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Microfluidic methods for forming liposomes

Dirk van Swaay and Andrew deMello*

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Liposome structures have a wide range of applications in biology, biochemistry, and biophysics. As a result, several methods for forming liposomes have been developed. This review provides a critical comparison of existing microfluidic technologies for forming liposomes and, when applicable, a comparison with their analogous macroscale counterparts. The properties of the generated liposomes, including size, size distribution, lamellarity, membrane composition, and encapsulation efficiency, form the basis for comparison. We hope that this critique will allow the reader to make an informed decision as to which method should be used for a given biological application.

1 Introduction

Liposomes, or lipid vesicles, are self-assembled lipid structures in the shape of closed membrane capsules (see Fig. 1). They can act as biomimetic compartments with a membrane that closely resembles that of living cells, encapsulating materials such as DNA, proteins, drugs, or other chemicals.[1,2] They can be formed, manipulated, and modified in a variety of ways,[2] and due to their similarity to cells and naturally occurring vesicles, they have been extensively studied.[3–5] Tools for observing and characterising their properties are well developed,[6–14] and moreover basic molecular dynamics simulations of their structure and behaviour have been refined in recent years.[15–18]

First synthesised in a laboratory in the late 1960s,[19–22] liposomes have become a standard tool in lipid and membrane science,[23–27] drug delivery,[28–30] compartmentalisation of biomolecules,[31–34] as well as the formation of rudimentary artificial cells.[35–43] Liposome-based artificial cells can be used to study cellular systems in isolation and in a simpler physical environment than in living cells. For example, Sackmann and coworkers, as well as other research groups, have studied the reconstitution of cytoskeletal components inside liposomes,[44–50] while Noireaux *et al.* used liposomes to encapsulate an *in vitro* transcription and translation reaction.[37,38]

The usefulness of liposomes in such a wide range of applications dictates the importance of developing robust production methods. Over the last three decades, a variety of macroscale methods have been developed for their production.[2] These include extrusion through porous membranes,[51,52] electroformation,[53,54] freeze-drying,[6,55] hydration or swelling,[56,57] double emulsions (water-in-oil-in-water, or W/O/W),[58,59] and budding[60] to name a few. Macroscale techniques, using fluidic volumes on the order of millilitres to hundreds of microlitres, are the most common way to form liposomes. However, such methods afford limited process control and are often accompanied by features such as poor reproducibility and the inefficient use of materials and reagents. In order to address these difficulties, microfluidic technologies have recently been developed either as adaptations of macroscale methods or as completely new techniques. "Microfluidic" refers to methods in which fluid handling procedures are, in the current context, performed in a geometrically constrained volume, typically defined by sub-millimeter length scales and low Reynolds Numbers. The adoption of microfluidic techniques allows the integration of laboratory procedures into planar chips or other small devices, reducing reaction volumes and the associated cost of chemical and biological experimentation by several orders of magnitude, while simultaneously increasing throughput and analytical performance.[61–65] This review will assess the merits of current techniques of liposome production and material encapsulation that are either entirely or partially performed within microfluidic devices, and appraise them according to application-specific criteria. As background for this discussion, a description of the desirable liposome and method characteristics follows.

1.1 Liposome and method specifications

Membrane composition and lamellarity. Vesicles have been made using many different surfactants besides phospholipids.[66] The choice or combination of surfactants greatly affects the properties of the membrane, such as shape, thickness, stability, elasticity, and permeability, as well as compatibility with biological materials.[67–70]

Naturally-occurring phospholipids such as L-α-phosphatidylcholine (Egg PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) have the advantage that they are more likely to be

Institute for Chemical and Bioengineering, ETH Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland. E-mail: andrew.demello@chem.ethz.ch; Tel: +41 (0)44 633 6610

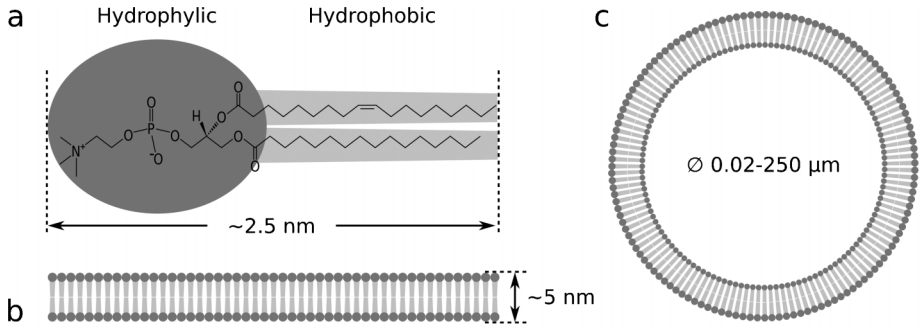

Fig. 1 (a) The structure of one POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid molecule, showing the hydrophilic head and hydrophobic tail. POPC is a naturally-occurring lipid and is commonly used in the production of synthetic liposomes. (b) The heads and tails of the lipid interact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

compatible with membrane-bound proteins. This is important since pore forming peptides such as α-hemolysin[71] allow for transport of molecules that would otherwise not be able to cross the membrane. This greatly extends the functionality of vesicles as bioreactors or artificial cells.[37]

One aspect of membrane composition that must be considered when producing vesicles is the presence of residue solvents or oil inside the lipid bilayer as a result of the formation process. Most vesicle preparation methods involve the dissolution of lipids in an organic solvent (typically chloroform), with subsequent removal of this solvent.[2] The solvent is usually removed by an evaporation, vacuum desiccation, or lyophilisation step prior to addition of the oil or buffer that will be used to prepare the liposomes. In some methods, water droplets are formed in oil and later transformed into liposomes by, for example, droplet transfer or by use of double emulsion templates (see sections 2.5 and 2.8). In the former, the oil may partition into the liposome bilayer during transfer, while in the latter, the removal step may not be completely efficient.

The unidentified or uncharacterised presence of foreign material within the membrane introduces an uncontrolled experimental factor, which may compromise the quality or reproducibility of results if not properly accounted for. For example, a membrane-spanning protein may adopt a different conformation if an organic solvent is present inside the lipid bilayer and thus behave unnaturally. It has also been shown that residual solvent affects mechanical or physical characteristics of the membrane such as rigidity or elasticity, parameters that have been shown to affect protein behaviour.[72–76] Moreover, since some oils or solvents may pool in specific regions of a membrane (oils, for example, eventually float to the top),[77] protein behaviour may vary depending on whether it is located in the oil-containing portion of the membrane or not.

Another important aspect of membrane structure is its lamellarity, a term which defines the number of bilayers by which it is composed. A membrane composed of a single bilayer is unilamellar, while a membrane composed of many bilayers is multilamellar (see Fig. 2). Unilamellar vesicles resemble living cells more closely both in structure and function, and are typically used in membrane protein studies.[78–81] Multilamellar vesicles, on the other hand, are primarily used for encapsulating substrates to be released upon disruption of the membrane at a later time, such as in drug delivery, or when membrane properties other than stability have no effect, such as the use of magnetic-particle containing vesicles as MRI contrast agents.[82] It is also worth noting that existing methods cannot control the number of bilayers in multilamellar vesicles.

Size and size distribution. Many vesicle preparation methods result in polydisperse vesicles—that is, a vesicle population containing vesicles of different sizes.[83] Although different sized vesicles may have different applications, most applications require vesicle populations to be monodisperse.[5] This is because variability in size causes variability in other application-critical factors such as stability, the amount of material encapsulated or bound to the membrane, membrane curvature, or rates of transport across the membrane.[5,84–86] As such, monodispersity is a key criterion in determining the usefulness of a technique, and one of the main motivations for using microfluidic systems to form liposomes.

Vesicles can range in diameter from a few tens of nanometres to a few hundred micrometres. A commonly used classification system is summarised in Fig. 2.

