

Lipid Bilayers and Liposomes on Microfluidics Realm: Techniques and Applications



Fatih Inci

1 Introduction

Liposomes and supported lipid bilayers (SLBs) can act as biomimetic snapshots of cellular compartments and intact cells as they depict capacity to integrate and encapsulate biomolecules such as DNA, enzymes, proteins, drugs, or other biochemicals [1, 2]. Due to their biomimicry, the formation and application of those entities have been extensively studied through different conventional methods. However, current techniques have limitations in terms of process control, poor reproducibility, inefficient use of materials and reagents required for their formation. To minimize and address most of those associated challenges, recent efforts on microfluidics hold great promise as it provides easy handling of fluids, the need for miniscule sample volume, and confined structure with low Reynold Numbers [3]. In addition to its technical achievements, microfluidics allows the integration of other micro/nanotechnology modalities such as sensors, and also eventually reduces assay cost and increases throughput and analytical performance [3, 4]. In this chapter, we extensively provide various fabrication techniques of microfluidic systems to form liposomes and SLBs for the application of biology, physics, and medicine. Lipid composition, encapsulation efficiency, versatility, size distribution, and applicability will be discussed in detail.

F. Inci (✉)

Department of Radiology, Stanford University, School of Medicine, Canary Center at Stanford for Cancer Early Detection, Palo Alto, CA, USA

e-mail: finci@stanford.edu

2 Electroformation and Hydration

Electroformation, one of the most common techniques, is based on the spreading lipids on a planar electrode surface, formation of a dry lipid film through desiccation, rehydration of the film, and consequently applying an electric field to peel off lipids from film structure to form liposomes spontaneously [3]. This strategy obviously results in generating polydisperse and multilamellar lipid vesicles. There have been considerable efforts on forming unilamellar vesicles [5, 6]. For instance, this technique was employed to produce various types of giant liposomes in microfluidic channels [7]. By applying an AC voltage, the vesicles were formed inside the channels defined between glass slides coated with indium tin oxide (ITO) electrodes. Despite the production of unilamellar structure and encapsulation of micro/nano-sized objects in the vesicles, large variations in the diameter were observed on this report. On the parallel track, silicon (Si) was employed as a substrate for the electroformation of giant phospholipid vesicles (Fig. 1) [8]. Through tuning silicon surface properties (chemical composition and microstructures), the researchers were able to manage the molecular organization of phospholipid film and the properties of final vesicles with a defined size control. Further adaptation of the same platform could be potentially employed for the molecular organization and transition from 2-D to 3-D self-assembly for other self-organized systems. As another example, functional voltage-gated potassium channel, KvAP, was integrated into cell-sized Giant Unilamellar Vesicles (GUVs) by using electroformation method on a microfluidic chip [9]. Therefore, multiple parameters such as membrane composition, tension, and geometry could be controlled. Microscopic and patch-clamp measurements confirmed the unity, selectivity, and functionality of the channel proteins. In general, potential obstacles on the electroformation strategy can be summarized as the limitation for the use of high/physiological level of salt

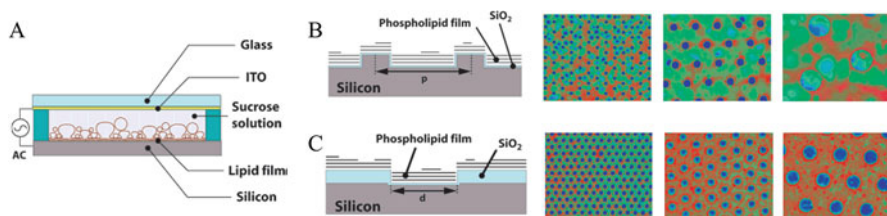


Fig. 1 Electroformation strategy. Schematic illustration depicts (a) the experimental setup and strategy for the electroformation on a silicon surface. (b) The schematic demonstrates the microstructures with 170 nm height and different pitch sizes, and the reflection microscopy images (in false colors) of the phospholipid film formed on various microstructured surfaces with a pitch size of 7, 15, and 60 μm are presented. (c) Further integration of an insulating patterned mask of SiO₂ on the silicon substrate is depicted. The schematic demonstrates the phospholipid film on the substrate. Reflection microscopy images (in false colors) of the phospholipid film organization are presented for hole sizes of 7, 12, and 24 μm , respectively. (Reprinted with permission. Copyright 2006, American Chemical Society [8])

