_1) = \left(\frac{\sin \theta_1}{\sin \theta_2} \right)^3 \left[1 + \left(\frac{R_1}{R_2} \right)^3 \right] \alpha(\theta_2) + \left(\frac{\sin \theta_1}{\sin \theta_3} \right)^3 \left(\frac{R_1}{R_2} \right)^3 \alpha(\theta_3). \quad (5.12)$$

In summary, Eq. 5.12 expresses θ_1 in terms of the free variables θ_2 and θ_3 ; Eq. 5.11 in turn expresses r_1 in terms of θ_1 ; and Eqs. 5.9 express r_2 and r_3 in terms of r_1 . We are therefore now in a position to evaluate ΔF for given values of θ_2 and θ_3 .

Solving the equations

Although it is not analytically tractable to calculate θ_1 for given values of θ_2 and θ_3 using Eq. 5.12, this is easily done by numerical methods with a computer. The free energy landscape was calculated by computing the free energy of bilayer formation ΔF for all possible combinations of θ_2 and θ_3 , using the following procedure:

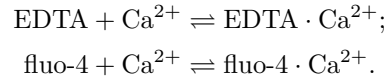
1. Choose values for R_1 , R_2 , γ_m and θ_{eq} that reflect the experimental conditions. R_1 and R_2 are determined by the chosen volumes for the oil drop and aqueous droplet, while γ_m and θ_{eq} depend only on the fluid and lipid compositions. All four quantities are kept constant throughout the procedure.
2. Choose values for θ_2 and θ_3 in the range of interest.
3. Calculate θ_1 using Eqs. 5.7 and 5.12, with the values chosen for θ_2 and θ_3 in the previous step. This was done with MATLAB's `fsolve` function.
4. Calculate r_1 using Eq. 5.11, using the value of θ_1 calculated in the previous step and the chosen values of θ_2 and θ_3 .
5. Calculate r_2 and r_3 using Eqs. 5.9, using the values of r_1 and θ_1 calculated in the previous steps and the chosen values of θ_2 and θ_3 .
6. Calculate ΔF using Eq. 5.3. This is the free energy of bilayer formation for a multisome with the final geometry described by the chosen values of θ_2 and θ_3 .

7. Repeat from step 2 with different values of θ_2 and θ_3 .

A MATLAB program was written to perform these steps for θ_2 at 4° intervals between 0° and 248° , and for θ_3 at 4° intervals between 0° and 188° , to produce the map of free energy of bilayer formation shown in Fig. 2.4b. The values chosen for the experimental parameters are given in the text. The value of $\gamma_b/\gamma_m = 0.68$ given in Ch. 2 was derived from measurements of the contact angle θ_{eq} of pairs of aqueous droplets joined by interface bilayers in bulk oil (see Appendix). θ_{eq} was measured as $70 \pm 6^\circ$ (mean $\pm$ s.d., $n = 10$) using droplets of buffer containing 500 mM KCl, and a bulk oil 9:1 (v/v) mixture of silicone oil and hexadecane containing 0.1 mg ml $^{-1}$ DPhPC. The interfacial tensions in systems with high contact angles could be measured accurately using the method of Needham and Haydon²⁵.

5.2 Competitive binding

This section describes the competitive binding of EDTA and fluo-4 to Ca^{2+} ions in the fluorescence microscopy experiments of Section 2.5. The Ca^{2+} ions that diffuse into a multisome droplet through αHL pores can bind to either EDTA or fluo-4:



Because the timescales for Ca^{2+} binding and unbinding to EDTA or fluo-4 (Section 2.5) are significantly faster than the timescale of the fluorescence measurements in Fig. 2.7, the binding and unbinding can be treated as being in quasi-equilibrium. The concentrations of free and bound EDTA and fluo-4 are therefore given by

$$\begin{aligned}E_f C_f &= E_b K_{d,E}; \\ F_f C_f &= F_b K_{d,F},\end{aligned}\tag{5.13}$$

where E_f and E_b represent the concentrations of free and Ca^{2+} -bound EDTA, and F_f and F_b are defined similarly for fluo-4. Expressing the concentrations of the various binding states of Ca^{2+} , EDTA and fluo-4 in terms of their total concentrations gives three more relations:

$$\begin{aligned}C_t &= C_f + E_b + F_b; \\ E_t &= E_b + E_f; \\ F_t &= F_b + F_f,\end{aligned}\tag{5.14}$$

where C_t is the total Ca^{2+} concentration inside the droplet, which increases with time, and E_t and F_t are respectively the total, constant concentrations of EDTA and fluo-4. The simultaneous equations in Eqs. 5.13 and 5.14 can be reduced by substitution to the following:

$$\begin{aligned}C_t &= \left(\frac{E_t}{E_f} - 1\right) \left(K_{d,E} + E_f + \frac{K_{d,E}}{K_{d,F}} F_f\right); \\ C_t &= \left(\frac{F_t}{F_f} - 1\right) \left(K_{d,F} + F_f + \frac{K_{d,F}}{K_{d,E}} E_f\right).\end{aligned}\tag{5.15}$$

For given values of the total concentrations C_t , E_t , F_t and the dissociation constants $K_{d,E}$ and $K_{d,F}$, Eqs. 5.15 yield E_f and F_f . The latter can be used in turn to evaluate F_b , the concentration of

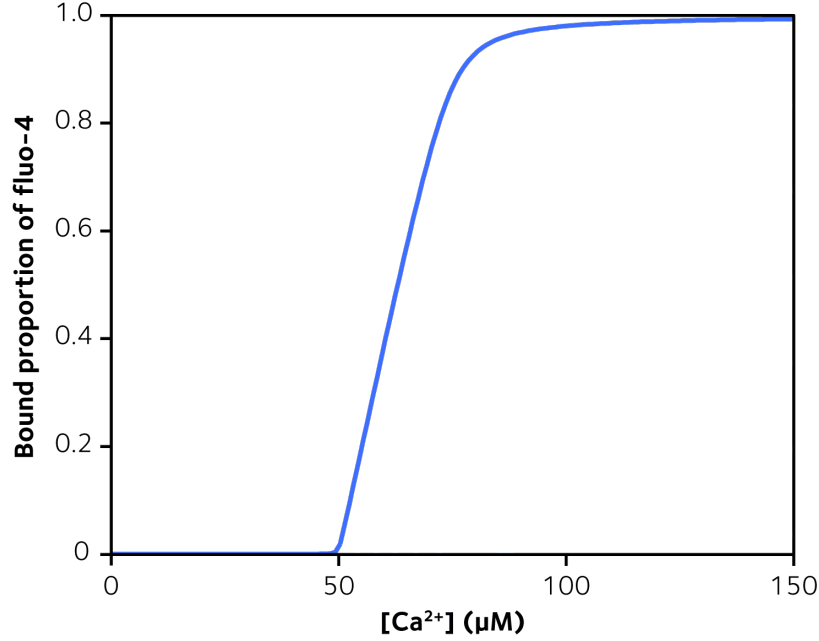


Figure 5.4 | Predicted fluorescence delay due to competitive binding. The proportion of fluo-4 bound to Ca^{2+} , calculated as F_b/F_t , is shown as a function of the total concentration of Ca^{2+} ions, C_t . Ca^{2+} ions become available to binding by fluo-4 only once the 50 μM EDTA in the solution has been saturated.

fluo-4 that is bound to Ca^{2+} , and which is expected to be proportional to the fluorescence intensity measured in Fig. 2.7.

In the experiment depicted in Fig. 2.7a, Ca^{2+} diffused through αHL pores from a bath of 100 mM Ca^{2+} into the droplet of a multisome, which contained EDTA and fluo-4 at total concentrations $E_t = 50 \mu\text{M}$ and $F_t = 25 \mu\text{M}$, respectively (Ch. 4). Using $K_{d,E} = 34 \text{ pM}$ (ref. 136) and $K_{d,F} = 520 \text{ nM}$ (ref. 70), Eqs. 5.15 can be used to calculate the bound proportion of fluo-4 as a function of the concentration of Ca^{2+} in the multisome (Fig. 5.4). Assuming a constant flux of Ca^{2+} into the droplet, the fluorescence as a function of time should have a similar profile to the curve in Fig. 5.4. This is indeed the case (Fig. 2.7a), and the delay in the onset of fluorescence is therefore expected to be due to competitive binding of Ca^{2+} by EDTA.

5.3 Mechanical properties of printed networks

In this section we derive some mechanical properties of droplet networks. First we calculate the elastic modulus and ultimate tensile strength of a pair of droplets joined by a bilayer, and extend this analysis to an arbitrarily long linear chain of droplets. The extension to fully three-dimensional droplet networks is then made by scaling arguments. Finally, we derive a theoretical upper limit for the number of layers in a self-supporting droplet network.

