Challenges and opportunities in achieving the full potential of droplet interface bilayers

Elanna B. Stephenson, Jaime L. Korner & Katherine S. Elvira

Challenges and opportunities in achieving the full potential of droplet interface bilayers

Elanna B. Stephenson‡, Jaime L. Korner‡, and Katherine S. Elvira

Department of Chemistry, University of Victoria, Canada

Abstract

Model membranes can be used to elucidate the intricacies of the chemical processes that occur in cell membranes, but the perfectly biomimetic, yet bespoke, model membrane has yet to be built. Droplet interface bilayers (DIBs) are a new type of model membrane that are able to mimic some features of real cell membranes better than traditional models such as liposomes and black lipid membranes. In this perspective we discuss recent work in the field that is starting to showcase the potential of these model membranes to enable the quantification of membrane processes, such as the behaviour of protein transporters and the prediction of in vivo drug movement, and their use as scaffolds for electrophysiological measurements. We also highlight the challenges remaining to enable DIBs to achieve their full potential as artificial cells, and as biological analytical platforms for quantifying molecular transport.

Unanswered questions about the chemistry of fundamental biological mechanisms often drive the development of new analytical techniques. One such example is the use of droplet interface bilayers (DIBs) as tools for the analysis and quantification of membrane processes. DIBs are a type of artificial bilayer created by amphiphilic molecules at the interface between two aqueous droplets in a surrounding immiscible (usually oil) phase (Figure 1A). Each droplet in a DIB is effectively a compartment on either side of the artificial bilayer, which can hence be used to quantify molecular transport across the bilayer.

From a classical perspective, DIBs can be said to provide answers to many of the shortcomings of other model membrane systems, such as liposomes (Figure 1B) and black lipid membranes (BLMs) (Figure 1C). DIBs are easier to make, their formation can be automated using microfluidic technologies, they can be arranged to create complex networks that exhibit emergent properties similar to those found in cells and tissues [1] and can be made with volumes similar to those of some human cells [2, 3]. They also have the potential to mimic features of real cell membranes such as asymmetry [4, 5] and curvature [6]. However, to the detriment of the field, DIBs have generally been made from one type of synthetic phospholipid (1,2-diphytanoyl-sn-glycero-3-phosphocholine, DPhPC) [7] and used mostly to study the transport of fluorescent dyes passively or through membrane pores such as α-hemolysin [7], alamethicin [8], gramicidin [9] and bacteriorhodopsin [10].

A quantitative analysis of all DIB publications shows that research into the formation and use of DIBs has been growing steadily over the last 15 years. Early work focused on the formation of DIBs using almost exclusively DPhPC [11, 12] and the use of electronic measurements to study the pore forming proteins and peptides alamethicin [13, 14] and α-hemolysin [15, 16]. This emphasis on electronic studies continued through the late 2000s and early 2010s with the insertion of the first ion channels [17] and pore forming antibiotics [18]. Another trend that arises from this analysis is that the type of analytical techniques available for use with DIBs guides the field. For example, the use of fluorescence or spectroscopic detection methods has an inverse relationship with electronic studies during this period: a drop-off in the insertion of new pores and channels following 2011 seems to align with a relative explosion in publications using fluorescence for quantification, primarily to studying molecular diffusion across these model membranes [19, 20]. Later in the 2010s, more exotic compounds such as membrane transport proteins [21] and mechanoselective ion channels [22, 23] were inserted into DIBs, with both electrical and fluorescent methods used for quantification of

molecular transport. Another trend seen since 2008 is the use of different lipids for DIB formation, such as cholesterol [24, 25]. We believe interest in this area will continue to grow as a result of a greater focus on biomimeticity, which shows an increasing interest in these model membranes as artificial cells. It is notable that the proportion of publications using microfluidic techniques [26, 27, 28] for DIB formation has remained constant. We think that this is primarily due to difficulties associated with developing and using microfluidic technologies in laboratories that lack the facilities and equipment for doing so, an issue often referred to as the "chip-in-a-lab" problem.

In the first review of the field [18], the authors predicted that DIBs would be used to facilitate precise membrane measurements to answer fundamental biophysical questions about membrane proteins, lipid diffusion and lipid flip-flop, and that asymmetric DIBs had not reached their full potential. In this perspective, we will discuss the progress that has taken place since then and highlight the major challenges facing the field, including the limitations associated with microfluidic methods for the formation of DIBs, how the lack of suitable detection techniques for quantifying molecular transport within DIBs is limiting their application in more diverse fields, and how to increase the biomimetic nature of DIBs. We will also explore what the future holds for DIBs.

Designing and building DIBs as model biomembranes

The ultimate goal when developing model membranes is to build a fully biomimetic bilayer from the bottom up. However, cell membranes are complex entities, and it is not always possible to determine how molecules interact with and pass through these membranes. Hence, being able to design and build model membranes that mimic key features present in cell biology provides a versatile and modular tool to study in vivo processes. Bespoke model membranes can be designed to highlight specific components of the membrane, from the molecular composition to the membrane proteins, which, while mimicking those found in nature, enable these components to be studied in biomimetic, but more controlled, environments.

Traditional model membrane systems can be broadly categorised into planar or curved. BLMs are the earliest example of planar model membranes [30], but some of their inherent characteristics have limited widespread adoption of these systems. BLMs are short lived and unstable [31], which means that they are mostly used to study fast processes carried out by ion channels and pores [31, 32]. Forming BLMs is also a particularly delicate process [32], requiring a high degree of skill that is not compatible with high throughput experimentation. Because they are planar bilayers formed between aqueous wells, BLMs fail to capture features of cellular membranes such as membrane curvature, and the vastly different ratio of cellular volume to bilayer surface area. Liposomes are the most commonly used curved model membranes and are relatively straightforward to make compared to BLMs, leading to their widespread adoption as model membranes for studying lipid interactions [33]. However, achieving consistency in terms of size and lipid distribution can be difficult, as can be modelling features of cellular membranes such as the asymmetric molecular composition of each of the monolayers that form the bilayer in cell membranes. DIBs represent a third category of model membranes, which have advantages associated with planar membranes, such as the ease of compartmentalisation of the fluid on each side of the membrane, and advantages associated with curved model membranes, such as the formation of asymmetric bilayers and consequential inducement of membrane curvature [5].

Manufacturing DIBs using microfluidic methods

Microfluidic technologies allow unprecedented control over how DIB systems are built and interrogated. Microfluidic technologies are designed to enable accurate control of fluids on the micron scale, for example to create aqueous droplets of volumes similar to the size of cells, which can then be used to build biomimetic DIBs. Over the last decade, around a third of publications have used microfluidic methods for DIB formation, highlighting a significant shift in the field since the first high-throughput method was published in 2010 [26]. Microfluidic technologies have enabled the creation of artificial tissues-on-a-chip, where multiple DIBs are arranged into interconnected networks, by refining control over droplet and bilayer size and their precise arrangement in the network [34, 35, 36, 37] and can hence be used for the measurement of passive molecular movement across the membranes [20, 29]. These applications require the isolation of distinct chemical

environments in separate droplets which is enabled by the highly tunable nature of microfluidic platforms, and showcase properties of DIBs that are not possible with other types of model membranes.

There are several key designs of on-chip features that allow this level of control and are consistently used in the field. The introduction of meanders [35] allows time for lipids to self-assemble as monolayers on the droplet surface before DIB formation, bypassing the long equilibration times (minutes) required when using manual micromanipulators [18]. This means that whereas manual methods for DIB formation are usually limited to tens of DIBs, microfluidic methods can generate hundreds or even thousands of DIBs per minute [26]. Droplet trapping geometries such as pillars [20] and rails [35] are commonly used to guide droplets to exact locations and hence provide control over the exact composition of DIB networks (Figure 1D and E). However, DIB formation techniques are far from homogeneous and more esoteric microfluidic DIB formation strategies include bilayers formed by solvent evaporation [7], flow-guiding [35], hydrodynamic traps [36, 38], "passive" droplet guidance that relies solely on on-chip architectural features [3, 27, 29], and "active" droplet guidance which additionally requires the use of off-chip features such as valves to control droplets [37].