concentrations and possible hindrance to integrate membrane proteins due to the electric field required. Recently, GUVs were produced using commercially available syringe needles as electrodes [10]. This method implied that the electroformation process could be possible with an inexpensive and off-the-shelf material within a short period of time in contrast to the earlier studies.

On the other hand, hydration technique resembles major steps of electroformation method and only excludes to apply an electrical field. A vortex or controlled flow with high flow rates is applied, and the lipid film hence is removed from the surface due to the shear stress. However, lipid type and composition and osmolarity are major obstacles of this method. Through integrating a microfluidic platform, there have been many studies reported, and eventually, unusual structures such as connecting networks between liposomes were observed [11]. Overall, although these two techniques have capability to generate various sizes of vesicles, low encapsulation efficiency, the need for lipid film deposition on a surface, and lengthy desiccation step minimize their utility and large-scale production with an accurate size.

3 Extrusion Strategy

This method utilizes similar strategy of commercially available products in a microfluidic platform. Basically, a polycarbonate filter with a refined pore diameter is integrated on the inlet of microfluidic chip, and a controlled flow interacts with the lipid film on the filter surface. Finally, liposomes and microtubule structures are formed through this extrusion process [3, 4]. Apart from the conventional extrusion methods, microfluidics offers more control and minute sample volumes. Ultimately, the researchers have further employed this strategy to isolate nano-sized secretomes such as exosomes for cancer diagnosis [12–14]. As experienced in electroformation and hydration techniques, this method however requires the desiccation of lipid film, increasing the complexity and reducing the utility of strategy.

4 Flow Focusing

The major features in microfluidics systems are to enable the precise control of flow rate and to integrate multiple fluids on a single chip. As an example, by utilizing these assets, three inputs on a microfluidic channel were projected to form liposomes with a diameter of 50–150 nm [15]. On this design, a phospholipid-alcohol solution was loaded into the central input, whereas the remaining inputs introduced buffer solution to create an intersection between these different interfaces. This simple configuration provided monodisperse and unilamellar vesicles, but producing larger sizes have been still in progress with this technique. Akin to this strategy, a planar lipid bilayer structure was achieved through contacting two monolayers at the inter-

face between water and organic solvent on the chip [16]. In this study, two different methodologies were presented: (i) water droplet-based strategy in a well and (ii) flow-control strategy with an external syringe pump. Capacitance measurements were used to evaluate the functionality and unity of bilayer structure by collecting ion channel signals from reconstituted antibiotic peptides. By combining these two strategies, an SLB formation was achieved in a well-formatted microfluidic chip through driving by the shear forces of a bulk flow (Fig. 2a) [17]. Only altering the pH of the bulk solution was enough to guide the SLB localization whether to seal the wells (pH 9.5), creating free-spanning membranes or to follow the contour of the wells (pH 8.0) (Fig. 2b). In addition, a single channel was adapted to create lipid bilayer structures through subsequent flow of lipid and buffer solutions [18]. The researchers therefore created <5 nm thickness of free-standing lipid layer structures reliant on a self-assembly process driven by solvent extraction in a microfluidic channel. This strategy also allowed to integrate membrane channel proteins at single-molecule conductance resolution.