Mechanical properties of a droplet pair

We first define the droplet pair system, and derive an expression for the free energy of bilayer formation for a given final geometry. The conservation of volume determines the attainable range of

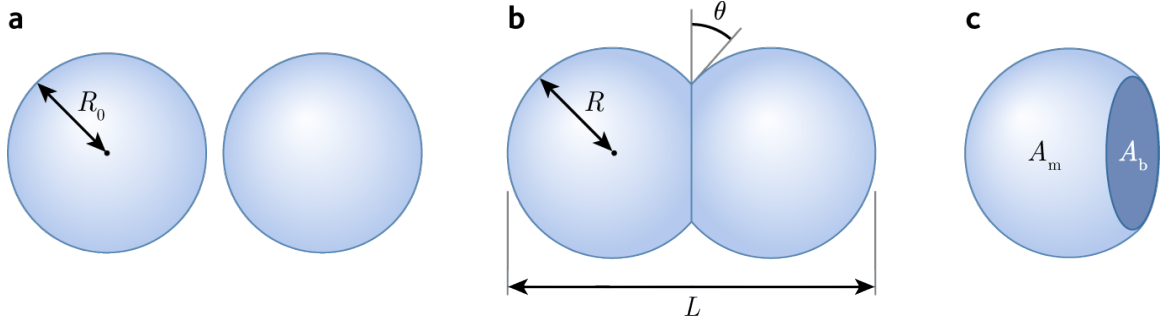


Figure 5.5 | Geometry of a droplet pair joined by a bilayer. (a) The droplet pair prior to bilayer formation. The two monolayer-coated droplets are identical, with initial radii R_0 . (b) The droplet pair after the formation of a bilayer, with an associated change in free energy ΔF . After bilayer formation, the monolayer-coated portion of each droplet has the geometry of a spherical cap of radius R . The contact angle θ is defined by the intersection of the bilayer and one monolayer. (c) Oblique view of one droplet in **b** after bilayer formation. The circular bilayer has area A_b , and each of the two monolayers has area A_m .

final geometries. We use this to derive the free energy of bilayer formation for the equilibrium final geometry, and then examine the system as it is perturbed from equilibrium. From the behaviour near equilibrium we calculate an effective spring constant between the droplets and their Young modulus, while the behaviour far from equilibrium is described by the stress-strain relationship and the tensile strength.

Definitions. We assume for simplicity that the two droplets are identical, so that prior to bilayer formation each droplet is spherical with a known radius R_0 (Fig. 5.5a). In the final state, the droplets adhere with a contact angle θ , so that each monolayer adopts the geometry of a spherical cap with curved area A_m and the bilayer is a disc of area A_b (Fig. 5.5b,c).

Free energy of bilayer formation. The free energy of bilayer formation ΔF is given by the difference in the surface energies of the final and initial states. Recalling that the surface energy per unit area is given by the interfacial tension, γ_m and γ_b for a monolayer and bilayer respectively, it follows that

$$\Delta F = \gamma_b A_b + \gamma_m 2A_m - \gamma_m 8\pi R_0^2, \quad (5.16)$$

where the first term corresponds to the bilayer in the final state, the second term to the two monolayers in the final state, and the last term to the two monolayers in the initial state.

For a pair of adhered droplets at equilibrium, the balance of interfacial tensions requires that (see Appendix)

$$\gamma_b = 2\gamma_m \cos \theta_{\text{eq}}, \quad (5.17)$$

where θ_{eq} is the equilibrium value of the contact angle θ defined in Fig. 5.5b. Because this relationship also holds out of equilibrium, we can without loss of generality express Eq. 5.16 as

$$\Delta F = 2\gamma_m (A_m + A_b \cos \theta_{\text{eq}} - 4\pi R_0^2).$$

The bilayer and monolayer areas can be expressed in terms of the contact angle θ and the radius of curvature R of the adhered droplets:

$$\begin{aligned} A_b &= \pi R^2 \sin^2 \theta; \\ A_m &= 2\pi R^2 (1 + \cos \theta). \end{aligned}$$

Substituting these into our expression for ΔF yields the free energy of bilayer formation in terms of R and θ :

$$\Delta F = 2\pi R^2 \gamma_m \left(2 + 2 \cos \theta + \cos \theta_{\text{eq}} \sin^2 \theta - 4 (R_0/R)^2 \right). \quad (5.18)$$

Conservation of volume. The two variables in Eq. 5.18, R and θ , are related by the conservation of volume of the aqueous droplets. Using the formula for the volume of a spherical cap derived in the Appendix, the conservation of volume for each droplet implies:

$$\frac{4\pi}{3} R_0^3 = \frac{\pi R^3}{12} (8 - \cos 3\theta + 9 \cos \theta),$$

which can be rearranged to give the following relation for R and θ :

$$\frac{R_0}{R} = 2^{-4/3} (8 - \cos 3\theta + 9 \cos \theta)^{1/3}. \quad (5.19)$$

We are now in a position to express ΔF in terms of a single independent variable. First we address the case where the two droplets have reached equilibrium, which is most conveniently done in terms of the easily measured contact angle θ . This is followed by an analysis of the response of the system to perturbation, which is most readily interpreted in terms of the end-to-end length L of the droplet pair (Fig. 5.5b).

Equilibrium case. We wish to calculate the free energy of bilayer formation for a droplet pair that has reached equilibrium. To do so we first use Eq. 5.19 to eliminate R in Eq. 5.18, which yields ΔF in terms of only θ :

$$\Delta F = 8\pi \gamma_m R_0^2 \left[2^{2/3} \left(\frac{2 + 2 \cos \theta + \cos \theta_{\text{eq}} \sin^2 \theta}{[8 - \cos 3\theta + 9 \cos \theta]^{2/3}} \right) - 1 \right]. \quad (5.20)$$

The free energy change for bilayer formation in the equilibrium geometry is then found straightforwardly by inserting $\theta = \theta_{\text{eq}}$ in the above equation, which simplifies to:

$$\Delta F = \gamma_m \pi R_0^2 \left([32 (8 - \cos 3\theta_{\text{eq}} + 9 \cos \theta_{\text{eq}})]^{1/3} - 8 \right). \quad (5.21)$$

This is a monotonically decreasing function of θ_{eq} in the range $0 \leq \theta_{\text{eq}} \leq 180^\circ$; note that contact angles greater than 90° are not possible with a nonzero monolayer interfacial tension, as evident from the force balance expression Eq. 5.17. A droplet pair with a greater equilibrium contact angle is therefore more strongly adhesive. For the conditions of the experiments presented in Ch. 3, $R_0 \approx 25 \mu\text{m}$, $\gamma_m \approx 1 \text{ mN m}^{-1}$ (refs. 66, 67) and θ_{eq} was measured as $47 \pm 4^\circ$ ($n = 7$), so that $\Delta F \approx -360 \text{ fJ}$. An intrinsic measure of the droplet adhesion energy for these values of θ_{eq} and γ_m can be obtained by dividing ΔF by the initial total area of monolayer: $\Delta F / (8\pi R_0^2) \approx -23 \mu\text{J m}^{-2}$. Note that this is significantly smaller than the energy of bilayer formation under these conditions, $-640 \mu\text{J m}^{-2}$ (Table 2.1), because the adhesion of the droplets distorts the interfaces that remain as monolayers, and this incurs a free energy cost.

Perturbation from equilibrium. We now turn to the mechanical behaviour of the adhered droplet pair under axial extension and compression. Specifically, we first examine the response to gentle perturbations from the equilibrium state, and derive an effective spring constant between the two droplets and the Young modulus of the system. We then calculate the ultimate tensile strength of the droplet pair under severe deformation.

To perform these analyses it is necessary to express ΔF in terms of the end-to-end length L of the adhered droplets (Fig. 5.5b). The simplest way to obtain ΔF in terms of L is to begin with Eq. 5.18, which expresses ΔF in terms of R and θ . We begin by expressing R in terms of L and θ :

$$R = \frac{L}{2(1 + \cos \theta)}. \quad (5.22)$$

Substituting into Eq. 5.18, we obtain ΔF in terms of L and θ :

$$\Delta F = \pi \gamma_m \left(\frac{L^2}{2} \left[\frac{2 + \cos \theta_{\text{eq}} (1 - \cos \theta)}{1 + \cos \theta} \right] - 8R_0^2 \right). \quad (5.23)$$

Now we relate L and θ by combining the expression for conservation of volume, Eq. 5.19, which relates R and θ , with Eq. 5.22, which relates R , L and θ , to obtain:

$$\frac{R_0}{L} = 2^{-7/3} \frac{(8 - \cos 3\theta + 9 \cos \theta)^{1/3}}{1 + \cos \theta},$$

which simplifies to

$$\cos \theta = \frac{2 - 32 (R_0/L)^3}{1 + 32 (R_0/L)^3}. \quad (5.24)$$

Finally, we substitute this into Eq. 5.23 to obtain ΔF in terms of L only:

$$\Delta F = \gamma_m \pi \left[\frac{L^2}{6} (2 - \cos \theta_{\text{eq}}) + \frac{32R_0^3}{3L} (1 + \cos \theta_{\text{eq}}) - 8R_0^2 \right]. \quad (5.25)$$

Effective spring constant. The mechanical behaviour of the droplet pair near equilibrium is characterized by the second derivative of the free energy about the minimum point. This quantity represents an effective spring constant k between the two droplets:

$$k = \frac{d^2 \Delta F}{dL^2} \bigg|_{L=L_{\text{eq}}},$$

where L_{eq} is the value of L at equilibrium. L_{eq} can be found straightforwardly from Eq. 5.24 with $\theta = \theta_{\text{eq}}$ or by minimizing ΔF with respect to L . Both approaches yield

$$L_{\text{eq}} = R_0 \left[32 \frac{1 + \cos \theta_{\text{eq}}}{2 - \cos \theta_{\text{eq}}} \right]^{1/3}. \quad (5.26)$$

The second derivative of Eq. 5.25 is

$$\frac{d^2 \Delta F}{dL^2} = \frac{\gamma_m \pi}{3} \left[2 - \cos \theta_{\text{eq}} + 64 (1 + \cos \theta_{\text{eq}}) \left(\frac{R_0}{L} \right)^3 \right],$$

and substituting in $L = L_{\text{eq}}$ from Eq. 5.26 we obtain the effective spring constant (Fig. 3.6a):

$$k = \gamma_m \pi (2 - \cos \theta_{\text{eq}}). \quad (5.27)$$

Young's modulus. The mechanical behaviour of the adhered droplet pair can be compared with that of other materials by calculating the Young modulus for this system. Young's modulus E is defined as the ratio of the stress applied to a material to the resulting strain in the direction of the applied stress; that is, $E = \sigma/\epsilon$, where σ and ϵ are the stress and strain, respectively.