Molecular design of DIBs

From a molecular perspective, the design of the membrane fundamentally limits the type of assays that can be performed in the DIB, both from the perspective of the molecules used to form the bilayer and the oil phase used to support the amphiphilic molecules prior to DIB formation. So far, DIBs have largely been built using single synthetic phospholipids, mainly DPhPC and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and are hence not very biomimetic. The few exceptions include polymers [39, 40], and more biomimetic bespoke lipid formulations [3, 41] and total lipid extracts [41, 42, 43, 44]. Other components of cell membranes, including neutral lipids such as cholesterol and monoglycerides, have also been used along with phospholipids for DIB formation [24, 41, 45, 46, 47, 48, 49]. The chemical structure of phospholipids is a factor in membrane permeability, with both acyl chain and polar head group type influencing bilayer packing and permeability [50, 51]. In biological systems, lipid packing depends partially on the relative space occupied by the head group and acyl chains [52, 53] (Figure 1F). Lipids with similarly sized head groups and acyl chains have an overall cylindrical shape and pack together as flat bilayers. Lipids with head groups that are much larger than the space occupied by the acyl chains form a cone shape that creates curvature when packing. Finally, lipids with small head groups are the opposite, where the cone is inverted because the head groups are much smaller than the splayed tails. These structural complexities create differences in molecular packing which are only recently beginning to be understood [50]. It is also important to note that bilayer formation in cells in not just dependent on self-assembly, but is also reliant on cellular machinery that is not currently present in these types of artificial cells.

From an energetic perspective, DIBs present a non-biomimetic system where the tail groups of the lipids are in a supportive oil phase, rather than in fully aqueous biological systems. This means that there needs to be an energetic advantage to forming a bilayer in a DIB [54] and this is dependent on the molecular composition of the lipids used to form the membrane. In fact, the length of the lipid tails has an effect on how likely the droplets are to adhere to each other and form a DIB [55]. Lipids available for use in building model membranes are either synthesised directly or extracted from natural sources, which leads to differences in the molecular composition of the reagents. Synthetic lipids are formed from molecules with the same head groups and acyl chains, and usually have high degrees of purity. Naturally derived lipids have been extracted from a natural source (mostly egg or animal tissues) and purified to ensure that all molecules present in the sample have the same head group. However, the sample contains a range of acyl chains lengths and degrees and location of unsaturation. The most biomimetic lipid source are total lipid extracts, where all head groups (and hence acyl chains) from a natural source are extracted. Both of the naturally derived lipids may also include other molecules present in cell membranes, such as polyunsaturated fatty acids.

The oil in which the aqueous droplets are formed also plays an important role in the energetic favourability of DIB formation, though this has been less studied. The oil must both support the self assembly of the monolayer covering the droplets by supporting the lipid tails and, as the DIB forms, be easily removed from the bilayer. Hexadecane in the most commonly used oil for DIB formation, though it is likely to stay in the membrane and hence cause unrealistically thick model membranes [56]. Squalene is likely to be excluded

completely from the membrane [57] and is therefore a more attractive choice for biomimetic model membrane formation. Hence, the choice of the lipids (and of the supporting oil) used to form DIBs has a direct effect on the degree of biomimicry of the model membrane.

However, being able to make DIBs from either bespoke naturally derived lipid formulations or biomimetic total lipid extracts is a key step for DIBs to fulfil their potential as an elegant model for complex cell membranes. Controlled heating of the aqueous droplets has been shown to facilitate complete monolayer self-assembly and encourage DIB formation [3, 41, 42]. We have also recently shown that the lipid-out method, where lipids are dosed in the oil phase, allows DIB formation at physiological temperatures with naturally derived lipids [3], whereas when the same lipids are dosed in the aqueous phase (lipid-in) higher temperatures are required to enable DIB formation. Hence, temperature is another factor that can affect packing dynamics in DIBs by allowing the molecules to rearrange themselves to accommodate their neighbours [51]. Generally, the lipid-out method is slower to form a monolayer because of the high energy required for inverse micelles to form a monolayer due to tail-tail interactions. Repulsive interactions between micelles in the water phase favours monolayer formation when using the lipid-in method. However, this does not hold for naturally derived lipids, where heating is required to overcome this energetic barrier.

DIBs allow the interplay between the molecular components of membranes and their biological behaviour to be elucidated. Fundamental research into water permeability across DIBs provides insight into the role that key lipid types play in membrane permeability. DIBs built between two droplets containing a NaCl concentration gradient allowed the observation of osmotic transport of water across the membrane using microscopy [45, 46, 47, 58] and Raman microspectroscopy [59]. By designing lipid formulations containing precise amounts of membrane components such as cholesterol [45], cholesterol sulphate [58], monoglycerides with varying acyl chain lengths and saturation levels [45, 46, 47] and asymmetric DIBs [58], it is possible to isolate the effects these wide-ranging membrane properties have on water permeability. For example, increasing monoglyceride unsaturation is correlated with increasing water permeability across the DIB [45, 46, 47] and addition of cholesterol is correlated with decreasing permeability [45, 47]. This is because unsaturated lipids have a cis bond which allows for looser packing. Cholesterol itself actually sits inside the bilayer causing the lipid tails to agglomerate around it and hence packing becomes tighter making the membrane less permeable [24]. Increasing monoglyceride acyl chain length, which leads to creation of thicker bilayers, is also correlated with decreasing water permeability [46]. Addition of cholesterol sulphate to a DPhPC bilayer correlates with decreasing permeability, while addition of this molecule to only one leaflet of the DIB creates an intermediate condition [58]. In fact, DIBs have enabled the investigation of the effect that cholesterol has on the formation of lipid rafts, which are not readily visible in other systems [49]. It is exciting to see that the ease with which both symmetric and asymmetric DIBs can be formed from bespoke lipid formulations means that they are used to precisely quantify how molecular transport across membranes is affected by changes in lipid composition.

DIBs to quantify molecular kinetics

DIBs represent a simple method to quantify molecular transport across an artificial bilayer because the droplets on either side of the bilayer serve as donor and acceptor compartments, with volumes small enough to enable detection at low concentrations and the potential to perform parallelised assays. This is one of the major advantages of DIBs when compared to other model membranes for chemical analysis. However, this has not been exploited to its full advantage and most research has been performed using dyes such as fluorescein [3, 20, 26, 29], carboxyfluorescein [60], calcein [22, 29] and resorufin [43, 61]. In fact, diffusion of fluorescein is regularly used in place of capacitance measurements [62] to verify that contacted droplets have formed a DIB, especially within microfluidic devices.

An interesting example shows the translocation of large molecules across a DIB with the horseradish peroxidase enzyme as a detector using the cell penetrating peptide Pep-1 [19]. Translocation across the bilayer was verified by merging the acceptor droplet with a droplet of fluorogenic substrate, demonstrating the possible utility of this technique for droplet sized fluorescence assays. Furthermore, by treating multiple encapsulated DIBs as a reactor unit, it is possible to use these “multisomes” as self-contained chemical

reactors for the synthesis fluorescent reporters (Figure 2 A and B) [28, 63] or to mimic how cells process external chemical signals [64]. This type of in situ modular processing opens the door to performing multi-step synthetic chemistry in biologically relevant environments, for example for the synthesis of drugs close to their membrane-bound targets and the study of membrane-bound signalling pathways. Since DIBs can be used to easily form asymmetric bilayers, it is possible to use these model membranes to study the effect that membrane asymmetry has on molecular transport and hence the design of intracellular drug delivery methods [65].

From a pharmacological perspective, early work showing the diffusion of caffeine in DIBs showed their potential to become an alternative to parallel artificial membrane permeability assays (PAMPA) [20], which are a commercial in vitro platform used in drug discovery to predict the ability of drugs to cross cell membranes in vivo. There is no true model membrane in PAMPA, instead, a plastic filter placed in a well and infused with lipids is used to quantify molecular transport. This work is also interesting because it addresses another current limitation of DIB research, namely the over reliance on fluorescence microscopy for detection, and instead uses UV microspectroscopy to monitor molecular movement. We have recently demonstrated that DIBs more accurately predict apparent permeability than PAMPA for fluorescein absorption in rat intestinal cells [3]. Our DIBs were made using naturally derived lipids that mimicked the lipid composition of intestinal cells. This means that DIBs are potentially a more reliable indicator of passive drug permeability than the current state of the art commercial technique.