Stability. The stability of vesicles depends not only on size and membrane composition, but also on properties of the internal and external phases such as osmolarity, pH, salinity, and temperature.[68,70,87] Since all of these properties are in some way affected by the method of formation, the method will also have a strong effect on vesicle stability. Physical instability of vesicles can manifest itself in the form of lysis, aggregation, coalescence, or budding (Fig. 3).[83]

Vesicles are stable on timescales ranging from a few minutes to a few weeks. Indeed, Tan *et al.* reported unilamellar vesicles formed by their emulsion transfer process lasting for more than 26 days.[88] Long-lasting vesicles allow for greater flexibility in their use after preparation, and thus greatly extends their breadth of applications. For example, liposomes used in drug delivery or as containers for MRI contrast agents typically need to be robust enough to survive on the skin or in the blood stream long enough to carry out their function, which may be anywhere from a few hours to a few days. Vesicles used for membrane protein studies or as artificial cells, on the other

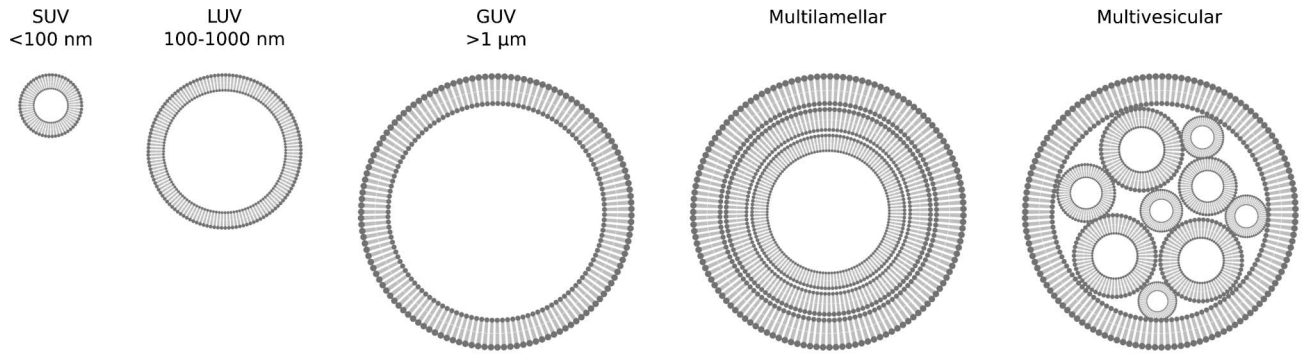

Fig. 2 The common vesicle size and lamellarity classification system. Small unilamellar vesicles (SUV) are less than 100 nm in diameter; large unilamellar vesicles (LUV) are between 100 and 1000 nm; and giant unilamellar vesicles (GUV) are larger than 1 micron. Multilamellar vesicles have many membrane layers, and multivesicular vesicles encapsulate smaller vesicles.

hand, must be able to withstand the experimental procedure. That may involve changing physical conditions, such as applying electric fields or chemical concentration gradients. Microfluidic techniques have the potential to create long-lasting vesicles due to a high degree of process control. For example, temperature, pH, salinity, osmolarity, fluid mechanical forces, and vesicle size can all be tuned very precisely using microfluidic tools. Moreover, all of these factors can be controlled during vesicle formation as well as during their subsequent manipulation or incubation.

If stability is a primary concern, techniques for enhancing vesicle lifetime after preparation have also been reported. For example, Angelini *et al.* improved stability by depositing PEGylated nanotubes on a membrane surface of charged multilamellar liposomes through charge interactions,[89] while Li and Palmer used actin polymerisation along the internal surface of the membrane.[6] The use of cross-polymerisable synthetic lipids to form the membrane, followed by initiation of polymerisation after liposome formation, have also been shown to greatly improve stability.[90–92] Although modifications like these are not ideal in many applications, they do result in a significant improvement in liposome stability. Indeed, Lee *et al.* carried out stability tests on vesicles prepared by electroformation, and found that they were strong enough to withstand electroporation and the mechanical stresses of microfluidic systems without reinforcing treatment after formation.[93]

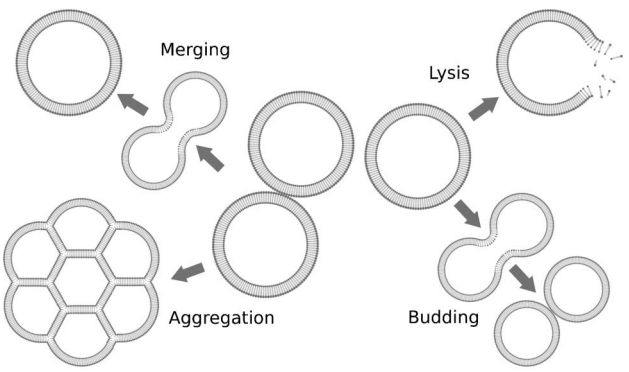

Fig. 3 Vesicle instability can manifest itself as merging, aggregation, budding, or lysis.

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

Encapsulation efficiency. Encapsulation efficiency is defined as the ratio of the concentration of material encapsulated to that found outside the vesicle after formation. Encapsulation efficiency can be inherently poor if the method does not separate the internal and external phases throughout the formation process. Stability also affects encapsulation efficiency, as ruptured or leaking vesicles release their contents into the external solution.[94,95] One of the main purposes of encapsulation of materials into vesicles is to isolate them from other components in an experiment. A process with a low encapsulation efficiency clearly defeats this purpose.

Several methods exist for determining encapsulation efficiency.[4] This is typically done by first separating loaded vesicles from the sample, followed by their disruption and then bulk quantification of the previously entrapped material by size exclusion chromatography or other separation techniques.[5,96,97] NMR spectroscopy can also be used in some cases, with the advantage that it does not require lysis of the liposomes or removal of the untrapped sample from the external medium.[98] Finally, individual vesicles can also be characterised using confocal single-molecule detection.[99] It should be noted that finding the true encapsulation efficiency value is a non-trivial problem, due to the difficulty in distinguishing between internal and external material, as well as material adsorbed to the membrane.[83]

In practice, the significance of the encapsulation efficiency of a method can be interpreted in different ways. The fact that all target material is contained within the liposome membrane does not necessarily mean it is functional or can be delivered. For example, the activity of a protein may be compromised during the formation process such that only a fraction of the sample is functional after encapsulation.[83] Methods involving high temperatures, large or rapid temperature changes, phase changes, high shear stress, electric fields, or incompatible chemical environments may disrupt or inactivate fragile biological samples. Therefore, when choosing a method for a particular application, one should consider that descriptions of encapsulation efficiency do not necessarily account for the function of the material being enclosed.

The problem of low encapsulation efficiency can often be worked around by employing techniques such as dialysis, filtration, or column separation of the resulting vesicles after they are formed.[100–102] Such methods can remove most if not all of the material that has not been encapsulated.[103,104] However, this clearly adds to process complexity, wastes material, and may compromise vesicle stability.

Another option is to use micro-injection to introduce a desired material into an already-formed liposome. This requires the trapping of a liposome and careful disruption its membrane with a microneedle.[105,106] The reliance on individual trapping and disruption of a vesicle membrane for injection of material is a major drawback in terms of throughput, however. Furthermore, due to the highly sensitive nature of the trapping and injection operations, it is likely that liposomes of different sizes, composition, or with different contents will respond differently to the procedure.