The stress σ may be expressed as the ratio of the applied force f to the area A over which the force is applied. We assume that the force is applied normal to the bilayer, and take A to be the

area of the droplet pair projected along the direction of the applied force. Therefore at equilibrium $A = \pi R_{\text{eq}}^2$, where R_{eq} is the value of R at equilibrium. We recall that the free energy of the droplet pair is quadratic about the minimum point, that is,

$$\Delta F \approx \frac{1}{2}k(L - L_{\text{eq}})^2 + \Delta F_0,$$

where ΔF_0 is the free energy at the minimum point. The restoring force exerted by the droplet pair is

$$f = -\frac{d\Delta F}{dL},$$

so that $f = k \delta L$, where $\delta L = L - L_{\text{eq}}$.

The strain is defined as the ratio of the change in length of the object to the original length, that is,

$$\epsilon = \frac{\delta L}{L_{\text{eq}}}.$$

Combining the above equations, we arrive at an expression for the Young modulus E :

$$E = \frac{kL_{\text{eq}}}{\pi R_{\text{eq}}^2}. \quad (5.28)$$

We now use Eq. 5.22 to express R_{eq} in terms of L_{eq} and θ_{eq} ; Eq. 5.26 for L_{eq} in terms of θ_{eq} ; and Eq. 5.27 for k in terms of θ_{eq} . Combining these results with the above equation, we obtain the Young modulus for an adhered droplet pair (Fig. 3.6b):

$$E = \frac{2^{1/3}\gamma_m}{R_0} (2 - \cos \theta_{\text{eq}})^{4/3} (1 + \cos \theta_{\text{eq}})^{5/3}. \quad (5.29)$$

Stress-strain relationship. Because the Young modulus is a measure of rigidity derived from a quadratic approximation to the free energy landscape, it strictly applies only to small perturbations. It is also possible to calculate the stress required to expand or compress the length of the droplet pair to any extent. As in the calculation of Young's modulus, the stress is $\sigma = f/A$, where $f = -d\Delta F/dL$ and A is the area of the droplet pair projected normal to the bilayer. Differentiating Eq. 5.25 and using Eqs. 5.22 and 5.24 to find A as a function of L , we obtain:

$$\sigma = \frac{12\gamma_m}{L} \left[2 - 32 \left(\frac{R_0}{L} \right)^3 (1 + \cos \theta_{\text{eq}}) - \cos \theta_{\text{eq}} \right] \left[1 + 32 \left(\frac{R_0}{L} \right)^3 \right]^{-2}. \quad (5.30)$$

Using $\epsilon = (L - L_{\text{eq}})/L_{\text{eq}}$, and using Eq. 5.26 to substitute for L_{eq} , the stress as a function of strain can then be found straightforwardly from Eq. 5.30. The stress-strain curve for a droplet pair under the conditions of this study is given in Fig. 3.6e.

Tensile strength. We now calculate the greatest stress that the droplet pair can withstand without separating irreversibly. The magnitude of this stress is here called the tensile strength, σ_t . The tensile strength is equal to the restoring force per unit area exerted by the droplet pair at the critical extension beyond which the droplets separate irreversibly. Because the free energy of bilayer formation as a function of L (Eq. 5.25) has a single minimum, no energetic barriers exist between the equilibrium state and the state in which the droplets are in infinitesimal contact. The critical value of L is therefore $L_{\text{lim}} = 4R_0$ (Fig. 5.5), so that $\sigma_t = \sigma(L = 4R_0)$. Using Eq. 5.30 then gives the tensile strength in terms of the equilibrium contact angle θ_{eq} :

$$\sigma_t = \frac{2\gamma_m}{R_0} (1 - \cos \theta_{\text{eq}}). \quad (5.31)$$

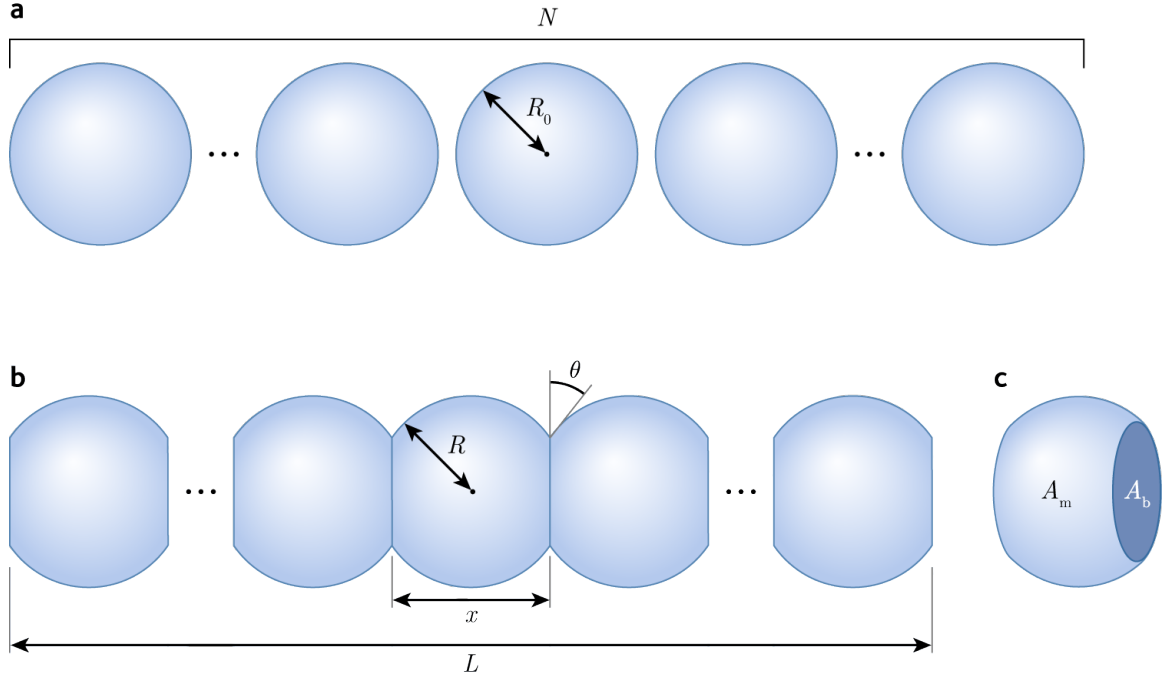


Figure 5.6 | Geometry of a linear chain of droplets joined by bilayers. (a) The droplets prior to bilayer formation. The N monolayer-coated droplets are identical, with initial radii R_0 . (b) The droplets after the formation of bilayers in a linear chain, with a total associated change in free energy ΔF . After bilayer formation, the monolayer-coated portion of each droplet has the geometry of a doubly truncated spherical cap of radius R . The contact angle θ is defined by the intersection of a bilayer and monolayer. The end effects of the two terminal droplets are neglected. (c) Oblique view of one droplet in **b** after bilayer formation. Each droplet contributes to two circular bilayers, each of area A_b . The curved, monolayer-coated surface of each droplet has area A_m .

Mechanical properties of a linear chain of droplets

We now investigate how the mechanical behaviour of a single pair of droplets differs from that of larger droplet networks. We choose a linear chain of droplets as a tractable system, and analyze it in a similar way as the droplet pair. As above, we first find an expression for the free energy of bilayer formation, and constrain it by using the conservation of volume. We then analyze the system away from equilibrium to calculate the effective spring constant, Young modulus, stress-strain relationship and tensile strength of the droplet chain.

Definitions. We assume that the linear chain consists of N identical droplets, so that prior to bilayer formation each droplet is spherical with a known radius R_0 (Fig. 5.6a). In the final state, each monolayer adopts the geometry of a doubly truncated spherical cap with curved area A_m , and each of the two bilayers formed by each droplet is a disc of area A_b (Fig. 5.6b,c). For simplicity, we use the same notation as for the case of two droplets.

Free energy of bilayer formation. The free energy of bilayer formation for each droplet in the chain is the difference in the surface energies of the final and initial states:

$$\Delta F_{\text{droplet}} = \gamma_b A_b + \gamma_m A_m - \gamma_m 4\pi R_0^2,$$

where only one of the two bilayers formed by the droplet contributes to $\Delta F_{\text{droplet}}$, to prevent double-counting. The free energy of adhesion for the full chain, neglecting the error introduced by the two terminal droplets, is therefore $\Delta F = N\Delta F_{\text{droplet}}$.