We have also recently shown that DIBs can be used to model the breakdown in membrane asymmetry that occurs during cancer, and how this affects the transport of the chemotherapy agent doxorubicin, giving new insight into the mechanism of chemoresistance [5]. In a similar manner, DIBs have also been used to investigate the effect that the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen has on the integrity of the membrane itself, showing that membrane permeability is increased when ibuprofen interacts with certain lipids in the membrane, but not when cholesterol is present [66]. These recent works illustrate the potential of DIBs to be used to study drug-membrane interactions to help understand the mechanisms behind drug in vivo behaviour.

DIBs to study integral membrane proteins

Being structurally analogous to a cell membrane, DIBs provide a useful scaffold to study membrane proteins [67]. Since DIBs are more stable than other artificial membrane systems they provide an attractive platform for this kind of analysis. The first protein to be inserted into a DIB was α-haemolysin [10, 62], a pore forming toxin from Staphylococcus aureus. Due to its relative ease of use, and ability to self insert in a membrane, α-hemolysin has dominated the field perhaps to the detriment of more exciting biological entities. In the past decade many more proteins and peptides have been successfully reconstituted and inserted into DIBs using three main methods: self-insertion, reconstitution in liposomes before DIB formation, and in vitro transcription/translation (IVTT). Proteins can sometimes be self-inserted into membranes without any external influence through self-assembly under favourable conditions. Usually, however, proteins need to be reconstituted using detergents to solubilise them, which are then exchanged for lipids using dialysis. In IVTT, cellular machinery (ribosomes, DNA, amino acids etc.) is inserted into a droplet of the DIB to express the protein in situ directly into the bilayer.

Notable examples of protein insertion into DIBs include the incorporation of the eukaryotic hERG potassium channel and the N-methyl-D-aspartate (NMDA) receptor in DIBs after expression in mammalian cells [68]. Electrical measurements of these, as well as endogenously expressed mammalian potassium channels, and prokaryotic KcsA were carried out with no purification. Since purification of membrane proteins is notoriously difficult [69], bypassing this step greatly increases the accessibility of methods to study them. The incorporation of ion channels into DIBs suggests that DIBs may also be used as an alternative to patch clamp experiments. The patch clamp technique is used to compartmentalise sections of live cells such that transport across ion channels can be measured while in their natural environment. The compartmentalisation inherent in DIBs means that these experiments can happen in model membranes that mimic the natural environment much more easily. The mechanoselective MscL channel, a stretch activated ion channel found in

E. coli, has been incorporated into DIBs and activated using the compound 2-(trimethylammonium)ethyl methane thiosulphonate bromide [22]. Further work has confirmed that MscL proteins incorporated into DIBs retain their stretch responsive properties by taking electrical measurements while mechanically stimulating the DIB in which MscL was incorporated [70]. It is interesting to see again that being able to create asymmetric DIBs provides more information on the behaviour of a biological system. In this case, membrane asymmetry allows control over the activation state of MscL, showing that the lipoidal composition of the membrane can be leveraged to generate physical forces to pull open the channel [71]. More recently, the spontaneous insertion of the human chloride channel CLIC1 has been shown in a DIB, and quantification of chloride flux using the quenching of a chloride responsive fluorescent dye [25]. This work is particularly interesting due to their unique approach to forming DIB pairs using a microfluidic device that created a multiplexed array of several replicates.

While most proteins successfully inserted into DIBs are pores or ion channels, so far only two examples of active transport across a DIB have been demonstrated through the insertion of the E. coli lactose permease transporter (LacY) (Figure 2 C) [21] and bacteriorhodopsin [10, 72]. The uphill transport of a fluorescent sugar using LacY was shown and through manipulation of the lipid composition, the authors were able to vary the activity level of LacY. In this case, adding DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) resulted in a faster rate of transport by increasing the activity of LacY. LacY was successfully incorporated in a DIB using both reconstitution in liposomes after expression in E. coli, and with IVTT. Bacteriorhodopsin is a light-driven proton pump that has been inserted into DIBs to use them as "bio-pixels" to detect images as the proton pumps are activated by light and produce a current [72]. The aim of this work was to show the potential of these DIBs to be used to stimulate real cells such as neurons.

Since the droplet on either side of a DIB can be used to mimic a cell [3], linking multiple DIBs together in a network can form an artificial tissue where it is possible to control the packing of each artificial cell in the tissue [73, 74, 75]. In these cases proteins are not only inserted into DIBs to enable their behaviour to be studied, but are used as well to demonstrate fundamental characteristics of the artificial tissues, such as intercellular communication [76] and collective properties [77]. Recent work has shown that DIBs created in this manner but encapsulated in a non-oil surrounding material (in this case a hydrogel) for stability can also be used to mimic communication between organelles in a cell through the bacterial pore cytolysin A (ClyA) [78]. This surrounding material can also be deformed, causing controllable changes in the membrane properties in these three dimensional networks [79].

However no human proteins have so far been inserted into DIBs. This is likely because it is not straightforward to create DIBs (or any type of model membranes) that are biomimetic enough to support the correct insertion of complex human proteins and ensure their functionality [80]. A good first step in this direction will be the creation of DIBs that mimic the native environment of the proteins, both in terms of the molecular composition of the membrane and in terms of creating favourable experimental conditions for the insertion of proteins. With screening of drugs against transporter libraries forming an important and costly part of early drug discovery [81], successfully inserting pharmacologically relevant transporters into DIBs will mark a turning point in the use of DIBs as biomimetic model membranes and we predict that interest in their use as a tool to enable cheaper and faster early drug discovery by supplementing or replacing cell cultures will be high.

DIBs as scaffolds for electrophysiological measurements

Electrochemical gradients provide the driving force for cellular processes such as nerve conduction and respiration. Studying the electrophysiological characteristics of lipid membranes is essential to fully understand this aspect of cellular physiology. The electrical properties of DIBs have been measured extensively, with capacitance measurements and measurement of current through α-hemolysin forming the bulk of these measurements. Fundamental research into the voltage-current relationship of DPhPC membranes and the effect of α-hemolysin and of alamethicin on this relationship has shown that α-hemolysin decreases membrane resistance through formation of pores, and alamethicin causes a voltage dependant increase in conductance [8]. The dependence of bilayer capacitance on bilayer area has been exploited to

accurately measure the specific capacitance of DPhPC based DIBs in hexadecane [56, 82], mixtures of hexadecane, silicone oil and decane [48] and tetradecane [24]. Capacitance measurements of planar bilayers can be used to accurately quantify membrane parameters to ensure biological similarity. For example, to show the formation of the bilayer through exclusion of the oil phase [57] and to quantify membrane asymmetry [83].

While electrodes used for these types of measurements in DIBs are frequently Ag/AgCl electrodes inserted into the droplets themselves, it has also been shown that electrodes that sit at the surface of the droplets can be used to induce or measure flow of ions between DIBs [84]. These electrodes are covered with a monolayer of lipids and form another bilayer with the monolayer covered droplet. This type of measurements has been developed for other model membrane systems in ways that could also be integrated with DIBs. Interesting work to create a membrane containing a single peptide pore and measure current across it has potential for high-throughput screening using gold [85], Ag/AgCl [86] or hydrogel electrodes [87]. The advantage of external electrodes is that they can be easily integrated into microfluidic devices for analysis of molecular transport across DIBs, potentially expanding the types of analyses that can be performed in DIBs, which is one of the current main limitations in the field.

Engineered networks of DIBs had already been shown to exhibit interesting emergent properties prior to the first review of the field, such as functioning as batteries or light sensors [10]. Since then, networks of DIBs have been shown to be capable of functioning as diodes when treated with modified α-hemolysin [77]. Droplets containing these modified α-hemolysin heptamers were arranged to create a half wave rectifier, a current limiter, and even a full wave bridge rectifier. Because DIBs can easily be built into networks, more complex signal processing may be carried out with systems like this, for example to perform computing, or to serve as an interface between electronics and tissue. A recent review of the field further expands these ideas by elegantly describing the potential of DIBs from an engineering and materials science perspective [88].