2 Microfluidic liposome formation

2.1 Electroformation and hydration

Electroformation is probably the most common method for vesicle production. The process was first described by Angelova and Dimitrov in 1986.[53] It involves spreading lipids dissolved in an organic solvent such as chloroform on the surface of a planar electrode, evaporation of the solvent by vacuum desiccation to form a dry phospholipid film, immersing the coated electrode in an aqueous solution, and finally applying an electric field across the lipid film and surrounding buffer. The lipids interact with the aqueous solution and electric field by ''peeling off'' the electrode surface in layers and self-assembling into giant but polydisperse, multilamellar vesicles. Later refinements of the method have allowed for unilamellar vesicles to be consistently produced.[107–109]

One drawback of the first implementations of the electroformation method was the requirement for low salt concentrations in the buffer. Since most proteins require high salt concentrations in order to function properly, this made it very difficult to encapsulate active proteins using electroformation.[107,110] Recent refinements to the technique have overcome this limitation and produced liposomes by electroformation using physiologically relevant salt concentrations.[54,111–113] There is also a concern that the electric field applied during the vesicle formation process may disrupt proteins that contain charge.[49] Though this may be the case, Girard *et al.* and Aimon *et al.* have shown functional proteins incorporated through electroformation.[114,115]

The electroformation method has been successfully implemented within microfluidic channels by Kuribayashi *et al.*[116] The process was a simple modification of the conventional method in which they coated an electrode with a film of dry lipids and then placed two slits made of silicone rubber over it. The slits were then covered with a second electrode plate, thus forming microfluidic channels through which buffer could flow. The remainder of the process was identical to the conventional method, and resulted in a polydisperse population of liposomes.

In a further development, LeBerre *et al.* showed that vesicle size and size distribution could be controlled by artificially fragmenting the lipid film through patterning of the electrode surface with elevated microstructures (Fig. 4).[117,118] This limited the film segments to the size similar to that of the microstructures. Since these were small, fragments did not break apart when peeling, resulting in vesicles with surface areas equal to that of the patterned structures. This was a significant improvement over the standard electroformation method because it allowed for the formation of monodisperse vesicles.

Hydration is another popular technique used for the formation of vesicles. The process is similar to that of electroformation, except that no electric field is applied. Instead, a vortex or controlled flow of aqueous buffer solution is passed over the coated surface. The shear stress acting against the lipid layers causes them to peel off, break, and self-assemble into liposomes. However, since there is no control over ''peeling'' and ''fragmentation'', the resulting vesicles are polydisperse and multilamellar.[22,56,83] In addition, the hydration method is extremely sensitive to the type of phospholipids

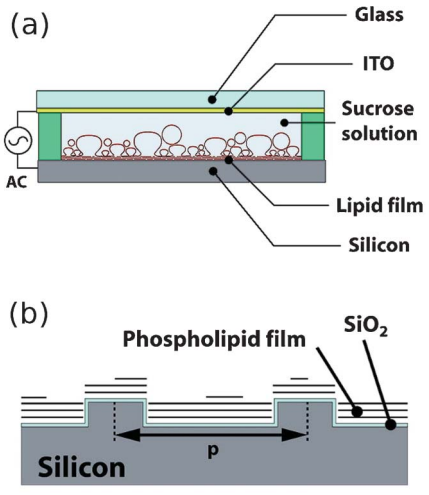

Fig. 4 The experimental setup for the electroformation of giant phospholipid vesicles on a silicon substrate (a), as demonstrated by Le Berre *et al.* (b) A section showing only the silicon electrode, patterned with an array of micropillars 170 nm in height and with pitch sizes *p* of 7, 15, and 60 μm. Adapted with permission from ref. 117. Copyright 2008, American Chemical Society.

used and physical conditions such as the osmolarity of the buffer solution, temperature, and pH.[22,57,83] For this reason, its use in applications where encapsulation is intended is limited, because the materials being encapsulated will often affect the formation process.

The use of hydration in a microfluidic device has been described by Lin *et al.* in the formation of microtubes, and under certain conditions, connected networks of microtubes and vesicles.[119,120] This method was similar to that of Kuribayashi *et al.* in that the dry lipid film was formed on a glass slide before being covered by a block of polydimethylsiloxane (PDMS) with microfluidic channels patterned on the surface. The microtubes and vesicles were formed by continuous flow of an aqueous buffer solution over the lipid film. The resulting liposomes, however, were not unilamellar or monodisperse (Fig. 5). Nonetheless, the ability to accurately control the buffer flow rate inside the microfluidic channel allowed them to find the optimum conditions for obtaining different types of structures—a notable advantage in comparison to the macroscale hydration method.

The processes of electroformation and hydration do not physically separate the solution that will eventually end up inside the vesicles from that which will be outside. Materials to be encapsulated in vesicles are dissolved or suspended in the aqueous solution in which the vesicles are formed. There is no way to isolate the contents of a vesicle from its external environment during the formation process. As a result, these two methods have a low encapsulation efficiency. This limitation is also present when the process is carried out inside a microfluidic channel.[116] A second limitation, related to the usability of the procedures described by LeBerre *et al.* and Lin *et al.*, is that every iteration of the process requires the deposition of a lipid film on a substrate followed by several hours of desiccation by vacuum before assembling the microfluidic channels. This prevents the continuous operation of the device, since lipids cannot be replenished during operation.

2.2 Extrusion

Extrusion of vesicles is typically carried out after their formation to modify properties such as lamellarity, size, and size distribution. The process was first described by Olson *et al.* in 1979.[121] It consists of passing a lipid or vesicle solution multiple times through the pores of a polycarbonate membrane or other mesh of small apertures. Extrusion makes vesicles unilamellar and monodisperse, with diameters controlled by the size of the pores, the pressure applied during the extrusion process, and the number of times the solution is passed through the membrane.[51,122] For example, Jousma *et al.* showed that a single pass through a membrane with a pore size of 200 nm resulted in liposomes with diameters of 320 $\pm$ 50 nm, while five passes yielded vesicles 143 $\pm$ 10 nm in diameter.[123]

The basic concept has been applied on a microfluidic chip by Dittrich *et al.* in the actual formation of liposomes (Fig. 6).[124] The technique involved the use of a free-standing silicon nitride membrane, on which a lipid film coating was produced by desiccation, covering apertures with diameter of approximately 3.5 microns. Aqueous flow around the silicon-

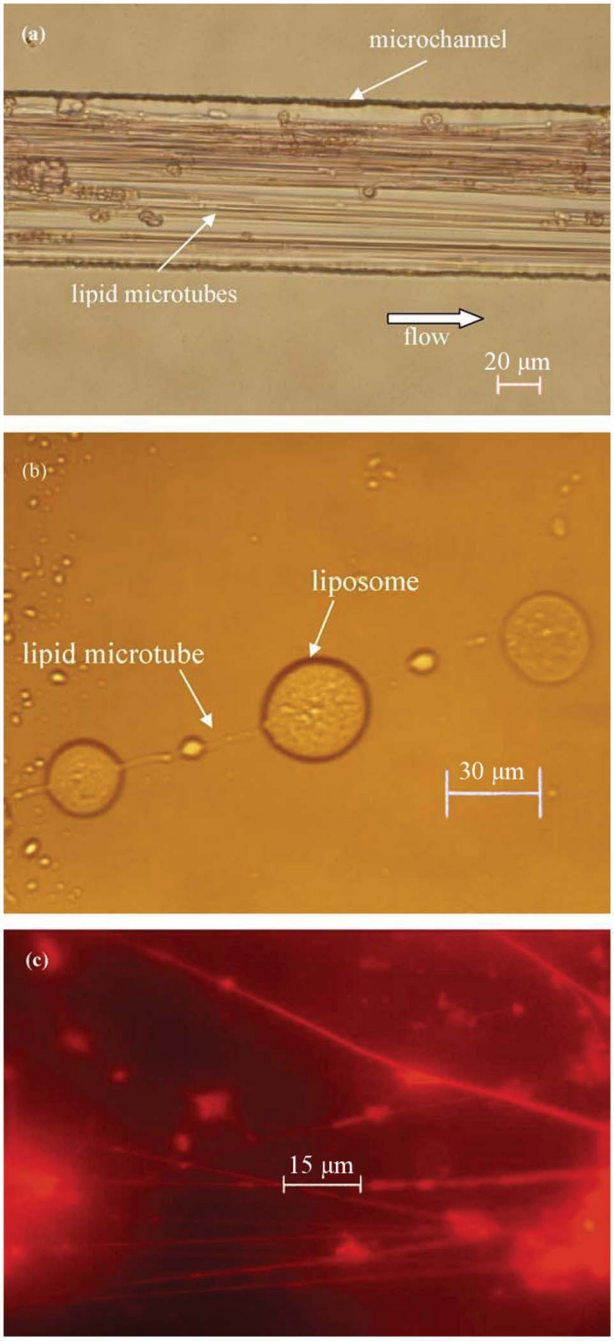

Fig. 5 Microtubule formation by hydration in a microfluidic channel, by Lin *et al.* (a) The DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) microtubules formed in a PDMS channel are stable and controllable. (b) The method sometimes also generated nanotube-vesicle networks. Vesicles are connected by nanotubules, allowing for material exchange between them. (c) A fluorescence image of a self-assembled network of lipid tubules and vesicles. Adapted with permission from ref. 120. Copyright 2006, Elsevier.