The force balance expression in Eq. 5.17 gives γ_b in terms of γ_m and θ_{eq} . The areas of a single bilayer A_b and of the monolayer around one droplet A_m can be expressed in terms of the contact angle θ and the radius of curvature R of the adhered droplets:

$$\begin{aligned} A_b &= \pi R^2 \sin^2 \theta; \\ A_m &= 4\pi R^2 \cos \theta, \end{aligned}$$

Combining these expressions, we can write the free energy of bilayer formation for the entire chain in terms of R and θ :

$$\Delta F = 2\pi N R^2 \gamma_m \left(2 \cos \theta + \cos \theta_{\text{eq}} \sin^2 \theta - 2 (R_0/R)^2 \right).$$

We are interested in the response of the droplet chain to axial extension and compression, so we now express ΔF in terms of the repeating distance of the chain, x . The variables R , θ and x are related by:

$$R = \frac{x}{2 \cos \theta}, \quad (5.32)$$

which can be used to eliminate R in the expression for ΔF , to give:

$$\Delta F = \frac{N\gamma_m\pi}{2} \left(x^2 [2 \sec \theta + \cos \theta_{\text{eq}} \tan^2 \theta] - 8R_0^2 \right). \quad (5.33)$$

Conservation of volume. In order to express ΔF in terms of a single independent variable, we now relate θ and x using the conservation of volume of the aqueous droplets. Using the formula for the volume of a doubly-truncated spherical cap, the conservation of volume for each droplet implies:

$$\frac{4\pi}{3} R_0^3 = \frac{\pi x^3}{48 \cos^3 \theta} (9 \cos \theta - \cos 3\theta),$$

which can be solved to give a relation between θ and x :

$$\cos \theta = \left(\frac{12}{4 + r^3} \right)^{1/2}, \quad (5.34)$$

where we define $r = 4R_0/x$ for brevity. We can now find the free energy of the droplet chain as a function of mechanical extension. Substituting the statement of conservation of volume, Eq. 5.34, into the expression for ΔF , Eq. 5.33, we obtain an expression for the free energy of bilayer formation for the chain as a function of x only:

$$\Delta F = N\gamma_m\pi \left(\frac{x^2}{2\sqrt{3}} [r^3 + 4]^{1/2} + \frac{x^2}{24} \cos \theta_{\text{eq}} [r^3 - 8] - 4R_0^2 \right). \quad (5.35)$$

Effective spring constant. As before, the stiffness of the system is defined as

$$k = \frac{d^2 \Delta F}{dL^2} \bigg|_{L=L_{\text{eq}}},$$

where $L = Nx$ is the total length of the droplet chain (Fig. 5.6), and L_{eq} is the equilibrium value of L . The equilibrium value of x is found straightforwardly from Eq. 5.34 by setting $\theta = \theta_{\text{eq}}$:

$$x_{\text{eq}} = \frac{4R_0}{(12 \sec^2 \theta_{\text{eq}} - 4)^{1/3}}. \quad (5.36)$$

Taking the second derivative of Eq. 5.35, and noting that

$$\frac{d^2 \Delta F}{dL^2} = \frac{1}{N^2} \frac{d^2 \Delta F}{dx^2},$$

we find

$$\frac{d^2 \Delta F}{dL^2} = \frac{\gamma_m \pi}{N} \left(\frac{1}{\sqrt{3}} (r^3 + 4)^{1/2} - \frac{3\sqrt{3}}{8} \frac{r^6}{(r^3 + 4)^{3/2}} + \frac{1}{12} \cos \theta_{eq} (r^3 - 8) \right).$$

Substituting the value of x_{eq} into the above yields the effective spring constant of the droplet chain (Fig. 3.6a):

$$k = \frac{\gamma_m \pi}{4N} (3 \sec \theta_{eq} + 2 \cos \theta_{eq} - \cos^3 \theta_{eq}). \quad (5.37)$$

Young's modulus. In a similar way as for the isolated pair, we can calculate Young's modulus for the chain of droplets using Eqs. 5.28, 5.32, 5.36 and 5.37 (Fig. 3.6b):

$$E = \frac{\gamma_m}{4R_0} (3 \cos \theta_{eq} + 2 \cos^3 \theta_{eq} - \cos^5 \theta_{eq}) (12 \sec^2 \theta_{eq} - 4)^{1/3}. \quad (5.38)$$

Stress-strain relationship. As for the droplet pair, we can calculate the stress required to deform the droplet chain far from its equilibrium geometry. Differentiating Eq. 5.35 to obtain the force f as a function of x , and using Eqs. 5.32 and 5.34 to find an expression for the projected area $A = \pi R^2$, we obtain the stress $\sigma = f/A$:

$$\sigma = \frac{48\gamma_m}{x(r^3 + 4)} \left[\frac{1}{\sqrt{3}} (r^3 + 4)^{1/2} - \frac{\sqrt{3}}{4} r^3 (r^3 + 4)^{-1/2} - \frac{\cos \theta_{eq}}{24} (r^3 + 16) \right].$$

The stress as a function of strain is then readily calculated by using $\epsilon = (x - x_{eq})/x_{eq}$. The stress-strain relationship for a linear chain of droplets under the conditions of this work is depicted in Fig. 3.6e.

Tensile strength. We now calculate the tensile strength σ_t of the droplet chain. As for the isolated droplet pair, σ_t is given by σ at the limiting value of x beyond which the droplets separate irreversibly. Inspection of Fig. 5.6 reveals that for the droplet chain the limiting value is $x = 2R_0$, and evaluating $\sigma(x = 2R_0)$ gives

$$\sigma_t = \frac{2\gamma_m}{R_0} (1 - \cos \theta_{eq}), \quad (5.39)$$

which is identical to the result for an isolated pair of droplets.

Mechanical properties of three-dimensional droplet networks

We can perform a simple dimensional analysis to gain insight into how the above results might apply to fully three-dimensional networks. As a first approximation, we consider the network to be composed of N_{col} independent columns of droplets, each composed of N_{row} droplets. Young's modulus E is defined as kL/A , where k is here the stiffness of the network as a whole, L is the length of a single column, and A is the total cross-sectional area of all the columns. Taking the cross-sectional area of a single column as A_{col} , we have $A = N_{col}A_{col}$; similarly, $L = N_{row}L_{row}$, where L_{row} is the length of a single droplet in a column.

The spring constant of identical springs in series is inversely proportional to the number of springs in the chain, which here is N_{row} . Conversely, for identical springs in series the effective spring constant

is proportional to the number of springs in parallel, here N_{col} . Therefore assuming that a force is applied parallel to the columns, we have $k \propto N_{\text{col}}/N_{\text{row}}$, and so:

$$E \propto \frac{L_{\text{row}}}{A_{\text{col}}},$$

which from the derivations of the droplet chain can be reduced to $E \propto R_0^{-1}$. Therefore in this simple model of the network, the tensile modulus is independent of the overall size of the network and inversely proportional to the droplet radius, which is also the case for the droplet pair (Eq. 5.29) and chain (Eq. 5.38).

Upper limit on number of layers

The mechanical analysis of an isolated droplet pair allows us to estimate the requirements for a droplet network to be self-supporting. Suppose that two adjacent droplets in the bottom layer of a printed droplet network have formed a bilayer, and that a column of droplets sits above them such that its weight acts to push the droplets apart. We aim to determine the conditions under which the droplet column is capable of forcing the separation of the two lower droplets.

To make a conservative estimate, we neglect the contributions of the other bilayers in the network, which would act to prevent the connected droplets from being forced apart. Further, we assume that the force from the upper column acts on only this pair of droplets arranged as in Fig. 5.5b, whereas in a typical network geometry the force would be distributed between at least three lower droplets. Finally, we wish to find conditions under which the separation of the droplets would be thermodynamically favourable, and not necessarily those that are kinetically accessible.

For the droplet column to separate the connected pair of droplets, the gravitational potential energy lost must be greater than or equal to the free energy of adhesion for the droplet pair given in Eq. 5.21. If the aqueous column has volume V and density ρ_{drop} , and the environment has density ρ_{env} , then the condition for the droplets to remain attached is:

$$(\rho_{\text{drop}} - \rho_{\text{env}}) V g \Delta h \leq \gamma_m \pi R_0^2 \left(8 - [32 (8 - \cos 3\theta_{\text{eq}} + 9 \cos \theta_{\text{eq}})]^{1/3} \right),$$

where g is the acceleration due to gravity and Δh is the distance that the column must drop to separate the two droplets. Assuming for simplicity that $\Delta h \approx 2R_0$, and that V is a column of N vertically stacked droplets with radius R_0 , we have:

$$N \leq \frac{3\gamma_m \left(8 - [32 (8 - \cos 3\theta_{\text{eq}} + 9 \cos \theta_{\text{eq}})]^{1/3} \right)}{8gR_0^2 (\rho_{\text{drop}} - \rho_{\text{env}})}. \quad (5.40)$$

5.4 Electrical simulations of printed networks

We consider a set of N droplets, some of which are joined pairwise by bilayers in a given configuration. The bilayers may have different areas and contain any number of protein pores, and the bilayer areas and numbers of pores may vary with time. We assume that two of the N droplets, labelled a and b , are impaled by electrodes and poised at known voltages u_a and u_b , respectively. The system is modelled as depicted in Fig. 5.7, with each bilayer and any pores it contains represented by a capacitor and resistor in parallel. Given a droplet network of known connectivity, and bilayers of known conductance and capacitance, we wish to calculate the current measured by the electrodes in droplets a and b .