From a more biomimetic perspective, bilayer capacitance and air flow-induced current measurements have been used to model the mechanotransduction properties of animal sensory hairs via DIBs made from DPhPC as model hair cells [89, 90]. In this work, an artificial hair was inserted into one of the droplets forming the DIB, allowing the effect of hair movement on the membrane to be investigated. This integration of macro and micro biological entities serves as a new kind of electromechanical sensor for small air movements and sound. DIBs made from DPhPC and DOPC have also been used as models for biophysical kinetic measurements by monitoring changes in transmembrane potential of asymmetric DIBs before and after insertion of alamethicin. It is not easy to form asymmetric bilayers using other types of model membranes, and few systems even exist that enable these types of studies. Being able to easily model this asymmetry using DIBs revealed new insights into how this pore interacts with the membrane by inducing lipid "flip flop". Hence, DIBs allowed the elucidation of the behaviour of the antibiotic in the membrane [38]. Prior work shows the use of a combination of interfacial tension (IFT) measurements and computational analysis to investigate the effects of lipid adsorption kinetics on phospholipid monolayer self-assembly at an oil-water interface [54]. The quantification of the success rate of DIB formation as well as resistance and the likelihood of rupture of the DIBs successfully formed using each of the aforementioned techniques showed a significant difference between DPhPC and the unsaturated lipid DOPC. The quantification of the success rate of DIB formation showed a significant difference between lipid-in and lipid-out methods for both DPhPC and DOPC. Studying the factors affecting the kinetics of monolayer formation not only provides explanations for success rates of DIB formation in future work, but also provides useful information for processes involving lipid emulsion formation. The authors apply these findings to use evaporation to cause monolayer compression to be able to form DIBs using unsaturated phospholipids [91], with more recent work showing the use of smaller droplets or different lipid mixtures to more easily form DIBs from unsaturated lipids [66, 87, 92]. The mechanics of membrane formation in DIBs requires further investigation. This is especially true since DIBs made from more biomimetic lipids and more complex lipid mixtures are needed to enable them to become the go-to model membrane system.

The future and potential of DIBs

The last decade has shown that DIBs are versatile and biomimetic model bilayers for applications ranging from the study of protein transport to artificial cells. The ease of formation of DIBs using manual or microfluidic methods means that they have the potential to become a widely used model membrane system. However, it is clear that they have yet to achieve their full potential. We believe that advances in the following research areas will be key drivers in terms of the development and application of these systems in coming years.

Fundamental bilayer behaviour

There is a lack of fundamental chemical insight into the kinetics and mechanics of monolayer and DIB formation. For example, we need a deeper understanding of the effect that the method of lipid delivery (lipid-in or lipid-out) has on the kinetics of DIB formation, what the kinetics are for lipid domain formation in DIBs and whether this enables the modelling of domain formation in real cells, how lipid flip flop and lateral diffusion within the bilayer occur and how this affects molecular permeability, and how small molecule diffusion through bilayers is affected by differing lipid (and oil) composition. The incorporation of fluorescently tagged lipids into DIBs may be a key component of this research, as will the use of techniques, both experimental and computational, more widely used by the membrane biophysics community to study the structure and function of these systems. Understanding these fundamental biophysical processes will enable more complex DIBs to be formed, and will enable comparison of the model membrane with their biological comparators.

Biomimetic artificial cells

Cells are complex entities. DIBs can function as bespoke artificial cells that can be built from the bottom up to enable the quantification of the behaviour of each component of the cell membrane. To function as artificial cells for the understanding of chemical interactions in the cell membrane, we must expand the library of lipids and biomimetic lipid mixtures that can be used for DIB formation. This should include all types of molecules found in cell membranes, such as glycolipids and peripheral proteins. Making DIBs more biomimetic also necessitates the development of strategies to reproducibly generate asymmetric DIBs and to insert a range of human membrane proteins into DIBs. Creating more complex DIB-linked droplet networks will enable the investigation of larger scale cell communication and molecular transport. Progress on these fronts will allow DIBs to be developed specifically as a screening tool for early-stage drug candidate permeability and activity.

Technological advances

Microfluidic technologies have enabled a host of new advances in the field. However, most microfluidic platforms are made using polydimethylsiloxane (PDMS), a choice of material that both limits assay length as the droplets evaporate over short time-frames and limits the commercialisation of microfluidic methods for DIB formation because other materials are used for the mass manufacture of microfluidic devices. Microfluidic devices made from materials such as polymers suitable for injection moulding or hot embossing will ensure that they are not simply a gimmick used by experts in academic laboratories. More high-throughput methods for DIB formation would enable rapid screening of drugs in DIBs for potentially dangerous interactions with channels and pore proteins. To increase the biomimetic nature of DIBs, microfluidic technologies that enable even smaller DIBs to be formed would allow more cell types to be modelled. Most fundamentally, the field would benefit from a streamlined strategy for the design of microfluidic platforms for DIB formation both in specialist and nonspecialist laboratories, and from better integration of a variety of detection methods into these microfluidic platforms. For example, we have recently developed plug-and-play microfluidic platforms for the creation of droplets within droplets (multiple emulsions) that could be used for DIB formation [93]. Major impact in this area would be provided by a commercially available 3D printer capable of printing 3D networks of DIBs of different compositions onto surfaces with integrated electrodes or sensors for detection. Techniques such as these would enable these technologies to be used in laboratories with no microfluidic expertise.

Detection methods

One of the greatest barriers to the widespread adoption of DIBs as model membranes for the quantification of molecular behaviour is the reliance on fluorescence microscopy. This is particularly true for microfluidic methods of forming DIBs, since removing droplets from a microfluidic device for analysis is a nontrivial task. There is a need for new measurement techniques that allow the quantification of molecular transport on-chip (and off-chip) of a wider range of molecules. Types of molecules it would be interesting to detect in DIBs include those that enter or exit cells and organelles, such as drugs, cell signalling molecules, and cellular nutrients, or more complex entities that enter cells using other mechanisms, such as viruses. As we mentioned at the start of this article, the field has changed based on the types of analytical techniques available for detection in DIBs. Recent exciting work shows the integration of microfluidic platforms for the formation of DIBs with off-chip label-free analysis using liquid chromatography–mass spectrometry [94]. Older, and in our opinion underutilised, work with high potential for drug detection includes the use of UV-vis spectroscopy [20]. It would be exciting to see further work with these detection techniques, but also to see the integration of simple colorimetric assays, or more complex biochemical assays such as enzyme-linked immunosorbent assays (ELISA) into DIBs. New detection methods can also be based on the development of universal molecular recognition agents or through the development of on-chip sensors.

Looking to the future, we believe that these advances will allow DIBs to be used for a host of new applications. By exploiting the electrical properties of engineered DIB networks, we might be able to assemble more complex structures such as logic gates that function as tuneable biosensors. Through advances in microfluidic technologies we could build interconnected, three dimensional networks of DIBs to create new biomaterials for application in the medicinal sciences. We can also imagine a future that includes automated synthesis and testing platforms for drug discovery (Figure 3), where combinatorial synthesis of drugs occurs in droplets which are then brought together to form DIBs to test molecular uptake through bespoke artificial cell membranes containing different types of lipids and transporter proteins. We look forward to looking back next decade to see whether DIBs have achieved their full potential.

Acknowledgements

KSE is a Canada Research Chair and a Michael Smith Foundation for Health Research Scholar in partnership with the Pacific Alzheimer Research Foundation and receives funding from both. EBS and JLK are funded through KSE's Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant.

Correspondence to Katherine Elvira.

Author contributions

Elanna Stephenson and Jaime Korner: literature review and writing. Katherine Elvira: conceptualisation, supervision, writing, reviewing and editing. ‡These authors contributed equally.

Competing interests

The authors declare no competing interests.

References

1. Hwang, W. L., Holden, M. A., White, S. & Bayley, H. Electrical behavior ofdroplet interface bilayer networks: Experimental analysis and modeling. *J. Am. Chem. Soc.* **129**, 11854–11864 (2007).

2. Ginzberg, M. B., Kafri, R. & Kirschner, M. On being the right (cell) size. *Science* **348**, 1245075 (2015).

3. Korner, J. L., Stephenson, E. B. & Elvira, K. S. A bespoke microfluidic pharmacokinetic compartment model for drug absorption using artificial cell membranes. *Lab Chip* **20**, 1898–1906 (2020).