nitride membrane and through the apertures produced vesicles and microtubules, depending on flow speed. The resulting vesicles, however, were polydisperse. Nonetheless, this issue may be addressed if the method is modified such that the vesicles are passed through the membrane multiple

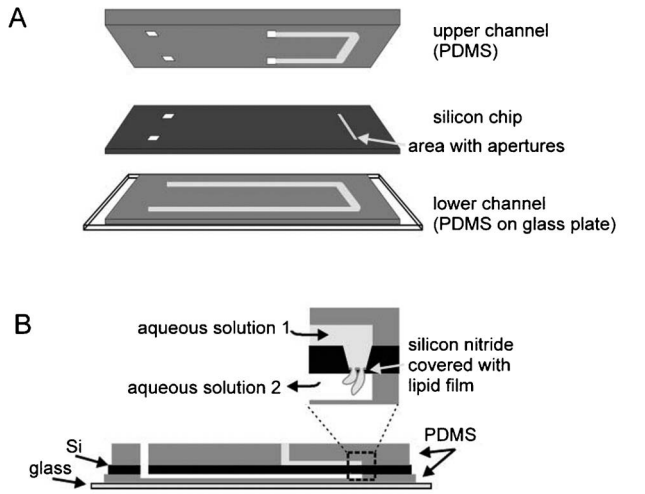

Fig. 6 A schematic of a microfluidic device for on-chip extrusion of vesicles and microtubules, developed by Dittrich *et al.* (a) The three layers in the device are thin PDMS layer, a silicon slide, and thick PDMS layer respectively. The white squares are the fluid reservoirs, and apertures are fabricated on the silicon substrate in a rectangular area of approximately 3 mm in length. (b) A cross-section of the assembled device and one of its 3.5 μm diameter apertures. Lipids are coated over the aperture, from which vesicles and tubules are extruded. Reprinted with permission from ref. 124. Copyright 2006, The Royal Society of Chemistry.

times. Unfortunately, the authors provide no discussion of lamellarity or the encapsulation efficiency of their method. But since there is no safe isolation between the solution to be encapsulated and that in which the vesicles will be contained in, it is likely that the encapsulation efficiency is low. By way of comparison, the work by Hope *et al.* reported an encapsulation efficiency of only 30%.[51] Similar to the microfluidic implementations of the electroformation and hydration methods, the coating of the free-standing silicon nitride membrane by phospholipid film adds complexity (in spite of vacuum desiccation not being necessary) and limits continuous use.

2.3 Flow focusing

The microfluidic flow-focusing method of vesicle production, demonstrated by Jahn *et al.*,[125] has no analogous protocol on the macroscale. This elegant technique consists of a central flow of a phospholipid-containing alcohol solution being intersected on either side by an aqueous solution. As the three flows merge into a main microchannel, the alcohol diffuses into the aqueous solution. As the alcohol dilutes past a critical concentration, the lipids spontaneously self-assemble into liposomes. Fig. 7 shows a schematic of the formation process. The vesicles produced have diameters between 50–150 nm, depending on flow rates, and are monodisperse.[125–127] It is noted that large vesicle sizes have not been formed using this method.

Microfluidic tweezing, described by Lin *et al.*,[119] is somewhat similar to flow focusing, and is also only feasible within a microfluidic format. The technique was developed for the creation of membrane tubes, but it may be possible to adapt it for the formation of vesicles, or to form vesicles from the tubes.[128] The technique involves dissolving phospholipids in

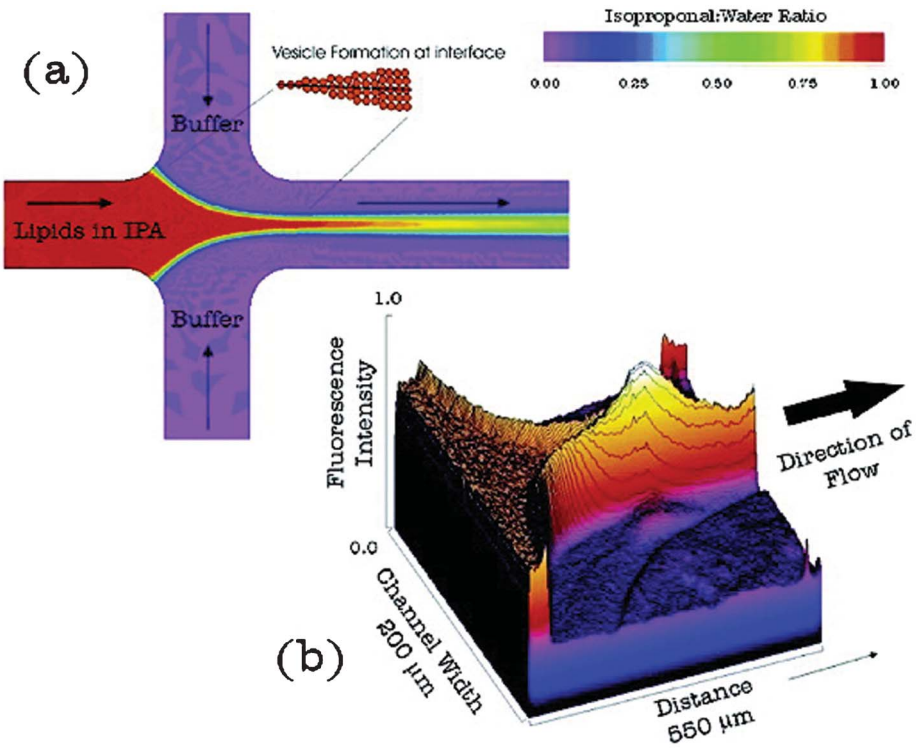

Fig. 7 Flow focusing vesicle production method, by Jahn *et al.* (a) Drawing of the vesicle forming microfluidic channel. The colour contours correspond to the concentration of isopropanol (IPA) and aqueous buffer. (b) A 3-D contour map of DiIC18 fluorescence intensity at the junction during vesicle formation. Reprinted from ref. 125.

water inside a chamber that is connected to a T-junction through a small aperture. The centre channel of the T-junction is oriented away from the aperture. Two fast flows arrive into the T-junction from opposite directions, and turn into the central channel, flowing away from the aperture. As the flows converge and turn, they pull the phospholipid solution through the aperture, forming membrane tubes.[119,128] However, the vesicles resulting from this method are not monodisperse or unilamellar.

Importantly, the flow focusing method can be used for high-throughput vesicle production.[129,130] The reaction solutions are prepared separately and stocked, and can be loaded into containers mounted onto the microfluidic device, allowing for continuous operation over long periods of time. There is no discussion of encapsulation efficiency in the work by Jahn *et al.* However, since membrane formation depends on a continuous, diffusive exchange of solvent into an aqueous phase, it is likely that some of the material to be encapsulated escapes to the exterior of the membrane before it forms. Furthermore, the solution that ends up inside the liposome also contains alcohol, with alcohol molecules being partitioned into the membrane. This means that the former may affect the behaviour of the encapsulated material of interest, and the latter will affect membrane properties such as stability.

2.4 Pulsed jetting

Pulsed jetting is a very elegant technique, first described by Funakoshi *et al.*[77] It mimics the action of blowing soap bubbles through a loop supporting a thin soap film. The method consists of creating a bilayer membrane by carefully contacting two lipid-stabilised water droplets,[131] and using a micro-nozzle or micro-pipette to shoot small jets of aqueous solution across it. The jetted volumes are wrapped by the membrane as they pass through, and as their momentum carries them further from the aperture, the membrane pinches off, forming a vesicle. Fig. 8 provides a simple illustration of the process.