As another strategy, designing the miniaturization of droplet-interface-bilayer (DIB) was aimed to form lipid bilayers in microchannels [19]. Herein, multiple biophysical parameters were also tested to control the position and storage of phospholipid-stabilized water-in-oil droplets for the scale-up and automation of process, such as lipid concentration, immiscible phase flow velocities, and the

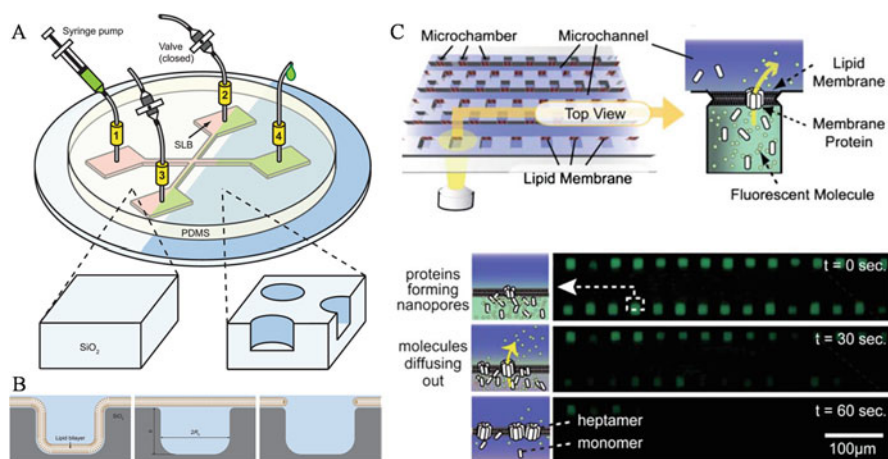


Fig. 2 Flow focusing strategy. (a) Illustration represents the combined version of well and flow-based bilayer formation platform. (b) A cross-section of the well is depicted. The lipid bilayer formation occurs in three distinct formats. It either follows the contour, spans the well, or stops at the edge of the well forming a pore in the lipid bilayer. (Reprinted with permission. Copyright 2010, American Chemical Society [17]). (c) Another flow focusing strategy is illustrated as a microchamber array. The lipid bilayers are generated perpendicular to an imaging plane. Fluorescent intensity is employed as a quantitative data to evaluate the translocation of confined fluorescent, non-charged molecules across the bilayer. Time-lapse images of the process are represented. (Reprinted with permission. Copyright 2011, The Royal Society of Chemistry [20])

device geometrical parameters. As a pilot data, diffusive transport of molecules and ions across on-chip DIBs was measured using fluorescence-based assays.

An array format of microfluidic device was also designed to form a large number of lipid membranes (Fig. 2c) [20]. This technique enabled to create multiple stable lipid membranes at a time and to regulate the concentration of solutions on each side of the membranes for molecular transport across the membranes. The same research group further leveraged this strategy to synthesize green-fluorescent proteins (GFPs) by confining an *in vitro* translation system [21]. The technique provided an array of microdroplets coated with a phospholipid membrane within a microfluidic platform, actively inhibiting the adsorption of proteins and DNA and minimizing the absorption of other molecules. The lipid-coated microdroplet array prolonged its stability for >20 hours and provided highly uniform structures.

5 Pulsed Jetting Strategy

Inspiring from the action of blowing soap bubbles and glass blowing has denoted an effective liposome generation method. This strategy requires two lipid-stabilized water droplets [16] and micro-nozzle/pipette [22] to create vesicles in the solution. The discharged volumes are encapsulated by lipid membrane, following a budding mechanism through their momentum, and this process consequently creates vesicles encapsulating materials in solution. Mostly, the formed vesicles are monodisperse, unilamellar giant vesicles, and the dimension of vesicle can be also manipulated by changing the nozzle diameter.

Via this technique, a pulsed microfluidic jet was deliberated to simultaneously create and load giant unilamellar vesicles (GUVs) [22]. This method formed multiple monodisperse, unilamellar vesicles encapsulating 500-nm particles, and ultimately demonstrated the incorporation of functional pore proteins into the vesicle membrane to mediate transport. Moreover, in another study, the vesicles formed by the pulsed jetting approach were comprehensively investigated using both optical dark-field microscopy and Raman micro-spectroscopy *in situ* [23]. As a result, majority of vesicles was found unilamellar, and n-decane thickness was considered as a critical parameter, which did not affect significantly the vesicle diameter, but could alter the bilayer properties. Recently, asymmetric giant vesicles were fabricated to investigate the dynamic responses of lipid molecules in the membrane [24]. Both utilizing the characteristics of distinct lipids and setting different nozzle parameters, the deformation of a sinusoidal undulation on the lipid microtubule was achieved. The giant vesicles were then jetted through inhomogeneous break-up of a lipid microtubule, which resulted in generating two main vesicle diameters (100–200 μm and 3–20 μm) (Fig. 3). Moreover, these asymmetric giant vesicles revealed the flip-flop behavior in the membrane, akin to the lipid flip-flop activity in apoptotic cells. Furthermore, *in vitro* synthesizing membrane proteins in the vesicles showed that the lipid asymmetry could improve the reconstitution ratio