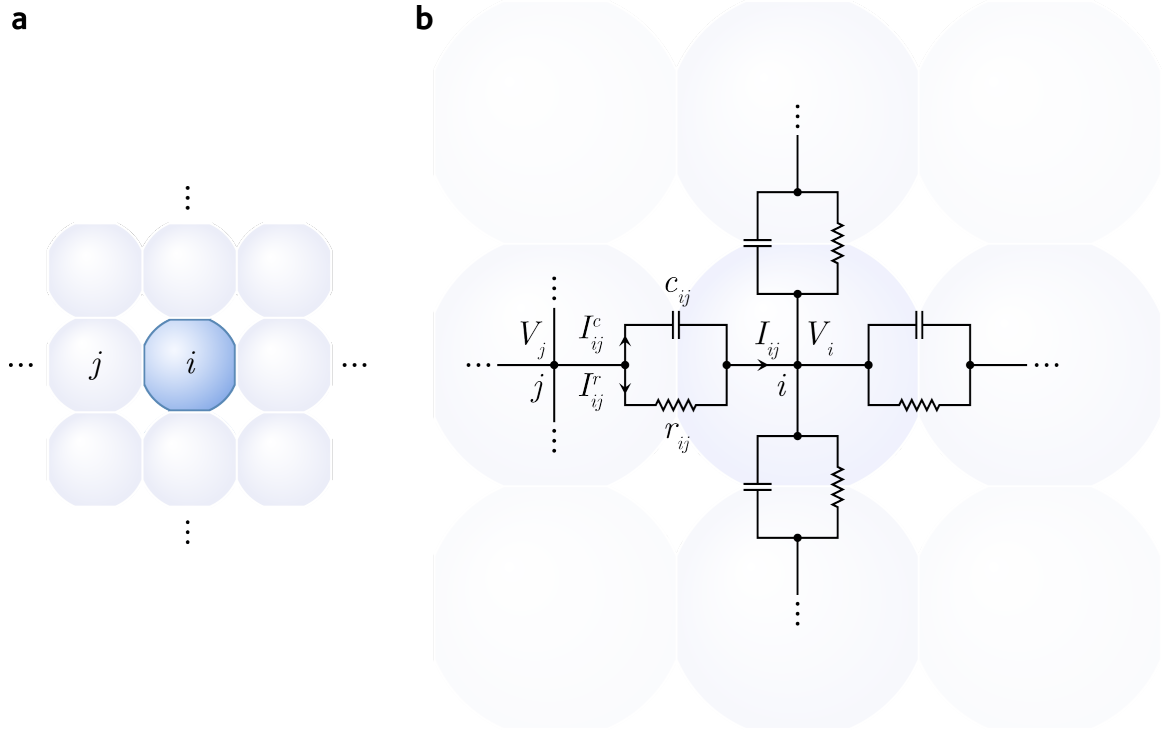


Figure 5.7 | Electrical representation of a general droplet network. (a) Schematic of a general droplet network, including two droplets labelled i and j . The droplets are depicted on a square grid only for clarity; the derivation in the text applies to a droplet network with any arrangement. (b) Electrical representation of part of the network in a. V_i and V_j are the electrical potentials at the droplets labelled i and j , respectively. c_{ij} and r_{ij} are, respectively, the capacitance and resistance between droplets i and j . I_{ij} is the ionic current flowing from j into i , and consists of a capacitive component I_{ij}^c and resistive component I_{ij}^r .

Mathematical formulation

The current flowing from droplet j into droplet i is given by $I_{ij} = I_{ij}^r + I_{ij}^c$, where:

$$\begin{aligned} I_{ij}^r &= g_{ij} (V_i - V_j); \\ I_{ij}^c &= c_{ij} (\dot{V}_i - \dot{V}_j). \end{aligned} \quad (5.41)$$

Here, V_i and V_j are the electrical potentials at the droplets i and j , $\dot{V}_i$ and $\dot{V}_j$ are the time derivatives of these potentials, $g_{ij} = 1/r_{ij}$ is the conductance between the two droplets, and c_{ij} is the capacitance between the two droplets. The net current into the i th droplet is given by the sum of the contributions from all droplets in the network:

$$I_i = \sum_{j \neq i} I_{ij}, \quad (5.42)$$

where the sum extends over the entire network and we define $g_{ij} = 0$ and $c_{ij} = 0$ if droplets i and j are not joined by a bilayer. Substituting Eqs. (5.41) into Eq. (5.42) gives

$$I_i = \sum_{j \neq i} \left(g_{ij} (V_i - V_j) + c_{ij} (\dot{V}_i - \dot{V}_j) \right),$$

which can be rearranged as

$$\begin{aligned} I_i &= V_i \sum_{j \neq i} g_{ij} - \sum_{j \neq i} g_{ij} V_j \\ &\quad + \dot{V}_i \sum_{j \neq i} c_{ij} - \sum_{j \neq i} c_{ij} \dot{V}_j. \end{aligned} \quad (5.43)$$

At this point it is convenient to define the matrices $\mathbf{G}$ and $\mathbf{C}$ as follows:

$$\begin{aligned} G_{ij} &= \begin{cases} -g_{ij}, & i \neq j, \\ \sum_{k \neq i} g_{ik}, & i = j. \end{cases} \\ C_{ij} &= \begin{cases} -c_{ij}, & i \neq j, \\ \sum_{k \neq i} c_{ik}, & i = j. \end{cases} \end{aligned}$$

With these matrices, Eq. (5.43) may be written succinctly as

$$\vec{I} = \mathbf{G}\vec{V} + \mathbf{C}\dot{\vec{V}}, \quad (5.44)$$

where $\vec{I}$ and $\vec{V}$ are N -dimensional vectors that respectively represent the current flowing into, and the voltage at, each droplet.

Finally, we assume that the droplets cannot act as sources or sinks of charge. The current flowing into each droplet is therefore zero, with the exception of the two droplets that are impaled by electrodes, which must source and sink the same current I :

$$|I_i| = \begin{cases} I, & i \in \{a, b\}, \\ 0, & \text{otherwise.} \end{cases} \quad (5.45)$$

Initial conditions

We assume that the system begins in a steady state. Then $\dot{\vec{V}} = \vec{0}$, so from Eq. (5.44) we have:

$$\mathbf{G}\vec{V} = \vec{I}. \quad (5.46)$$

To solve for $\vec{V}$, we recall that the two terminal droplets are voltage-clamped. We can therefore eliminate the two equations corresponding to droplets a and b , and use Eq. (5.45) to obtain:

$$\tilde{\mathbf{G}}\vec{V} = \vec{0}, \quad (5.47)$$

where $\tilde{\mathbf{G}}$ represents the matrix $\mathbf{G}$ without the two rows corresponding to droplets a and b .

Eq. (5.47) represents $N - 2$ equations in N variables. The two additional equations required to solve this system express the known voltages of the terminal droplets: $V_a = u_a$ and $V_b = u_b$. We include these equations by constructing a further matrix $\tilde{\mathbf{G}}'$, defined by replacing the rows in $\mathbf{G}$ that were removed to form $\tilde{\mathbf{G}}$ with the row vectors $\vec{x}$ and $\vec{y}$, respectively, defined by $x_i = \delta_{ia}$ and $y_i = \delta_{ib}$, where δ_{ij} is the Kronecker delta. $\tilde{\mathbf{G}}'$ is therefore defined as:

$$\tilde{G}'_{ij} = \begin{cases} \delta_{ai}\delta_{aj} + \delta_{bi}\delta_{bj}, & i \in \{a, b\} \\ -g_{ij}, & i \notin \{a, b\} \text{ and } i \neq j, \\ \sum_{k \neq i} g_{ik}, & i \notin \{a, b\} \text{ and } i = j. \end{cases}$$

Multiplying $\tilde{\mathbf{G}}'$ by $\vec{V}$ then gives

$$\tilde{\mathbf{G}}'\vec{V} = \vec{v},$$

where $v_i = u_a\delta_{ia} + u_b\delta_{ib}$. This system of N equations in N variables is easily solved computationally, and yields the initial voltage of each droplet. The initial current is then found straightforwardly from Eq. (5.46).