This method forms unilamellar, monodisperse giant vesicles, though under certain operation regimes, a smaller satellite vesicle is also formed.[77,132] The size of the vesicles produced can be controlled by the jet dispensing time. The encapsulation efficiency is reportedly also very high, with effectively all the material jetted across the membrane being encapsulated. Indeed, efficiency is only lost if liposomes rupture after forming. However, it is not clear whether all biological material can survive the high shear stress of the jetting process without damage. It is possible that, although encapsulated within the liposomes, some proteins or other biomolecules are not functional after formation.

The technique was used by Funakoshi *et al.* to compartmentalise both DNA and Jurkat cells (though no discussion was provided in regard to cell viability). More recently, Stachowiak *et al.* used the same technique to encapsulate 500 nm nanoparticles as well as the pore-forming protein α-hemolysin[132] and, in continuing work, refined the apparatus so that an inkjet system was used to control liposome formation (Fig. 9).[133,134] One problem with this technique is that a residual amount of the solvent used in the phospholipid

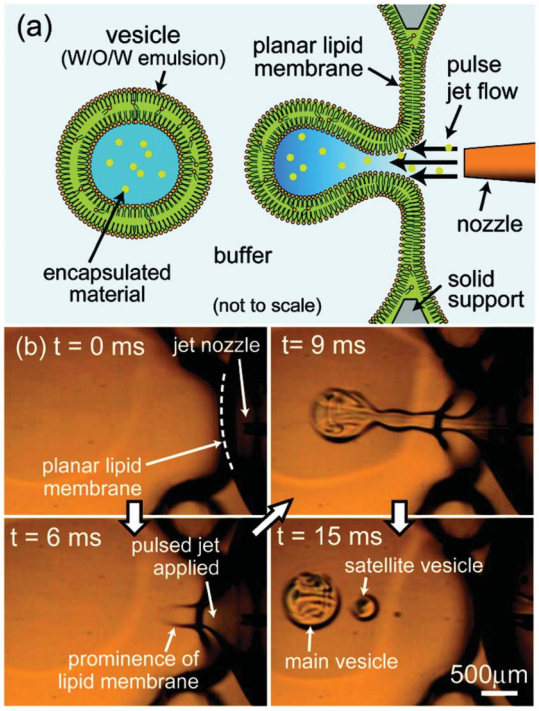

Fig. 8 Pulsed jetting, by Funakoshi *et al.* (a) A schematic of the vesicle formation method. The green area represents the organic solvent. (b) A sequence of images of vesicle formation captured by a high-speed camera. The planar membrane is stretched, forming a balloon that breaks out into spherical vesicles within 10 ms. Reprinted with permission from ref. 77. Copyright 2007, American Chemical Society.

solution is found inside the membrane, in between the monolayers.[77] Kirchner *et al.* determined, using Raman spectroscopy, that the decane solvent used in membrane preparation is present throughout the membrane, forming a layer up to tens of nanometres thick.[135] This is almost certain to affect the behaviour of some membrane-bound proteins and the passive diffusion of other materials across the membrane. Stachowiak *et al.* do not address this issue in their work, so it is not clear whether this problem can be solved.[132–134] Although this problem has not been reported previously in other techniques, it is likely to be a factor in other vesicle formation methods.

Two major shortcomings of this method are that it is non-trivial to set up, and very sensitive to both the operating conditions and the materials being used. Indeed, a micro-manipulator stage is needed to position the micro-nozzle in the right location with respect to the membrane. Since the droplets that form the interface bilayer are created by manual pipetting, it is very difficult to reproduce their exact location. This means that the micro-nozzle must be repositioned every time the apparatus is used. Also, since liposome formation depends on the membrane being deformed by the momentum of a fluid jet, the whole process is highly dependent on the viscosity of the solutions, operating temperature, and membrane composition. As a result, each time the materials being used are changed (lipids, and internal and external phases), it is likely that the protocol must be modified in some way.

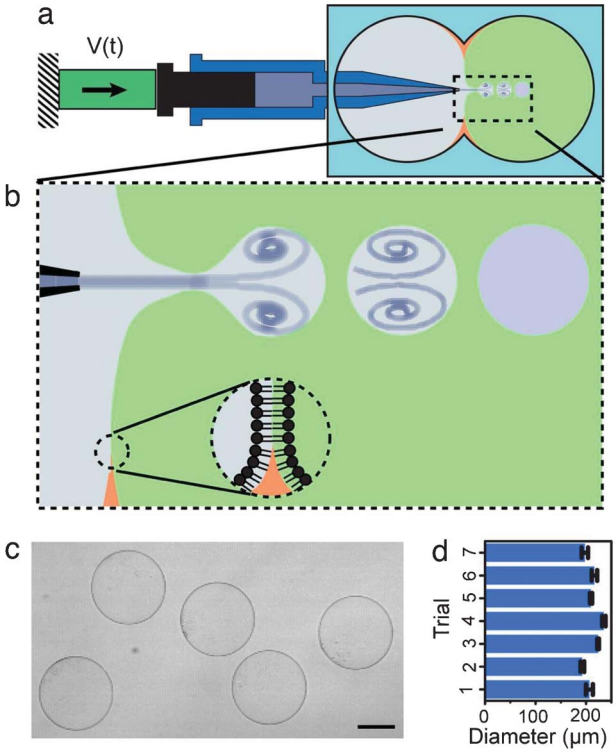

Fig. 9 Blowing vesicles by pulsed-jetting, after Stachowiak *et al.* (a) A schematic of the device and apparatus assembly. A micropipette is inserted close to a lipid membrane, and a piezo-electric actuator is used to generate pulses of fluid. (b) The pulsed jet crosses the membrane, forming vesicles. (c) A micrograph of the resulting liposomes (scale bar: 100 μm, and (d) the resulting diameters of vesicles formed in seven trials. Reprinted with permission from ref. 132. Copyright 2008, The National Academy of Science.

Another possible problem with the pulsed-jetting method is the high shear stress experienced by the sample during the jetting process. This may disrupt large or delicate proteins or other biomolecules, limiting the applications of this technique. However, to our knowledge, the consequences of this shear stress have not yet been investigated.

2.5 Double emulsion templates

Vesicle preparation by solvent removal from double emulsion templates is a high-throughput method that produces monodisperse giant unilamellar vesicles with high encapsulation efficiencies. It was first described by Shum *et al.*[136] Briefly, the method consists of the preparation of a lipid-stabilised water-in-oil-in-water emulsion in glass microcapillaries[137,138] (Fig. 10) or PDMS channels,[139] from which the oil phase is evaporated with the assistance of an organic solvent mixture of toluene and chloroform.[136,140] As the oil phase is removed, the lipid monolayers at the internal and external oil–water interfaces come together into a bilayer, thus forming a liposome. Unfortunately, not all of the solvent is removed during the evaporation step. The toxicity of toluene and chloroform and their ability to dissolve membranes greatly limits the breadth of applications of vesicles formed using this method. However, it may be possible to remove the oil phase without the use of organic solvents: Lorenceau *et al.* used this

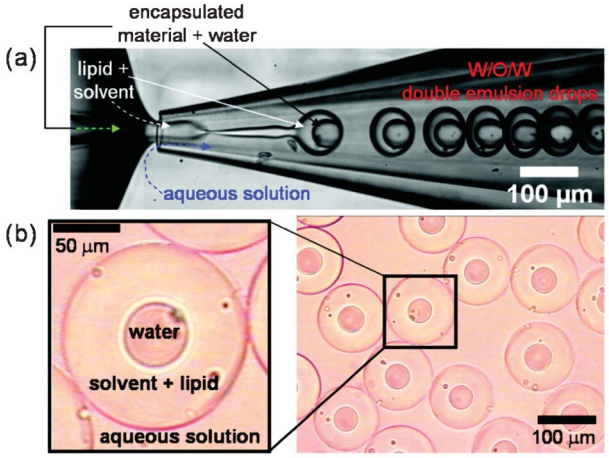

Fig. 10 Double emulsion templates, by Shum *et al.* (a) A glass microcapillary device forming a phospholipid-stabilised W/O/W double emulsion. (b) The collected double emulsion as seen through a microscope. The droplets have an aqueous centre surrounded by a phospholipid suspension. Reprinted with permission from ref. 136. Copyright 2008, American Chemical Society.