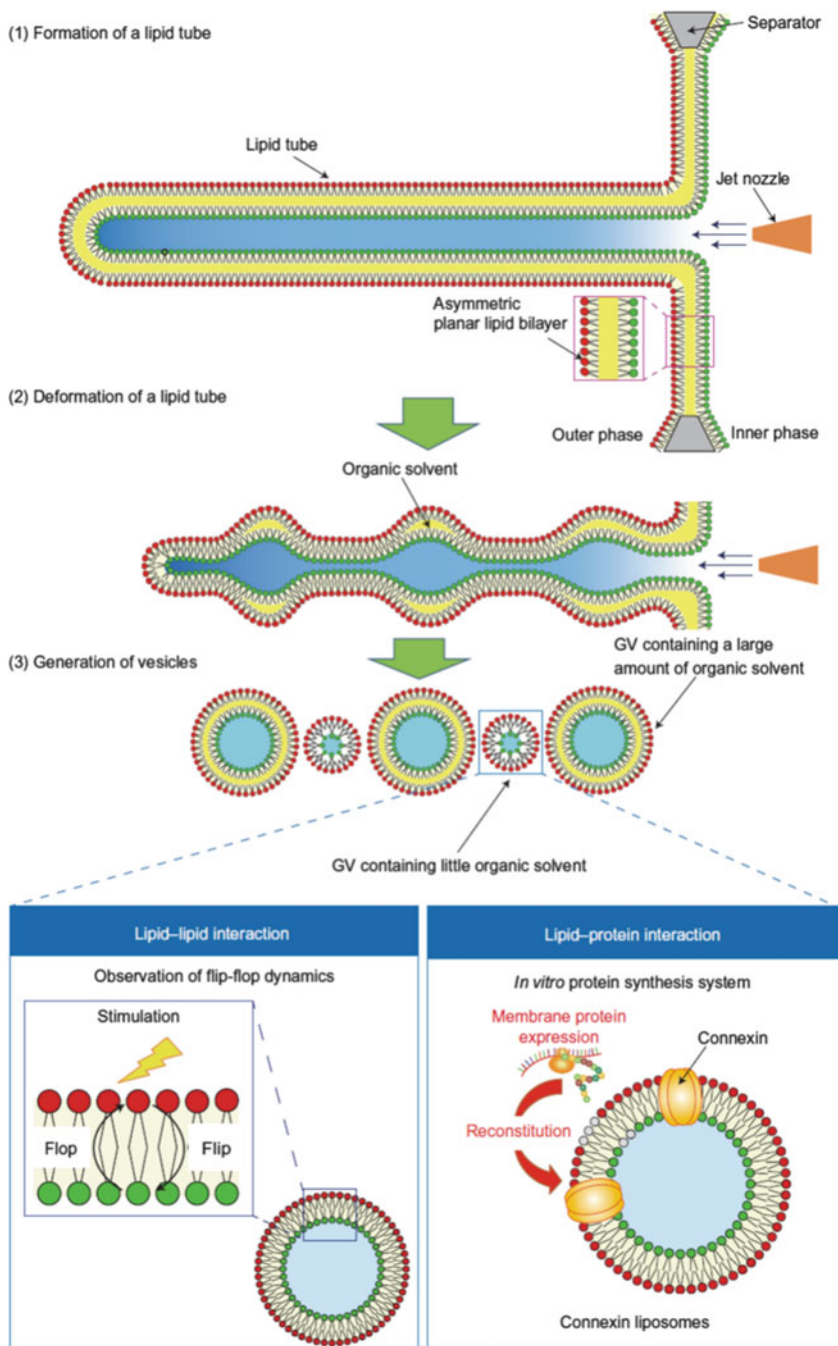


Fig. 3 Pulsed jetting strategy. The lipid microtubule is formed from the planar bilayer through the jet-flow method. The microtubule is then deformed and subsequently generates asymmetric vesicles from the unstable break-up of the deformed microtubule. As a result, two different sizes of vesicles are formed. Via this technique, lipid flip-flop and lipid-membrane protein interaction are easily investigated on the asymmetric giant vesicles. (Reprinted with permission. Copyright 2016, Springer Nature Group [24])

of membrane proteins. Such a crucial application will enhance our capability to investigate biophysical properties of lipids and membrane proteins.

In general, although this strategy has largely contributed to the microfluidic strategies by enabling high throughput, uniformity, and automation, there are considerable technical and biological drawbacks: (i) the fabrication of set-up is not facile and needs the sophisticated design parameters; (ii) the vesicle formation is intrinsically reliant on the operating conditions, material type, viscosity of solution, temperature, and membrane composition. Furthermore, (iii) manually positioning the nozzle for each fabrication is really difficult to reproduce the same vesicles at the same position, and the aforementioned parameters therefore need to be revisited for different assays. (iv) Encapsulation of cells and proteins, however, is another unmet challenge as high shear stress could potentially hinder their viability and 3-D structure. Addressing some of those challenges will significantly accelerate the deployment of acquired knowledge into novel strategies for the realms of drug delivery and cell therapy.

6 Emulsion-based Techniques

The emulsion is shortly described as a mixture of two immiscible liquids, where one liquid is dispersed in small drops in another liquid that generates a continuous phase [25]. The emulsion-stemmed strategies can be investigated under two major categories [3, 4, 25]: (i) double/higher order emulsion method and (ii) droplet emulsion transfer. The first category comprises of a lipid-stabilized water-in-oil-in-water emulsion inside glass microcapillaries or microfluidic channels. The oil phase is principally removed with the aid of an organic solvent mixture such as toluene