4. Hwang, W. L., Chen, M., Cronin, B., Holden, M. A. & Bayley, H. Asymmetric droplet interface bilayers. *J. Am. Chem. Soc.* **130**, 5878–5879 (2008).

5. Stephenson, E. B. & Elvira, K. S. Biomimetic artificial cells to model the effect of membrane asymmetry on chemoresistance. *Chem. Commun.* **57**, 6534–6537 (2021).

6. Barlow, N. E. *et al.* Measuring bilayer surface energy and curvature in asymmetric droplet interface bilayers. *J. R. Soc. Interface* **15**, 20180610 (2018).

7. Malmstadt, N., Nash, M. A., Purnell, R. F. & Schmidt, J. J. Automated formation of lipid-bilayer membranes in a microfluidic device. *Nano Lett.* **6**, 1961–1965 (2006).

8. Sarles, S. A. & Leo, D. J. Tailored current–voltage relationships of droplet-interface bilayers using biomolecules and external feedback control. *J. Intell. Mater. Syst. Struct.* **20**, 1233–1247 (2009).

9. Aghdaei, S., Sandison, M. E., Zagnoni, M., Green, N. G. & Morgan, H. Formation of artificial lipid bilayers using droplet dielectrophoresis. *Lab Chip* **8**, 1617 (2008).

10. Holden, M. A., Needham, D. & Bayley, H. Functional bionetworks from nanoliter water droplets. *J. Am. Chem. Soc.* **129**, 8650–8655 (2007).

11. Renner, S., Geltinger, S. & Simmel, F. C. Nanopore translocation and force spectroscopy experiments in microemulsion droplets. *Small* **6**, 190–194 (2010).

12. Poulos, J. L., Nelson, W. C., Jeon, T.-J., Kim, C.-J. Schmidt, J. J. Electrowetting on dielectric-based microfluidics for integrated lipid bilayer formation and measurement. *Appl. Phys. Lett.* **95**, 013706 (2009).

13. Creasy, M. A. & Leo, D. J. Non-invasive measurement techniques for measuring properties of droplet interface bilayers. *Smart Mater. Struct.***19**, 094016 (2010).

14. Sarles, S. A. & Leo, D. J. Physical encapsulation of droplet interface bilayers for durable, portable biomolecular networks. *Lab Chip* **10**, 710–717 (2010).

15. Sarles, S. A. & Leo, D. J. Regulated attachment methodfor reconstituting lipid bilayers of prescribed size within flexible substrates. *Anal. Chem.* **82**, 959–966 (2010).

16. Punnamaraju, S. & Steckl, A. J. Voltage control of droplet interface bilayer lipid membrane dimensions. *Langmuir* **27**, 618–626 (2011).

17. Syeda, R., Holden, M. A., Hwang, W. L. & Bayley, H. Screening blockers against a potassium channel with a droplet interface bilayer array. *J. Am. Chem. Soc.* **130**, 15543–15548 (2008).

18. Bayley, H. *et al.* Droplet interface bilayers. *Mol. BioSyst.* **4**, 1191– 1208 (2008).

19. Huang, J., Lein, M., Gunderson, C. & Holden, M. A. Direct quantitation of peptide-mediated protein transport across a droplet–interface bilayer. *J. Am. Chem. Soc.* **133**, 15818–15821 (2011).

20. Nisisako, T., Portonovo, S. A. & Schmidt, J. J. Microfluidic passive permeability assay using nanoliter droplet interface lipid bilayers. *Analyst* **138**, 6793–6800 (2013).

21. Findlay, H. E., Harris, N. J. & Booth, P. J. In vitro synthesis of a major facilitator transporter for specific active transport across droplet interface bilayers. *Sci. Rep.***6**, 1–9 (2016).

22. Barriga, H. M. G. *et al.* Droplet interface bilayer reconstitution and activity measurement of the mechanosensitive channel of large conductance from Escherichia coli. *J. R. Soc. Interface* **11**, 20140404 (2014).

23. Najem, J. S. *et al.* Multifunctional, micropipette-based method for incorporation and stimulation of bacterial mechanosensitive ion channels in droplet interface bilayers. *J. Vis. Exp.* **105**, e53362 (2015).

24. El-Beyrouthy, J., Makhoul-Mansour, M. M., Taylor, G., Sarles, S. A. & Freeman, E. C. A new approach for investigating the response of lipid membranes to electrocompression by coupling droplet mechanics and membrane biophysics. *J. R. Soc. Interface* **16**, 20190652 (2019).

25. Zhang, Y., Bracken, H., Woolhead, C. & Zagnoni, M. Functionalisation of human chloride intracellular ion channels in microfluidic droplet-interface-bilayers. *Biosens. Bioelectron.* **150**, 111920 (2020).

26. Stanley, C. E. *et al.* A microfluidic approach for high-throughput droplet interface bilayer (DIB) formation. *Chem. Commun.* **46**, 1620–1622 (2010).

27. Carreras, P., Law, R. V., Brooks, N., Seddon, J. M. & Ces, O. Microfluidic generation of droplet interface bilayer networks incorporating real-time size sorting in linear and non-linear configurations. *Biomicrofluidics* **8** (2014).

28. Elani, Y., Casadevall i Solvas, X., Edel, J. B., Law, R. V. & Ces, O. Microfluidic generation of encapsulated droplet interface bilayer networks (multisomes) and their use as cell-like reactors. *Chem. Commun.* **52**, 5961–5964 (2016).

29. Schlicht, B. & Zagnoni, M. Droplet-interface-bilayer assays in microfluidic passive networks. *Sci. Rep.***5**, 9951 (2015).

30. Mueller, P., Rudin, D. O., Ti Tien, H. & Wescott, W. C. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature* **194**, 979–980 (1962).

31. White, S. H. The physical nature of planar bilayer membranes. In Miller, C. (ed.) *Ion Channel Reconstitution*, 3–35 (Springer US, Boston, MA, 1986).

32. Oiki, S. Planar lipid bilayer method for studying channel molecules. In Okada, Y. (ed.) *Patch Clamp Techniques: From Beginning to Advanced Protocols*, Springer Protocols Handbooks, 229–275 (Springer Japan, Tokyo, 2012).

33. Peetla, C., Stine, A. & Labhasetwar, V. Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery. *Molecular pharmaceutics* **6**, 1264–1276 (2009).

34. Elani, Y., deMello, A. J., Niu, X. & Ces, O. Novel technologies for the formation of 2-D and 3-D droplet interface bilayer networks. *Lab Chip* **12**, 3514–3520 (2012).

35. Carreras, P. *et al.* A microfluidic platform for size-dependent generation of droplet interface bilayer networks on rails. *Biomicrofluidics* **9**, 064121 (2015).

36. Nguyen, M.-A., Srijanto, B., Collier, C. P., Retterer, S. T. & Sarles, S. A. Hydrodynamic trapping for rapid assembly and in situ electrical characterization of droplet interface bilayer arrays. *Lab Chip* **16**, 3576–3588 (2016).

37. Czekalska, M. A. *et al.* A droplet microfluidic system for sequential generation of lipid bilayers and

transmembrane electrical recordings. *Lab Chip* **15**, 541–548 (2015).

38. Taylor, G. *et al.* Electrophysiological interrogation of asymmetric droplet interface bilayers reveals surface-bound alamethicin induces lipid flip-flop. *Biochim. Biophys. Acta Biomembr.* **1861**, 335–343 (2019).

39. Tamaddoni, N., Taylor, G., Hepburn, T., Kilbey, S. M. & Sarles, S. A. Reversible, voltage-activated formation of biomimetic membranes between triblock copolymer-coated aqueous droplets in good solvents. *Soft Matter* **12**, 5096–5109 (2016).

40. Bai, Y. *et al.* A double droplet trap system for studying mass transport across a droplet-droplet interface. *Lab Chip* **10**, 1281–1285 (2010).

41. Taylor, G. J. *et al.* Capacitive detection of low-enthalpy, higher-order phase transitions in synthetic and natural composition lipid membranes. *Langmuir* **33**, 10016–10026 (2017).

42. Taylor, G. J. & Sarles, S. A. Heating-enabled formation of droplet interface bilayers using Escherichia coli total lipid extract. *Langmuir* **31**, 325–337 (2015).

43. Barlow, N. E. *et al.* Engineering plant membranes using droplet interface bilayers. *Biomicrofluidics* **11**, 024107 (2017).