same method without solvents to produce vesicle-like polymerosomes, where the membrane was composed of diblock copolymers.[141]

A very similar method was developed by Tan *et al.*, who used a microfluidic device and oleic acid to produce a lipid-stabilised water-in-oil emulsion, and transferred it into an aqueous mixture of ethanol and water.[142] The oleic acid phase dissolved in the ethanol, forcing the phospholipids to rearrange into a bilayer membrane, thus forming a vesicle. Such vesicles are unilamellar, monodisperse, of controllable size, and very stable—they were shown to last for more than 26 days—and the procedure is simple and fast to implement. Fig. 11 provides an illustration of the method. More recently, the same group demonstrated the entire process performed inside a microfluidic device.[143] Since in this case the solvent used for removing the oleic acid from the membrane is ethanol, the method is much more biocompatible than those previously described.

Although they did not discuss encapsulation efficiency in detail, Tan *et al.* demonstrated that their method can successfully encapsulate nano-sized proteins, microbeads, and HeLa cells.[142] They also stated that encapsulation efficiency is variable, and will depend on the flow rate during the emulsification process and on the concentration of alcohol in the external aqueous mixture when the droplets are transferred. Their method separates the internal aqueous solution from the external one throughout the initial stages of the process, and so it is likely that encapsulation efficiencies are good. The final stage is where leakage may occur, *i.e.*, when droplets are transferred to the aqueous mixture of water and ethanol and the phospholipids rearrange into a bilayer. At this point, rupture and mixing of droplets with the external phase may occur before the lipids rearrange into a bilayer membrane. Loss of efficiency due to the emulsification step is likely to occur because the size of vesicles affects their stability, while the formation rate affects the lipid concentration at the

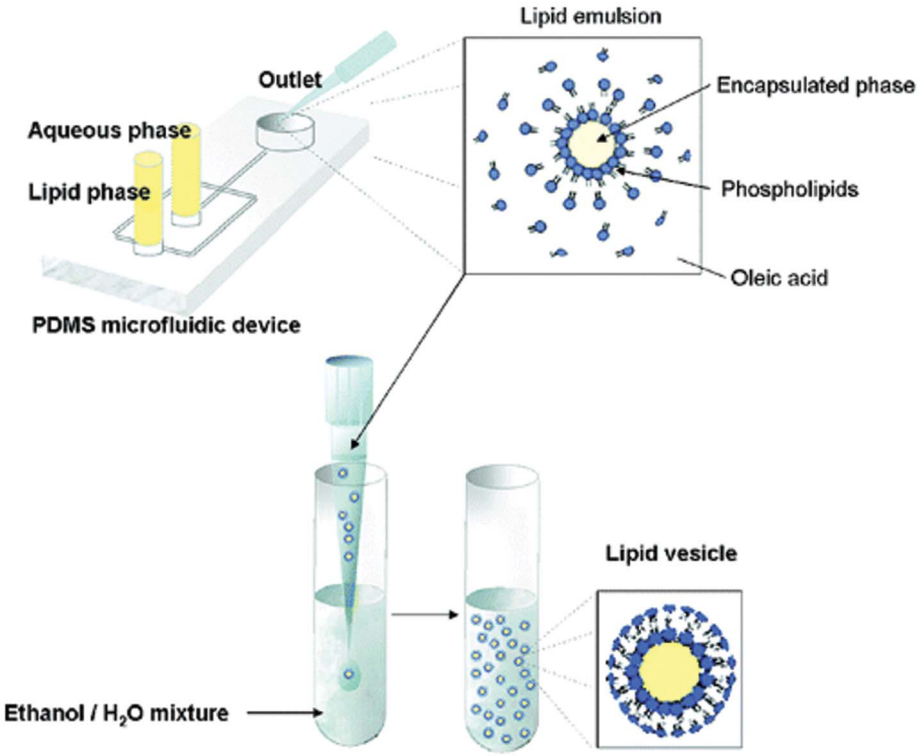

Fig. 11 Droplet emulsion transfer, by Tan *et al.* A schematic of the lipid vesicle preparation process. Reprinted with permission from ref. 142. Copyright 2006, American Chemical Society.

oil–water interface. Hence, if the droplets are too small or form too quickly (not giving enough time for lipids to stabilise the oil–water interface), the resulting vesicles are more likely to rupture and release their contents into the external medium, thus lowering the overall encapsulation efficiency.

2.6 Ice droplet hydration

The method of "lipid-coated ice droplet hydration" was developed by Sugiura *et al.*,[144] and results in monodisperse, giant multilamellar vesicles, with a low encapsulation efficiency (around 30%). The technique uses a microfluidic device to form a monodisperse water-in-oil emulsion stabilised by span-80 and stearylamine. This is then cooled such that the water droplets freeze. The droplets are then removed from the supernatant, after which surfactants are replaced by phospholipids. Next, the oil phase is evaporated, while the water droplets are still frozen, and an aqueous phase is added. The vesicles can then be extruded through a polycarbonate membrane to form unilamellar vesicles.[144,145] Fig. 12 illustrates the procedure.

The low encapsulation efficiency and the fact that resulting vesicles must be extruded in order to have unilamellar membranes are major drawbacks, especially since simpler methods exist which are less cumbersome and yield similar or better results.

2.7 Transient membrane ejection

An elegant method for forming vesicles that has only been demonstrated using microfluidic technology is that of transient membrane ejection, first demonstrated by Ota *et al.* (Fig. 13).[146] The technique consists of forming a lipid bilayer at a microchannel junction, and then disrupting that membrane to form vesicles. In this work, a laser is used to heat up a gas bubble in the aqueous solution on one side of the membrane. The resulting expansion of the bubble is enough to displace the fluid and push against the lipid bilayer to deform it. The bilayer then breaks off into liposomes in a way similar to the pulsed-jetting described in section 2.4. A modification of the technique by Kurakazu *et al.* uses pneumatic valves to decrease the volume of the channel instead.[147] The main advantages of this method are that it is fully integrated within a microfluidic platform, and results in monodisperse unilamellar vesicles with tunable size. However, the membrane used to form vesicles is eventually depleted, and needs to be replaced. This is achieved by pumping a lipid-containing oil phase into the junction, where a lipid-stabilised oil–water interface forms. The oil phase is then pumped out by a second aqueous phase. As the two oil–water interfaces come into contact, a bilayer membrane forms. This depletion and replacement of the membrane imposes a complication: the user must deal with intermittent oil phases separating batches of liposome samples. This membrane replacement action (and resulting two-phase output) along with the pneumatic or light actuation result in significant usability shortcomings in this method.