Time evolution

The time evolution of the system can be calculated in a manner similar to the initial conditions. We again begin with Eq. (5.44), and remove the two rows of $\mathbf{G}$ and $\mathbf{C}$ that correspond to droplets a and b to create $\tilde{\mathbf{G}}$ and $\tilde{\mathbf{C}}$, where $\tilde{\mathbf{C}}$ is defined analogously to $\tilde{\mathbf{G}}$. Eq. (5.44) then becomes:

$$\tilde{\mathbf{G}}\vec{V} + \tilde{\mathbf{C}}\dot{\vec{V}} = \vec{0}.$$

We then replace the two rows removed from $\tilde{\mathbf{G}}$ and $\tilde{\mathbf{C}}$ with $\vec{x}$ and $\vec{y}$ to create $\tilde{\mathbf{G}}'$ and $\tilde{\mathbf{C}}'$, where:

$$\tilde{C}'_{ij} = \begin{cases} \delta_{ai}\delta_{aj} + \delta_{bi}\delta_{bj}, & i \in \{a, b\} \\ -c_{ij}, & i \notin \{a, b\} \text{ and } i \neq j, \\ \sum_{k \neq i} c_{ik}, & i \notin \{a, b\} \text{ and } i = j. \end{cases}$$

Recalling that the voltages $V_a = u_a$ and $V_b = u_b$ are constant, we obtain:

$$\tilde{\mathbf{G}}'\vec{V} + \tilde{\mathbf{C}}'\dot{\vec{V}} = \vec{v}. \quad (5.48)$$

The evolution of $\vec{V}$ through time was calculated as follows using MATLAB's `ode45` ordinary differential equation solver:

1. At each time point t , $\mathbf{G}(t)$ is updated to reflect any changes in conductance that took place since the last time point to simulate pore insertions. We assume that there are no changes in bilayer sizes and that no new bilayers are formed, although these could be easily simulated by also updating $\mathbf{C}(t)$ at this step.
2. $\vec{V}(t)$ and the updated $\mathbf{G}(t)$ and $\mathbf{C}(t)$ are used in Eq. (5.48) to calculate $\dot{\vec{V}}(t)$.
3. $\vec{V}(t)$ and $\dot{\vec{V}}(t)$ are used to calculate the voltages at the next time point, $\vec{V}(t + \delta t)$. The interval δt is determined by the `ode45` solver.

Once $\vec{V}(t)$ has been calculated for the time period of interest, the current $I(t)$ can be found straightforwardly from Eq. (5.44).

Consistency of simulation conditions

The simulation conditions listed in Section 3.5 that created good agreement with experimental results can be verified further as follows. We use the electrical measurements to deduce first the conductance of a single α HL pore, and then the capacitance of a single droplet bilayer.

Conductance of α HL pore. The conditions stipulate that from the beginning of the measurement, α HL pores insert only into bilayers between one of the large drops, a , and the droplets on the pathway, and that there is a constant number of pores inserted into bilayers within the pathway. The system can therefore be approximately modelled as two resistors in series: one of constant resistance R_{path} that represents drop a and the droplet pathway, and one of variable resistance R_{drop} that represents the interface between the pathway and the other large drop, b . The total measured resistance is therefore

$$R_{\text{total}} = R_{\text{path}} + R_{\text{drop}}.$$

We further assume that R_{drop} can be modelled as N resistors of resistance R_{pore} in parallel, where each resistor represents a single α HL pore inserted into a bilayer between drop b and a droplet on the pathway. Using $R_{\text{drop}} = R_{\text{pore}}/N$, the above equation can be rearranged as follows:

$$R_{\text{pore}} = N [R_{\text{total}}(N) - R_{\text{path}}],$$

where $R_{\text{total}}(N)$ is the measured resistance for a given N . The constant R_{path} can be eliminated using the two simultaneous equations obtained for the cases of N and $N + 1$ pores, which give

$$R_{\text{pore}} = N(N + 1) [R_{\text{total}}(N) - R_{\text{total}}(N + 1)]. \quad (5.49)$$

Assuming that each of the five current steps in Fig. 3.10c corresponds to the insertion of a single pore, we have values of $R_{\text{total}}(N)$ for $N = \{1, 2, 3, 4, 5\}$. Therefore Eq. 5.49 with $N = \{1, 2, 3, 4\}$ yields four measures of R_{pore} and R_{path} , as quoted in Section 3.5.

Capacitance of droplet bilayer. The arrangement photographed in Fig. 3.10d can be modelled (Fig. 3.12c) by one large drop, a , connected to the droplet pathway by multiple α HL pores, and two layers of droplets without α HL that separate the pathway from the other large drop, b . The system can be modelled more simply as three electrical components in series: a resistor of resistance R_{path} that represents the pathway and drop a ; a capacitor of capacitance C_{bilayer} that represents the bilayer between two of the insulating droplets; and a variable resistor of resistance R_{pore} that represents any pores inserted into a bilayer between one of the insulating droplets and drop b (Section 3.5 and Fig. 3.12). For simplicity, we neglect the current contribution of other insulating droplets, which is expected to be small because of their low conductance compared to that of the pathway.

We denote the voltage across the resistor R_{path} by V_{path} , and similarly for the other two components. The measured current is equal to the current through each of the components:

$$I = \frac{V_{\text{path}}}{R_{\text{path}}} = C_{\text{bilayer}} \frac{dV_{\text{bilayer}}}{dt} = \frac{V_{\text{pore}}}{R_{\text{pore}}}. \quad (5.50)$$

The applied voltage V_{app} is equal to the sum of the voltages across each of the components:

$$V_{\text{app}} = V_{\text{path}} + V_{\text{bilayer}} + V_{\text{pore}}. \quad (5.51)$$

Assuming that V_{app} is constant, we can use Eqs. 5.50 and 5.51 to obtain an ordinary differential equation for one of the voltages, for example V_{path} , as a function of time:

$$\frac{dV_{\text{path}}}{dt} = -\frac{V_{\text{path}}}{(R_{\text{path}} + R_{\text{pore}}) C_{\text{bilayer}}}.$$

Therefore V_{path} decays exponentially with a time constant $\tau = (R_{\text{path}} + R_{\text{pore}}) C_{\text{bilayer}}$, and from Eqs. 5.50, so does the measured current. The value of τ can be estimated from Fig. 3.10e by assuming that each current spike corresponds to the insertion of a single pore into a bilayer that did not previously contain pores. Using the values of R_{path} and R_{pore} calculated as described in the previous section (Section 3.5), the measurements of τ can be used to estimate the value of C_{bilayer} as quoted in Section 3.5.

5.5 Water permeability of droplet bilayers

We consider a pair of droplets labelled 1 and 2, joined by a bilayer of area A . Let the initial volumes of the droplets be V_1 and V_2 , and their salt concentrations at time t be $C_1(t)$ and $C_2(t)$. The volume of water that flows per unit time from droplet 1 to droplet 2 across the bilayer can be expressed as⁶⁷

$$-\frac{dV_1}{dt} = iPA\bar{V}\phi(C_2(t) - C_1(t)), \quad (5.52)$$

where P is the permeability coefficient (with dimensions of length per unit time), $\bar{V}$ is the molar volume of water, ϕ is the osmotic coefficient, and i is the van 't Hoff factor of the salt. Equation (5.52) suggests a simple way to estimate the permeability P from experimental measurements:

$$P = [iA\bar{V}\phi(C_1(0) - C_2(0))]^{-1} \left. \frac{dV_1}{dt} \right|_{t=0}, \quad (5.53)$$

where $C_1(0)$ and $C_2(0)$ are the initial values of C_1 and C_2 , respectively. Because the volume of water is conserved, we may equivalently write:

$$P = [iA\bar{V}\phi(C_2(0) - C_1(0))]^{-1} \left. \frac{dV_2}{dt} \right|_{t=0}. \quad (5.54)$$

Single droplets containing 1 M KCl were made to form bilayers pairwise with droplets containing 250 mM KCl, and three such pairs were photographed through a microscope at intervals of 2 min over >2 h (Fig. 3.13). The droplet and bilayer diameters were measured using the IMAGEJ software package, and the volumes of the droplets calculated from these diameters by assuming that each droplet had the geometry of a spherical cap. The initial rates of change of V_1 and V_2 for each pair were calculated independently from the first two volume measurements. The permeability was then calculated according to Eq. (5.53) or Eq. (5.54) as $27 \pm 5 \mu\text{m s}^{-1}$ (mean $\pm$ s.d., $n = 6$), assuming $\bar{V} = 18.0 \text{ ml mol}^{-1}$, $i = 2$ for KCl, and $\phi = 0.90$ from the literature¹³⁷.

5.6 Model of folding droplet networks

Formulation of the model

The qualitative behaviour of spontaneously folding networks can be reproduced by a simple model that consists of two coupled components: a mechanical part, which models the motion of droplets in

a network, and an osmotic part, which models the transfer of water between droplets.

Mechanical component. Droplets are treated as point masses with an associated radius. If a pair of droplets of radii R_i and R_j approach each other within a distance $R_i + R_j$, they become connected by a Hookean spring of natural length L ($R_i + R_j$). The spring represents the bilayer formed between the two droplets, and the parameter $L < 1$ approximates the deformation of the droplets caused by their adhesion.