44. Najem, J. S. *et al.* Memristive ion channel-doped biomembranes as synaptic mimics. *ACS Nano* **12**, 4702–4711 (2018).

45. Michalak, Z., Muzzio, M., Milianta, P. J., Giacomini, R. & Lee, S. Effect of monoglyceride structure and cholesterol content on water permeability of the droplet bilayer. *Langmuir* **29**, 15919–15925 (2013).

46. Lopez, M., Evangelista, S. E., Morales, M. & Lee, S. Enthalpic effects of chain length and unsaturation on water permeability across droplet bilayers of homologous monoglycerides. *Langmuir* **33**, 900–912 (2017).

47. Lopez, M. *et al.* Effects of acyl chain unsaturation on activation energy of water permeability across droplet bilayers of homologous Monoglycerides: Role of cholesterol. *Langmuir* **34**, 2147–2157 (2018).

48. Taylor, G. J., Venkatesan, G. A., Collier, C. P. & Sarles, S. A. Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer. *Soft Matter* **11**, 7592–7605 (2015).

49. de Wit, G., Danial, J. S. H., Kukura, P. & Wallace, M. I. Dynamic label-free imaging of lipid nanodomains. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 12299–12303 (2015).

50. Faugeras, V., Duclos, O., Bazile, D. & Thiam, A. R. Membrane determinants for the passive translocation of analytes through droplet interface bilayers. *Soft Matter* **16**, 5970–5980 (2020).

51. Korner, J. L. & Elvira, K. S. The role of temperature in the formation of human–mimetic artificial cell membranes using droplet interface bilayers (DIBs). *Soft Matter* **17**, 8891–8901 (2021).

52. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: Where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* **9**, 112–124 (2008).

53. McMahon, H. T. & Boucrot, E. Membrane curvature at a glance. *J. Cell Sc.* **128**, 1065–1070 (2015).

54. Venkatesan, G. A. *et al.* Adsorption kinetics dictate monolayer Self-assembly for both lipid-in and lipid-out approaches to droplet interface bilayer formation. *Langmuir* **31**, 12883–12893 (2015).

55. Yanagisawa, M., Yoshida, T.-A., Furuta, M., Nakata, S. & Tokita, M. Adhesive force between paired microdroplets coated with lipid monolayers. *Soft Matter* **9**, 5891–5897 (2013).

56. Gross, L. C. M., Heron, A. J., Baca, S. C. & Wallace, M. I. Determining membrane capacitance by dynamic control of droplet interface bilayer area. *Langmuir* **27**, 14335–14342 (2011).

57. Beltramo, P. J., Scheidegger, L. & Vermant, J. Toward realistic large-area cell membrane mimics: Excluding oil, controlling composition, and including ion channels. *Langmuir* **34**, 5880–5888 (2018).

58. Milianta, P. J., Muzzio, M., Denver, J., Cawley, G. & Lee, S. Water permeability across symmetric and asymmetric droplet interface bilayers: Interaction of cholesterol sulfate with DPhPC. *Langmuir* **31**, 12187–12196 (2015).

59. Braziel, S., Sullivan, K. & Lee, S. Quantitative Raman microspectroscopy for water permeability parameters at a droplet interface bilayer. *Analyst* **143**, 747–755 (2018).

60. Valet, M., Pontani, L.-L., Voituriez, R., Wandersman, E. & Prevost, A. Diffusion through nanopores in connected lipid bilayer networks. *Phys. Rev. Lett.* **123**, 088101 (2019).

61. Barlow, N. E. *et al.* Rheological droplet interface bilayers (rheo-DIBs): Probing the unstirred water layer effect on membrane permeability via spinning disk induced shear stress. *Sci. Rep.***7**, 1–12 (2017).

62. Funakoshi, K., Suzuki, H. & Takeuchi, S. Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. *Anal. Chem.* **78**, 8169–8174 (2006).

63. Booth, M. J., Cazimoglu, I. & Bayley, H. Controlled deprotection and release of a small molecule from a compartmented synthetic tissue module. *Commun. Chem.* **2**, 1–8 (2019).

64. Cazimoglu, I., Booth, M. J. & Bayley, H. A Lipid-based droplet processor for parallel chemical signals. *ACS Nano* **15**, 20214–20224 (2021).

65. Li, X., Huang, J., Holden, M. A. & Chen, M. Peptide-mediated membrane transport of macromolecular cargo driven by membrane asymmetry. *Anal. Chem.* **89**, 12369–12374 (2017).

66. Wood, M. *et al.* Ibuprofen and the phosphatidylcholine bilayer: Membrane water permeability in the presence and absence of cholesterol. *Langmuir* **37**, 4468–4480 (2021).

67. Allen-Benton, M., Findlay, H. E. & Booth, P. J. Probing membrane protein properties using droplet interface bilayers. *Exp. Biol. Med.* **244**, 709–720 (2019).

68. Leptihn, S., Thompson, J. R., Ellory, J. C., Tucker, S. J. & Wallace, M. I.In vitro reconstitution of eukaryotic ion channels using droplet interface bilayers. *J. Am. Chem. Soc.* **133**, 9370–9375 (2011).

69. Smith, S. M. Strategies for the purification of membrane proteins. In *Protein Chromatography*, Methods in Molecular Biology, 485–496 (Humana Press, 2011).

70. Najem, J. S. *et al.* Activation of bacterial channel MscL in mechanically stimulated droplet interface bilayers. *Sci. Rep.***5**, 13726 (2015).

71. Strutt, R. *et al.* Activating mechanosensitive channels embedded in droplet interface bilayers using membrane asymmetry. *Chem. Sci.* **12**, 2138–2145 (2021).

72. Restrepo Schild, V. *et al.* Light-patterned current generation in a droplet bilayer array. *Sci. Rep.***7**, 46585 (2017).

73. Alcinesio, A. *et al.* Controlled packing and single-droplet resolution of 3D-printed functional synthetic tissues. *Nat. Commun.* **11**, 2105 (2020).

74. Graham, A. D. *et al.* High-resolution patterned cellular constructs by droplet-based 3D printing. *Sci. Rep.***7**, 7004 (2017).

75. Challita, E. J., Makhoul-Mansour, M. M. & Freeman, E. C. Reconfiguring droplet interface bilayer networks through sacrificial membranes. *Biomicrofluidics* **12**, 034112 (2018).

76. Villar, G., Heron, A. J. & Bayley, H. Formation of droplet networks that function in aqueous environments. *Nat. Nanotechnol.* **6**, 803–808 (2011).

77. Maglia, G. *et al.* Droplet networks with incorporated protein diodes show collective properties. *Nat. Nanotechnol.* **4**, 437–440 (2009).

78. Bayoumi, M., Bayley, H., Maglia, G. & Sapra, K. T. Multi-compartment encapsulation of communicating droplets and droplet networks in hydrogel as a model for artificial cells. *Sci. Rep.* **7**, 45167 (2017).

79. Challita, E. J., Najem, J. S., Monroe, R., Leo, D. J. & Freeman, E. C. Encapsulating networks of droplet interface bilayers in a thermoreversible organogel. *Sci. Rep.***8**, 1–11 (2018).

80. Shen, H.-H., Lithgow, T. & Martin, L. L. Reconstitution of membrane proteins into model membranes: Seeking better ways to retain protein activities. *Int. J. Mol. Sci.* **14**, 1589–1607 (2013).

81. Giacomini, K. M. *et al.* Membrane transporters in drug development. *Nat. Rev. Drug Discov.* **9**, 215–236 (2010).

82. Rofeh, J. & Theogarajan, L. Instantaneous tension measurements in droplet interface bilayers using an inexpensive, integrated pendant drop camera. *Soft Matter* **16**, 4484–4493 (2020).

83. Liu, P., Zabala-Ferrera, O. & Beltramo, P. J. Fabrication and electromechanical characterization of freestanding asymmetric membranes. *Biophys. J.* **120**, 1755–1764 (2021).

84. Creasy, M. A. & Leo, D. J. Non-invasive measurement techniques for measuring properties of droplet interface bilayers. *Smart Mater. Struct.***19**, 094016 (2010).

85. Okuno, D. *et al.* A Simple method for ion channel recordings using fine gold electrode. *Anal. Sci.* **32**, 1353–1357 (2016).