Ota *et al.* state that the lipid membrane produced by their method contains oil residue between the two lipid layers, though the discussion they provide on this topic claims that they did not experience problems related to this issue.[146]

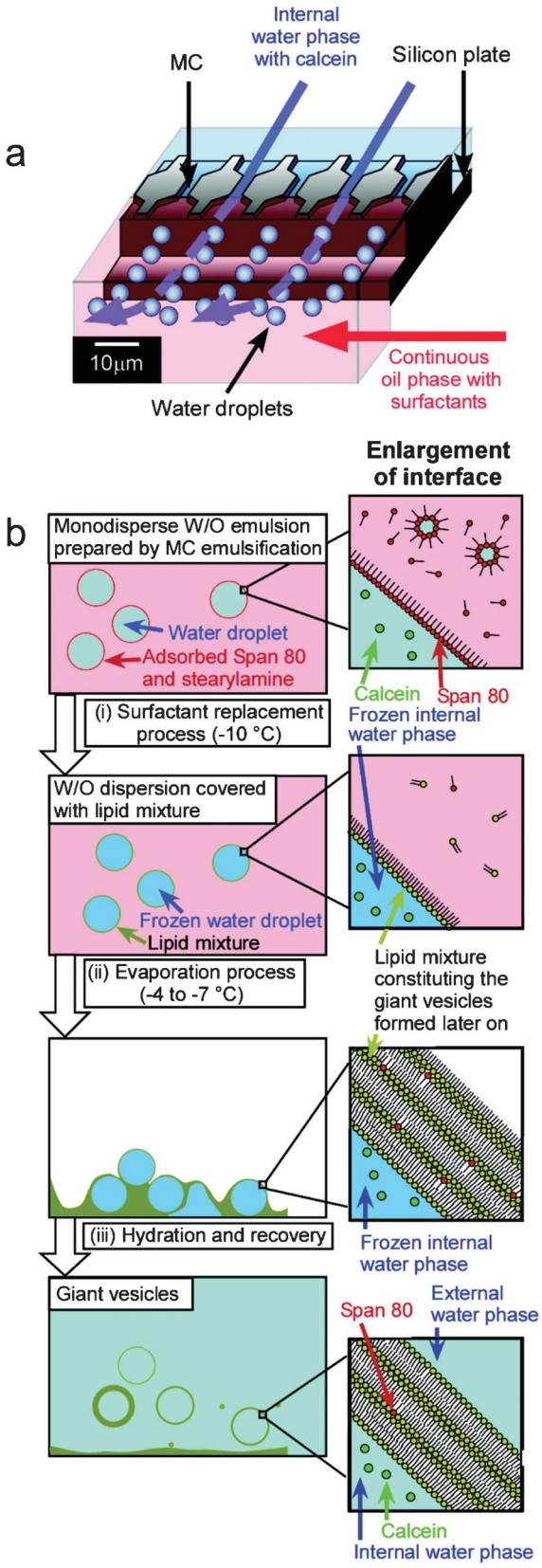

Fig. 12 Vesicle preparation by ice droplet hydration in a microfluidic device, by Sugiura *et al.* (a) A schematic of the microfluidic device, and (b) the process of ice droplet hydration for surfactant replacement. Reprinted with permission from ref. [144]. Copyright 2008, American Chemical Society.

Nonetheless, the membrane pore protein α-hemolysin was successfully incorporated into the membrane, and an *in vitro* gene-expression system based on *E. coli* cell-extract was encapsulated and used to express green fluorescent protein.

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

2.8 Droplet emulsion transfer

The droplet emulsion transfer method has been described previously on the macroscale by Pautot *et al.* (Fig. 14).[59] It consists of stabilising a water-in-oil emulsion (formed by vortexing or pipetting) with phospholipids, and then transferring the droplets to an aqueous medium. As the droplets cross the interface between the oil and aqueous phases, they pick up a second lipid layer, forming a unilamellar bilayer membrane.[58,59,148,149] This method was used by Noireaux *et al.* to carry out vesicle encapsulated gene expression, and proposed as a first step towards artificial cells.[38]

Since the original macroscale implementation of this method by Pautot *et al.* carried out the emulsification process by vortexing, the resulting vesicles were not monodisperse.[59] However, emulsification can be performed by other means. For example, the method has been significantly improved by using a microfluidic device to form the droplet emulsion, which is then collected and transferred in bulk across a preformed oil–water interface.[150–152] The use of a well-established microfluidic droplet formation technique eliminated the polydispersity issues experienced in the macroscale implementation.

Another droplet emulsion transfer method, this time fully integrated into a microfluidic device, was described by Matosevic *et al.*[153] In this work, they formed lipid-stabilised water-in-oil droplets which were then displaced by a ramp-shaped obstacle into a co-flowing aqueous stream. When pushed from the organic into the aqueous phase, the droplets picked up a second monolayer of lipids at the oil–water interface (see Fig. 15). This displacement stage, however, proved to be too disruptive, causing 95–99% of the droplets to burst as they crossed the interface. As a result, encapsulation efficiency (which should be close to 100% dropped to 83%), yield, and throughput were compromised. This method, however, seems very promising if the displacement step can be optimised to prevent droplets from bursting at the interface.

A major concern with the emulsion transfer method is the likely presence of oil residue inside the lipid bilayer after vesicle formation. Since the process involves the transfer of droplets through an oil phase, it is reasonable to suspect contamination of the final liposome membrane. As discussed in Section 1.1, the presence of oil in the lipid bilayer may have a significant impact in the behaviour of the membrane and associated proteins. It is therefore important to address this matter.

In their work first describing emulsion transfer, Pautot *et al.* performed a thin-layer chromatography assay on the final vesicles comparing them to pure lipids and pure oil samples, and could not detect the presence of oil in the vesicle sample.[59] This indicated that if any oil was present, it was

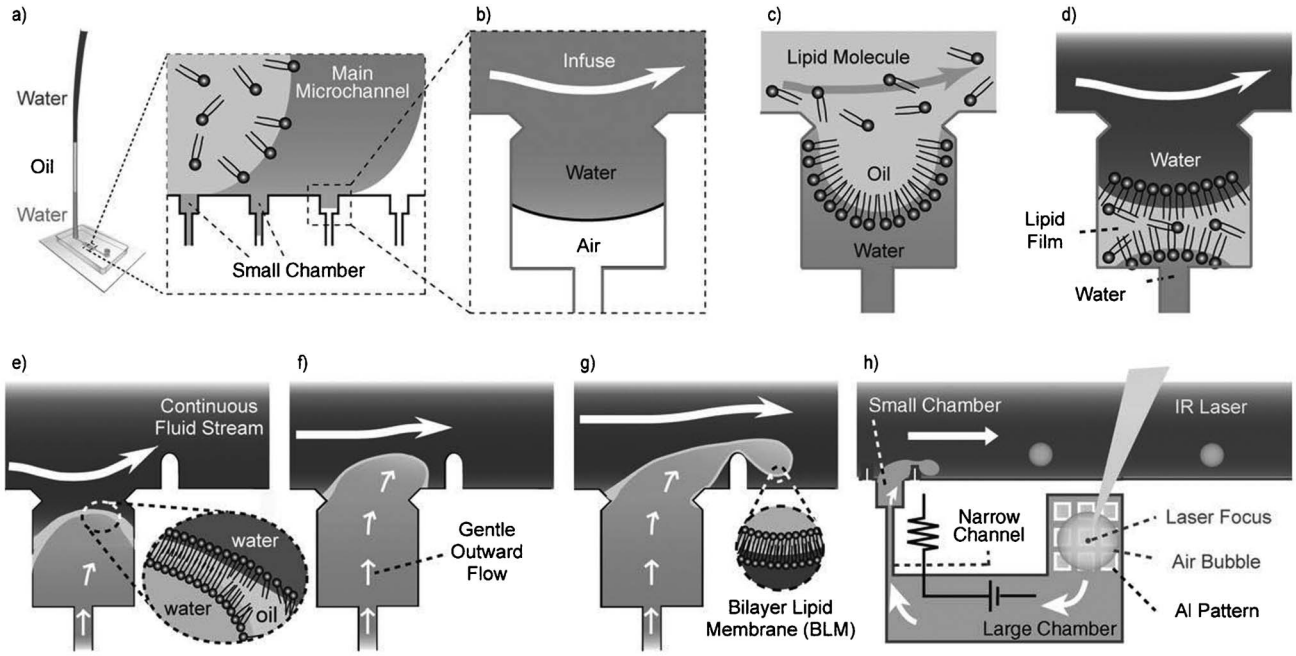

Fig. 13 The transient membrane ejection method invented by Ota *et al.* (a) Water, oil containing lipids, and water are sequentially passed through a main microfluidic channel flanked by many small chambers. (b) The first water phase enters the chambers, pushing the air out through the PDMS. (c) As the lipid-containg oil passes through the main channel, it forms a lipid-stabilised oil–water interface at the entrance of the chambers, enclosing small volumes of water in them. (d) As the second water phase displaces the oil, the two lipid-stabilised water–oil interfaces come into contact at the chamber entrance, forming a lipid bilayer membrane. (e-f) A gentle flow generated from the chamber out into the main channel displaces the membrane, causing it to bud off into vesicles. (h) This gentle outward flow is generated by the controlled expansion of an optically generated microbubble. Reprinted with permission from ref. 146. Copyright 2009, Wiley-VCH Verlag GmbH.