Given that the characteristic timescale of folding in our experiments was on the order of minutes to hours, we estimate the Reynolds number during folding to be on the order of 10^{-5} . Droplets may therefore be expected to obey Stokes' law, and we impose a drag force on each droplet that is proportional to its velocity. The net force on each droplet i is therefore given by

$$\vec{F}_i = \sum_{j \in C_i} k(r_{ij} - L) \hat{\vec{r}}_{ij} - \gamma \vec{v}_i, \quad (5.55)$$

where C_i is defined as the set of droplets connected to the i th droplet, $\vec{r}_{ij}$ is the vector from droplet i to droplet j with magnitude r_{ij} , k is the spring constant, γ is a damping coefficient, and v_i is the velocity of the i th droplet. The position of each droplet i through time, $\vec{r}_i(t)$, is then calculated using

$$\vec{F}_i = m \frac{d^2 \vec{r}_i}{dt^2}, \quad (5.56)$$

where m is the mass of the droplet. For reasons discussed below, we assume that the mass, spring constant and damping coefficients are constant and identical for all droplets.

Osmotic component. The osmotic component simulates the transfer of water between droplets of different osmolarities according to Fick's first law. The volume of water transferred per unit time from a droplet i with osmolarity C_i to a droplet j with osmolarity C_j was calculated as

$$J_{ij} = A_{ij} D (C_j - C_i), \quad (5.57)$$

where A_{ij} is the area of the bilayer between the two droplets, and the parameter D represents a permeability coefficient that was assumed to be constant and identical for all bilayers.

Parameter values

Table 5.1 lists the dimensionless values of the parameters used in the model. The values of D , m and k were chosen to make the timescale of water transfer slow compared to the timescale of mechanical relaxation, because the experimentally observed timescales for these processes were, respectively, tens of minutes and a few seconds. The value of γ was chosen to prevent oscillations of the springs while maintaining a relatively short mechanical relaxation time, because droplets are not experimentally observed to oscillate upon adhesion. C_{low} and C_{high} represent the lower and higher osmolarities of the droplets in the network. The ratio $C_{\text{high}}/C_{\text{low}}$ was chosen to approximate the experimental ratio of osmolarities. The remaining variables were chosen to reduce the computation time while maintaining numerical stability. Because the timescales of mechanical relaxation and water transfer were chosen so that folding takes place in a mechanical quasi-equilibrium, the simplifying assumption of identical and constant values of m and γ for all droplets, and of k for all bilayers, is not expected to have a significant effect on the simulations.

Parameter	Symbol	Value
Rate of water transfer	D	2×10^{-3}
Lower osmolarity	C_{low}	0.067 (Fig. 3.16b) 0.1 (Fig. 3.17c)
Higher osmolarity	C_{high}	1
Initial droplet volume	V_0	10
Droplet mass	m	0.2
Spring constant	k	10^3
Time step	Δt	10^{-2}
Damping	γ	1.1

Table 5.1 | Parameters used in folding model. The parameters D , m and k were chosen to make water transfer take place on a much longer timescale than mechanical equilibration of the network. The value of γ was chosen to prevent oscillations of the springs. $C_{\text{high}}/C_{\text{low}}$ was chosen to reflect the experimental ratio of osmolarities between the two types of droplets. $C_{\text{high}} - C_{\text{low}}$, Δt and V_0 were chosen to reduce the computation time while maintaining numerical stability.

Solving the equations

A computer program was written to simulate the behaviour of the system as follows. The program reads a series of images that define the network to be simulated, similarly to the printing program. The droplets are initially positioned according to the images in a hexagonal close-packed arrangement, which approximates the observed packing of droplets in printed networks. The droplets are then allowed to equilibrate mechanically without exchanging water, to ensure that any motion subsequently simulated is not due to mechanical equilibration of the droplets from their initial arrangement. Once this equilibration is complete, the osmotic component of the model is activated. Figs. 3.16b and 3.17c do not show the initial stage in which the network is allowed to equilibrate mechanically without water transfer. At each time point t , the program performs the following calculations:

1. Any two droplets i and j that have come within a distance $R_i + R_j$ are connected by a spring of natural length $L(R_i + R_j)$.
2. If the osmotic component is active, the volume of water transferred between each pair of droplets joined by a bilayer is calculated according to Eq. (5.57), and the size of each droplet is updated accordingly.
3. The position of each droplet at time $t + \Delta t$ is calculated according to Eqs. (5.55) and (5.56) using a fourth-order Runge-Kutta scheme.
4. The position of each droplet is updated according to the results of the calculations in step 3.

Visualization

The simulation program was also made to show the time evolution of the system. The droplets and the bilayers in a folding network could be visualized separately as described below.

Droplets. Each droplet in a simulated network was visualized as a sphere of the same volume as the droplet. Each droplet was coloured according to its osmolarity as described in Figs. 3.16b, 3.17c. The simulated network in Fig. 3.17c was rendered using the Visual Molecular Dynamics software package¹³⁸ by exporting the values for the position and osmolarity of every droplet at each time point to a text file in the appropriate format.

Bilayers. A simulated droplet network could also be visualized in terms of its bilayers. The bilayer between each connected pair of droplets was represented by a straight line that joined the centres of the two droplets. This visualization was combined with a representation of the droplets in the network to produce Fig. 3.16c.

5.7 Energetics of folding droplet networks

We first consider the free energy change of mixing a solute into a solvent, in our case KCl and water, respectively. For an ideal solution the free energy of mixing is purely entropic, and is given by

$$\Delta F_{\text{mix}}(x) = nRT [x \ln(x) + (1-x) \ln(1-x)],$$

where n is the amount of solute and x is the mole fraction of solute. Here $x = c/c_w$, where c is the concentration of solute and $c_w = 55.5 \text{ M}$ is the concentration of water. Therefore the amount of solute is $n = xc_w V$, where V is the volume of the solution. Combining this with the above expression, we obtain:

$$\Delta F_{\text{mix}}(x, V) = xVc_w RT [x \ln(x) + (1-x) \ln(1-x)]. \quad (5.58)$$

We now wish to calculate the change in free energy when two compartments that contain such solutions exchange water by osmosis. Each compartment may represent a single droplet or a collection of droplets within a droplet network. If the initial mole fractions in the two compartments are denoted by x_1 and x_2 and the volumes are V_1 and V_2 , and the corresponding final values are denoted by primed variables, then the overall free energy change is given by

$$\Delta F_{\text{osmosis}} = \Delta F_{\text{mix}}(x'_1, V'_1) + \Delta F_{\text{mix}}(x'_2, V'_2) - [\Delta F_{\text{mix}}(x_1, V_1) + \Delta F_{\text{mix}}(x_2, V_2)]. \quad (5.59)$$

Using the conservation of volume, and the condition that $x'_1 = x'_2$ at equilibrium, we obtain V'_1 and V'_2 in terms of known quantities:

$$\begin{aligned} V'_1 &= (V_1 + V_2) \left(1 + \frac{x_2 V_2}{x_1 V_1} \right)^{-1}; \\ V'_2 &= (V_1 + V_2) \left(1 + \frac{x_1 V_1}{x_2 V_2} \right)^{-1}. \end{aligned} \quad (5.60)$$

In the case of droplet interface bilayers the solute does not travel between compartments, so the final solute mole fractions are simply given by $x'_1 = x_1 V_1 / V'_1$ and $x'_2 = x_2 V_2 / V'_2$. Equations 5.58, 5.59 and 5.60 then allow the calculation of $\Delta F_{\text{osmosis}}$ for known initial mole fractions and volumes.

If x is small compared to unity and $V_1 = V_2 = V$, then Eq. 5.59 reduces to:

$$\Delta F_{\text{osmosis}} = Vc_w RT \bar{x}^2 \left[2(\ln(\bar{x}) - 1) - \left(\frac{x_1}{\bar{x}} \right)^2 (\ln(x_1) - 1) - \left(\frac{x_2}{\bar{x}} \right)^2 (\ln(x_2) - 1) \right],$$

where $\bar{x} = (x_1 + x_2)/2$ is the average solute mole fraction. It is evident that, for a given ratio x_1/x_2 (and therefore also $x_1/\bar{x}$ and $x_2/\bar{x}$), the free energy available from osmosis is approximately proportional to $\bar{x}^2$. For the folding networks presented in Ch. 3, $V \approx 200 \text{ nL}$, $T \approx 293 \text{ K}$, $x_1 \approx 3.5 \times 10^{-3}$ and $x_2 \approx 2 \times 10^{-4}$, so that $\Delta F_{\text{osmosis}} \approx 860 \text{ nJ}$. By comparison, the energy required to lift the centre of mass of such a network against gravity by 1 mm is only $\sim 220 \text{ pJ}$.

5.8 Lipid recruitment by folding networks

In this section we estimate the amount of lipid required by a swelling droplet in a folding network, and the timescale for diffusion of the lipid through the oil phase.