86. Shoji, K., Kawano, R. & White, R. J. Recessed Ag/AgCl microelectrode-supported lipid bilayer for nanopore sensing. *Anal. Chem.* **92**, 10856–10862 (2020).

87. Challita, E. J. & Freeman, E. C. Hydrogel microelectrodes for the rapid, reliable, and repeatable characterization of lipid membranes. *Langmuir* **34**, 15166–15173 (2018).

88. Makhoul-Mansour, M. M. & Freeman, E. C. Droplet-based membranous soft materials. *Langmuir* **37**, 3231–3247 (2021).

89. Sarles, S. A., Madden, J. D. W. & Leo, D. J. Hair cell inspired mechanotransduction with a gel-supported, artificial lipid membrane. *Soft Matter* **7**, 4644 (2011).

90. Tamaddoni, N., Freeman, E. C. & Sarles, S. A. Sensitivity and directionality of lipid bilayer mechanotransduction studied using a revised, highly durable membrane-based hair cell sensor. *Smart Mater. Struct.* **24**, 065014 (2015).

91. Venkatesan, G. A. *et al.* Evaporation-induced monolayer compression improves droplet interface bilayer formation using unsaturated lipids. *Biomicrofluidics* **12** (2018).

92. Dupin, A. & Simmel, F. C. Signalling and differentiation in emulsion-based multi-compartmentalized in vitrogene circuits. *Nat. Chem.* **11**, 32–39 (2019).

93. Farley, S., Ramsay, K. & Elvira, K. S. A plug-and-play modular microcapillary platform for the generation of multicompartmental double emulsions using glass or fluorocarbon capillaries. *Lab Chip* (2021).

94. Bachler, S., Ort, M., Krämer, S. D. & Dittrich, P. S. Permeation studies across symmetric and

asymmetric membranes in microdroplet arrays. *Anal. Chem.* **93**, 5137–5144 (2021).

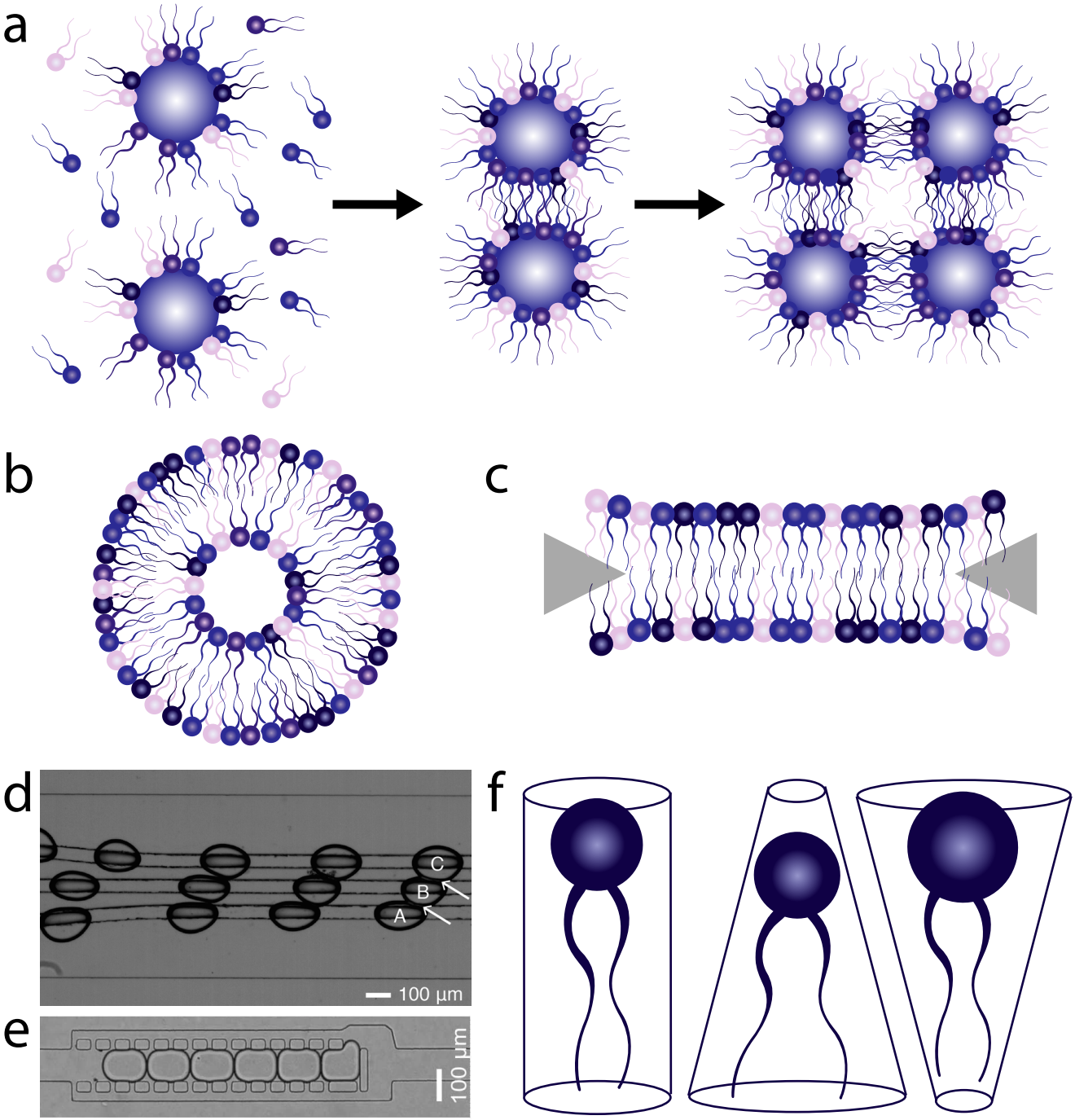
95. Czekalska, M. A., Kaminski, T. S., Horka, M., Jakiela, S. & Garstecki, P. An automated microfluidic system for the generation of droplet interface bilayer networks. *Micromachines* **8**, 93 (2017).

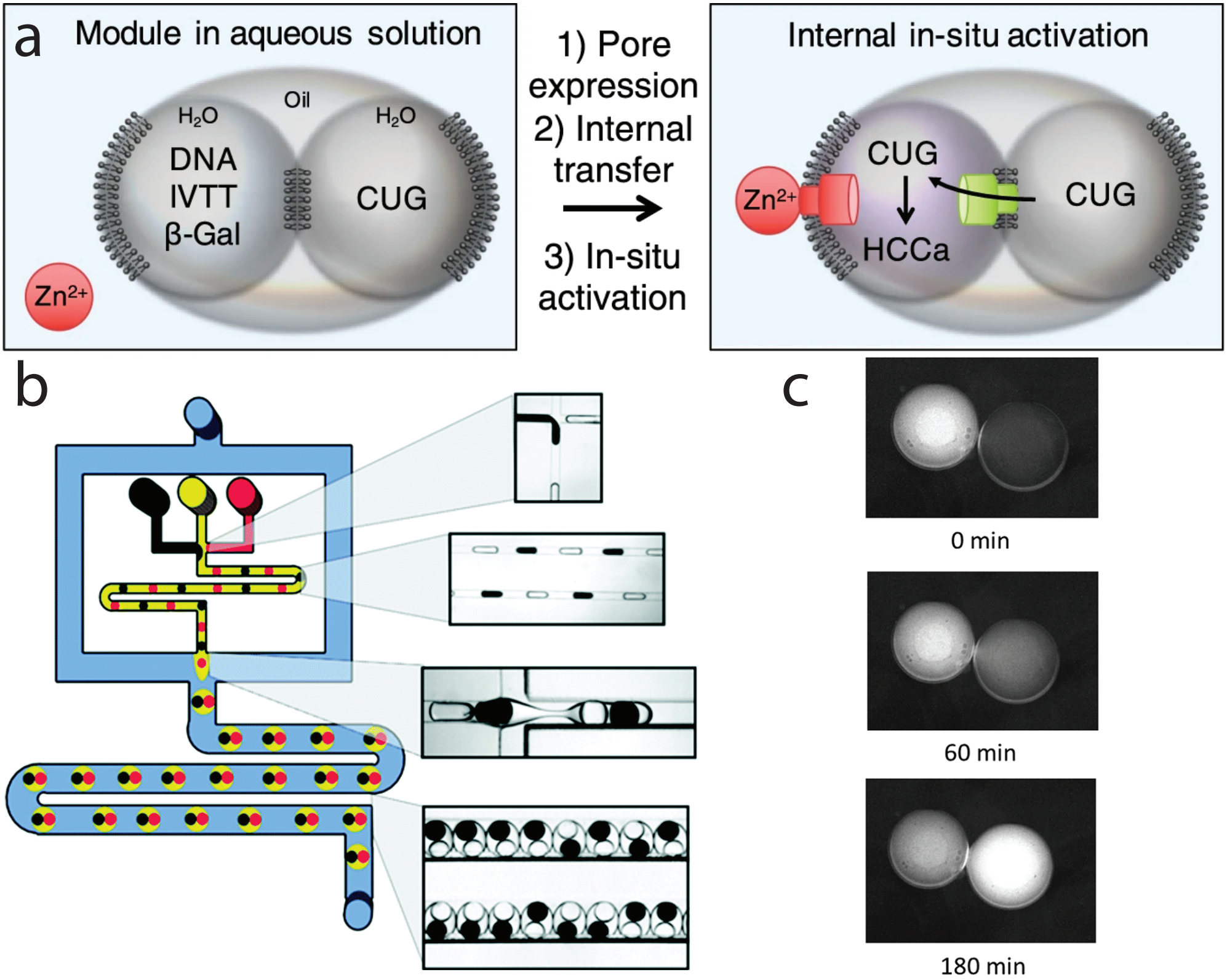
96. E. Barlow, N. *et al.* Multiplexed droplet interface bilayer formation. *Lab Chip* **16**, 4653–4657 (2016).