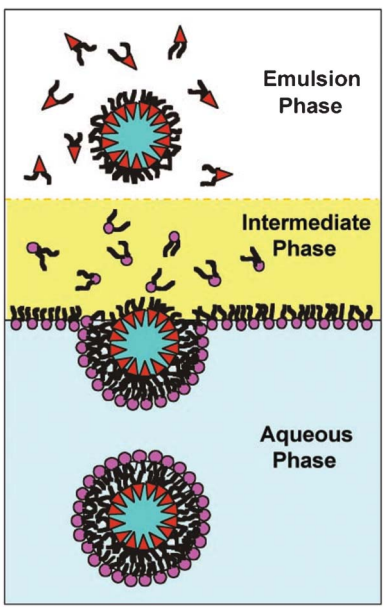

Fig. 14 Droplet emulsion transfer method for vesicle formation, developed by Pautot *et al.* Water droplets are formed in a lipid-in-oil suspension, and then passed through a oil–water interface which is itself stabilised by phospholipids. As the droplets cross the interface into the aqueous phase, they pick up a second layer of phospholipids, forming a bilayer membrane, and thus become vesicles. Reprinted with permission from ref. 58. Copyright 2003, The National Academy of Sciences.

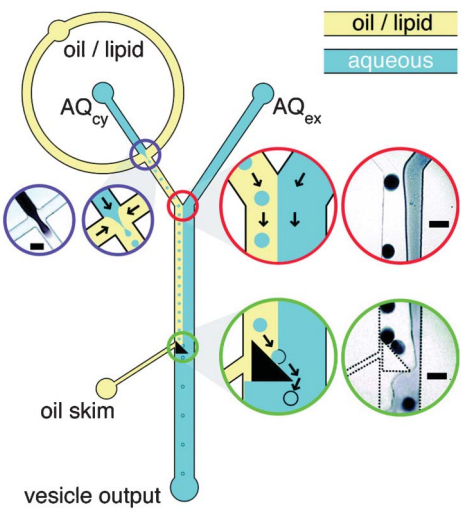

Fig. 15 Fully-integrated microfluidic droplet transfer method for vesicle formation, developed by Matosevic *et al.* Lipid-stabilised water-in-oil droplets are formed by flow-focusing of water into an oil stream at a microfluidic junction, and then forced across an oil–water interface by a ramp-shaped barrier. Similar to the macroscale method, the droplets pick up a second layer of lipids as they cross the interface, forming a bilayer membrane, and thus becoming liposomes. Scale bars: 100 μm. Reprinted with permission from ref. 153. Copyright 2011, American Chemical Society.

less than 5% of the membrane (that being the sensitivity limit of their detection technique). They also suggested that squallene, a complex mixture of long-chain alkanes, is a good candidate for forming oil-free liposomes by emulsion transfer because it is immiscible in fully hydrated bilayers above the phase transition temperature.[59,72]

However, Kubatta *et al.* showed that a large amount of oil was clearly visible in the membrane of vesicles formed by emulsion transfer.[154] It should be noted, though, that the vesicles were between 3 and 5 millimeters in diameter—much larger than typical liposomes. On the other hand, Abkarian *et al.* claimed that their vesicles formed by emulsion transfer decreased in volume in a time-scale comparable to electroformed ones when exposed to osmotic overpressure, and also that their membranes showed fluctuations with similar amplitude.[155] Additionally, they could not detect any membrane defects using light microscopy at 300 nm resolution, and they also managed to incorporate the pore-forming protein α-hemolysin.

It is clear that the matter of oil residue presence in the membrane of liposomes formed by emulsion transfer is not resolved. Different implementations and different materials appear to yield different results, and when the issue is addressed, it is often not done satisfactorily. However, emulsion transfer can circumvent many of the difficulties encountered in other liposome formation methods, such as low encapsulation efficiency, unphysiological conditions, and polydispersity. Therefore, it would be of great utility if future work published with this method employed more sensitive techniques such as Raman spectroscopy or small-angle X-ray scattering (SAXS) to analyse the membrane of their liposomes.[4,135,156]

3 Conclusions

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

While progress has been rapid, a method that is capable of addressing all the requirements of the various applications of liposome technology has yet to be realised. For each method there is a trade-off. For example, hydration, electroformation, and extrusion result in excellent quality liposomes, but have very low encapsulation efficiency. Of those that achieve high encapsulation efficiencies, pulsed jetting and transient membrane ejection are difficult to implement, while double emulsion templates and ice droplet hydration are not very compatible with biological processes. Finally, droplet emulsion transfer, in spite of being a promising technique, has yet to be implemented in a reliable fashion.

Due to their qualities and drawbacks, different methods remain useful only in different application niches. For membrane protein biology membrane quality is paramount, while encapsulation efficiency or volume characteristics are less important. In such applications, electroformation and extrusion are well suited for vesicle production and hence their immense popularity. However, when liposomes are used as cell models, encapsulation efficiency and monodispersity are just as important as membrane quality. This demand has resulted in the invention of the other techniques, such as pulsed jetting, transient membrane ejection, and droplet emulsion transfer (Table 1). Finally, the use of liposomes as enclosures for drugs or other chemical species tends to require specific membrane compositions or stability over defined periods of time, with different specific of demands depending on the intended use.

In all cases, however, microfluidic technologies for liposome formation are already establishing themselves as useful tools. Looking ahead, there are numerous opportunities for developing the processes that occur either upstream or downstream of liposome formation itself. For example, there is an array of droplet-based technologies available for manipulation, mixing, dilution, and concentration of the

Table 1 Liposome formation method comparison

	Electroform. or hydration	Extrusion	Flow focusing	Pulsed jetting	Double emulsion templates	Ice droplet hydration	Transient membrane ejection	Droplet emulsion transfer
Lamellarity	Multi- and unilamellar	Unilamellar	Unknown	Unilamellar	Unilamellar	Unilamellar	Unilamellar	Unilamellar
Usability	Medium	Medium	Easy	Hard	Medium	Medium	Easy	Medium
Solvent in membrane?	No	No	Unknown	Yes	Unknown	Unknown	Yes	Yes/Maybe
Encapsulation efficiency	Low[a]	Low	Low	High[a]	High	High	High	High
Size range	LUV-GUV	SUV-LUV	SUV	GUV	GUV	LUV-GUV	GUV	GUV
Size distribution	Polydisperse	Monodisperse	Monodisperse	Monodisperse	Monodisperse	Monodisperse	Monodisperse	Monodisperse
References	116, 117, 119, 120	116	119, 125–130	77, 132–134	136, 140–143	144, 145	146, 147	150–153

[a] As discussed in Sections 2.1 and 2.4, it is possible that the electroformation and pulsed-jetting processes may disrupt the function of encapsulated material.

reagents to be encapsulated. Due to the similarity between liposomes and microdroplets or living cells, many established microfluidic technologies can also be adapted for use with liposomes downstream of their formation.

Already many downstream operations are being performed with liposomes. For example, Nishimura *et al.* have described sorting of vesicles by flow cytometry.[33,157] Operations such as liposome merging[158–160] and splitting[161–163] have also been demonstrated, as well as trapping,[164,165] anchoring, and perfusing with reagents.[166] Notably, Robinson *et al.* have developed a microfluidic platform in which a population of liposomes can be mechanically trapped into individual chambers, allowing single-liposome observation and manipulation.[167]

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

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