Lipid adsorption

We assume that a droplet of initial radius r_i and radius r_f after folding exposes a fraction of its surface f as a monolayer, and bilayers with other droplets with the rest of its surface. We assume for simplicity that the droplet is spherical, and that f remains constant during folding. If each lipid molecule occupies an area A_{lipid} in the monolayer, then the number of lipid molecules N that must adsorb onto the monolayer during folding to maintain a constant surface density of lipids is

$$N = \frac{4f\pi(r_f^2 - r_i^2)}{A_{\text{lipid}}}. \quad (5.61)$$

If the oil contains lipid at number density C_n , then the minimum volume of oil required to supply N lipid molecules is $V = N/C_n$. The number density is related to the mass density C_m by $C_n = C_m/m_w$, where m_w is the molecular mass of the lipid, so that $V = Nm_w/C_m$. Finally, to compare V to the droplet size, we consider a cube of side $L = V^{1/3}$. Using Eq. 5.61 we obtain the size of the cube:

$$L = \left[\frac{4f\pi(r_f^2 - r_i^2)m_w}{A_{\text{lipid}}C_m} \right]^{1/3}.$$

From Fig. 3.17 we estimate $r_i = 25 \mu\text{m}$ and $r_f = 37 \mu\text{m}$. With $m_w = 846 \text{ u}$ (ref. 139), $A_{\text{lipid}} = 69 \text{ \AA}^2$ (ref. 55) and $C_m = 0.5 \text{ mg ml}^{-1}$ (Ch. 4), we obtain $L \approx f^{1/3} \times 40 \mu\text{m}$. From Fig. 3.8 we estimate $f \approx 0.1$, so that $L \approx 20 \mu\text{m}$.

Lipid diffusion

The timescale t for diffusion in three dimensions over a length scale L is $t = L^2/6D$, where D is here the diffusion coefficient of the lipid in oil. The diffusion coefficient of phosphatidylcholines in n -heptane is $\sim 1.5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ (ref. 140), and n -heptane has a viscosity of 0.40 mPa s (ref. 141). The oil used in our experiments was an equal mixture of hexadecane and silicone oil (Ch. 4). Assuming that the viscosity of the mixture is the average of the viscosities of the two components, our oil has a viscosity of 12 mPa s . The Stokes-Einstein equation states that D is inversely proportional to the viscosity of the fluid, so that we estimate the diffusion coefficient of DPhPC in our oil to be $D \approx 5 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$. For diffusion over half the thickness of the droplet network in Fig. 3.17a, $L \approx 120 \mu\text{m}$, so that the diffusion timescale is $\sim 8 \text{ min}$.

5.9 Appendix

Buoyancy compared to interfacial tension

In this section we use scaling arguments to show that for the aqueous droplets in the multisomes presented in Ch. 2, buoyancy effects are negligible compared to the effects of interfacial tension. We

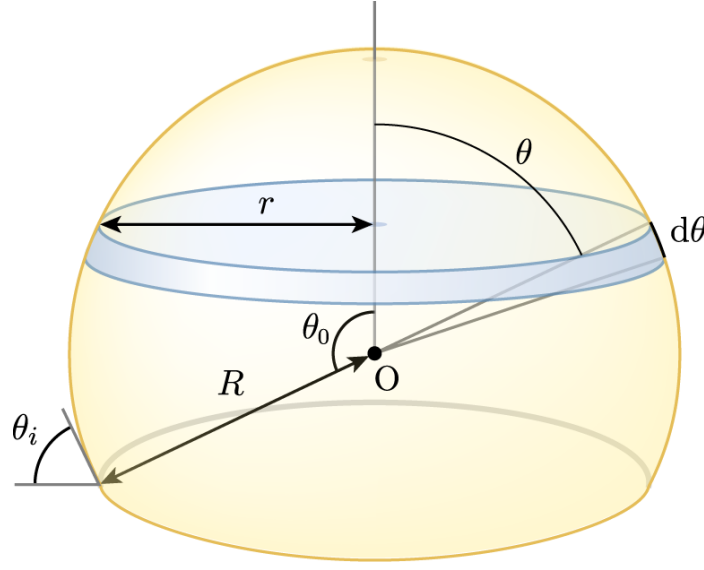


Figure 5.8 | Spherical coordinate system. Geometrical definitions for the calculation of the curved area and volume of a spherical cap of radius R .

also show that buoyancy forces are significant, however, for the oil drops used to print encapsulated droplet networks as presented in Ch. 3.

A characteristic value for the ratio of buoyancy forces to interfacial tension forces can be calculated from scaling arguments as the dimensionless Bond number¹⁴²:

$$\text{Bo} = \frac{gL^2\Delta\rho}{\gamma},$$

where $g = 9.81 \text{ m s}^{-2}$ is the acceleration due to gravity, L is a characteristic length scale, $\Delta\rho$ is the difference in density between the two phases and γ is the interfacial tension between the phases. For an aqueous droplet in a multisome as in Ch. 2, we may take $L \approx 200 \text{ }\mu\text{m}$ as the radius of the droplet, and the interfacial tension as $\gamma = \gamma_m \approx 1 \text{ mN m}^{-1}$. The density of the aqueous phase is $\sim 1.05 \text{ g cm}^{-3}$ and that of the oil mixture is $\sim 0.98 \text{ g cm}^{-3}$, so that $\Delta\rho \approx 0.07 \text{ g cm}^{-3}$, which yields $\text{Bo} \approx 0.03$. That $\text{Bo} \ll 1$ in this case indicates that buoyancy forces on the aqueous droplet are negligible compared to interfacial tension forces.

In contrast, for the oil drops used to print encapsulated networks in bulk aqueous solution as in Ch. 3, we have $L \approx 500 \text{ }\mu\text{m}$ as the radius of the oil drop, and the density of the oil mixture is $\sim 0.89 \text{ g cm}^{-3}$, so that $\Delta\rho \approx 0.16 \text{ g cm}^{-3}$, which gives $\text{Bo} \approx 0.4$. In accordance with the experimental observations in Ch. 3, that Bo is in this case comparable to unity indicates that buoyancy forces on the oil drop are significant compared to interfacial tension forces.

Area of a spherical cap

Consider a spherical cap, or truncated sphere, of radius R (Fig. 5.8); we wish to find the area of its curved surface. To do this, we first find the curved surface area of the volume element shown in blue in the figure. A horizontal disc whose circumference is at an angle θ from the vertical axis has radius $r = R \sin \theta$, so that the circumference of the volume element is $2\pi R \sin \theta$. An arc on the sphere perpendicular to this circumference, subtending an infinitesimal angle $d\theta$ at the origin, has length $R d\theta$. The curved area of the volume element is simply the product of these two lengths,

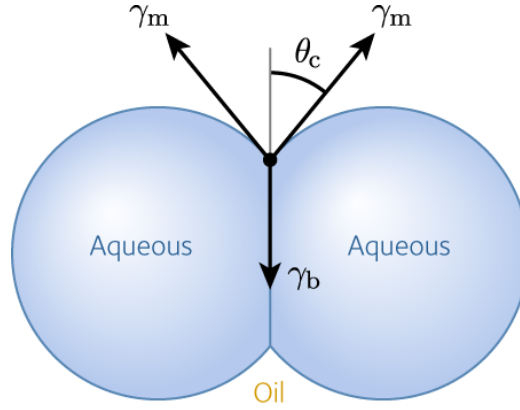


Figure 5.9 | Interfacial tensions in a droplet interface bilayer. Interfacial tensions acting on a short line segment at the interface between the oil phase and the two aqueous droplets. θ_{eq} is the droplet interface bilayer contact angle at equilibrium.

$2\pi R^2 \sin \theta d\theta$. The area of the entire curved surface of the spherical cap is then the integral of this area element over the range of θ subtended by the truncated sphere:

$$A = \int_0^{\theta_0} 2\pi R^2 \sin \theta d\theta = 2\pi R^2 (1 - \cos \theta_0).$$

As shown in Fig. 5.8, the contact angles θ_i defined in Fig. 5.1a are related to θ_0 by $\theta_i = \pi - \theta_0$, so that the curved surface area of a spherical cap with contact angle θ_i and radius of curvature r_i is:

$$A_i = 2\pi r_i^2 (1 + \cos \theta_i). \quad (5.62)$$

Contact angle in a droplet interface bilayer in bulk oil

Interfacial tension can be interpreted as a force per unit length that acts in the plane of an interface. Consider the circle around a droplet interface bilayer in bulk oil, defined by the region where the two aqueous phases simultaneously meet the oil phase and each other. The sum of interfacial tensions acting at each infinitesimal line element on this circle must balance at equilibrium¹⁴³. Referring to Fig. 5.9, balancing the vertical components of the monolayer and bilayer interfacial tensions gives:

$$\gamma_b = 2\gamma_m \cos \theta_{eq}. \quad (5.63)$$

The ratio of the bilayer and monolayer interfacial tensions can therefore be expressed in terms of the easily measured contact angle of a droplet interface bilayer in bulk oil.

Volume of a spherical cap

Here we wish to find the volume of the truncated sphere shown in Fig. 5.8, and we use the volume element shown in blue as the element of integration. From the calculation of the area of a spherical cap, we know that the circular end of the volume element has area $\pi r^2 = \pi R^2 \sin^2 \theta$. It can be shown that the infinitesimal height of the volume element is $R \sin \theta d\theta$. The volume of the element is simply the product of the circular area and the infinitesimal height: $\pi R^3 \sin^3 \theta d\theta$. The volume of the truncated sphere is then given by the following integral:

$$V = \int_0^{\theta_0} \pi R^3 \sin^3 \theta d\theta = \frac{\pi R^3}{12} (8 + \cos 3\theta_0 - 9 \cos \theta_0).$$

Expressed in terms of the parameters defined in Fig. 5.1, the volume of a truncated sphere with contact angle θ_i and radius of curvature r_i is therefore:

$$V_i = \frac{\pi r_i^3}{12} (8 - \cos 3\theta_i + 9 \cos \theta_i). \quad (5.64)$$



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