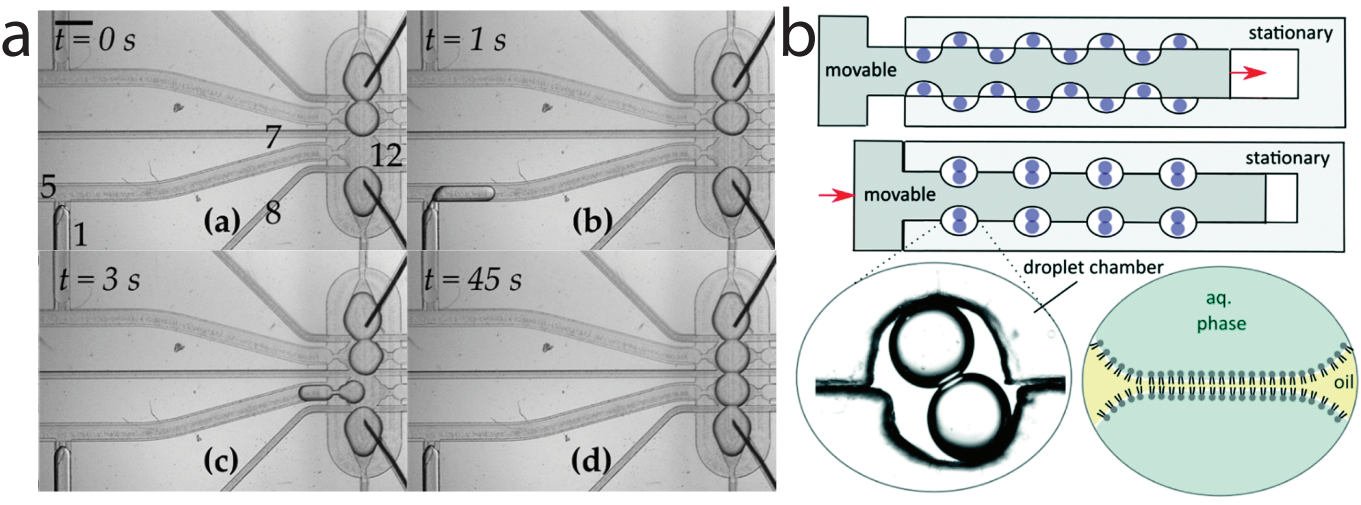
Figure 1: Formation and structure of DIBs. a) Schematic showing the formation of DIBs. Lipids self-assemble at the interface of aqueous droplets to form monolayers. Droplets are then brought into contact with each other manually or using microfluidic technologies to encourage bilayer formation between the droplets. DIBs can then be formed into networks in both two and three dimensions. b) Schematic showing the arrangement of lipids to form a liposome and c) a black lipid membrane. Triangles denote the solid support used to build the bilayer. Phospholipid size is exaggerated to show assembly. Microfluidic methods for the formation of DIBs usually rely on on-chip features such as d) rails [3] or e) pillars [29] to align the droplets and slowly bring them into contact with each other. This design allows three droplets of different composition (A, B and C) to be brought into contact with each other to form DIBs (highlighted with arrows). f) Cylindrical and conical shapes used to describe the packing characteristics of different types of lipids.

Figure 2: DIBs to study molecular transport. a) Use of IVTT to insert a variant of the pore α-hemolysin (green cylinder) into a multisome (water-in-oil-in-water droplets), allowing the precursor compound 3-carboxyumbelliferyl-β-D-galactopyranoside (CUG) to diffuse through the pore to the droplet containing the enzyme β-galactosidase, which converts CUG to the fluorescent reporter 7-hydroxycoumarin-3-carboxylic acid (HCCa). The structure of the multisomes is key for this reaction. The two aqueous droplets are created in an oil phase to form a DIB to support the membrane pore, but are surrounded by a third droplet that enables a bilayer to also form between the outer aqueous phase (light blue) and the aqueous droplets. Hence, zinc ions can be used to activate the membrane pore [63]. b) Multisomes have also been made in large quantities using microfluidic methods, creating several self-contained reaction vessels, also used in this work to create a fluorescent reporter. The diagram of the microfluidic platform shows the various phases used to make the multisomes. Where red and black represent two different aqueous phases that form the inner droplets, yellow represents the oil phase that encapsulates them allows a DIB for form between them, and blue represents the outer aqueous phase. Microscopy images of each step are shown at relevant regions of the microfluidic device [28]. c) IVTT has also been used for the expression of an ATP powered transporter protein from the major facilitator superfamily, shown here pumping the fluorescent sugar 4-methylumbelliferyl-β-galactopyranoside from the droplet on the left into the droplet on the right through the lactose permease (LacY) protein supported in the DIB. [21].

Figure 3: Automated DIB assays for drug transport applications. a) An automated microfluidic platform that uses electronic control of valves to assemble and disassemble networks of DIBs on demand [95]. Snapshots over the course of 45 s start (a) by showing three droplets held in place in a hydrodynamic trap. The top and bottom droplets are accessed by electrodes (dark lines) for further analysis. A fourth droplet is then formed at a T-junction (b) and moved into place in the network (c) and (d). For each manoeuvre, different valves are used. Oil (5) and aqueous (1) flows form the droplet, extra oil flow through guidance channels (7 and 8) move the droplet to its location within the network and extraneous liquid is ejected through the outlet (12). The scale is 1 mm. Complex experiments involving DIBs may potentially be done using such a system to increase throughput and allow networks to be reconfigured. b) A machined polycarbonate device for assembly of many DIB pairs on demand [96]. The diagram shows the simplicity of the device. Single droplets are loaded into the hemispherical chambers (top image), and brought together to form DIBs by manually sliding the inset into the stationary outer casing so that the chambers align (bottom image). The microscopy image shows a DIB formed in this manner in the aligned chamber, together with a schematic of the placement of the lipids in the DIB. With multiplexed arrays of DIBs, single bilayer experiments may be carried out in replicate much like traditional macroscopic experiments in well plates.


a
b
c
d
C
B
A
100 µm
e
100 µm
f


a
Module in aqueous solution
Oil
H2O
H2O
DNA
IVTT
β-Gal
CUG
Zn2+
1) Pore expression
2) Internal transfer
3) In-situ activation
Internal in-situ activation
CUG
Zn2+
HCCa
CUG
b
c
0 min
60 min
180 min


a
t = 0 s
t = 1 s
t = 3 s
t = 45 s
7
12
5
1
8
(a)
(b)
(c)
(d)
b
movable
stationary
movable
stationary
droplet chamber
aq.
phase
oil