and chloroform. Following this change, the lipid monolayers at the internal and external oil–water interfaces converge and form a liposome structure. Due to the toxicity of toluene and chloroform and their dissolving effect on the membranes, the researchers have modified the procedure by replacing the organic solvent mixture with either polymers [26] or ethanol–water solutions [27]. As an example, by utilizing a shear-focusing-based droplet forming method, the vesicle encapsulation was achieved for a range of application from nano-sized proteins to cancer cells [27]. This method assessed the technical parameters such as flow rate and concentration of ethanol in the system. As there was no need for toxic solvents, it prolonged the shelf life of the vesicles for >26 days. Despite having a unique capability to produce monodisperse giant unilamellar vesicles with high encapsulation efficiencies in a high-throughput manner, loss of efficiency due to the rupture of immature liposome products is still a major drawback, reducing the overall efficiency of encapsulation.

The latter method, the droplet emulsion transfer, describes the stabilization of water-in-oil emulsion with lipids, and followed by a transfer of droplets in an aqueous solution [3, 4, 25]. Through the transition from emulsion to intermediate phases, the droplet is shelled by the lipid at the intersection. As vortexing or pipet-

ting is mainly employed in this process, the production of monodisperse vesicles is highly challenging. As an important critique to be noted, the oil contamination is a remaining bottleneck even though various strategies and materials have been tested [3].

7 Array Strategies

The array format of assays provides an elegant feasibility to multiplex biological fashions on the same platform while testing the effects of various parameters intended in the research design. In the lipid bilayer realm, this creates a multitude of experimental options at unprecedented convenience and flexibility. As an example, vesicles were exposed on a pre-patterned surface to create a gradient of vesicles of various compositions (Fig. 4a) [28]. This was achieved by the flow and irreversible binding of vesicles to the substrate followed by the subsequent fusion into a bilayer form. The salient features of this method could be listed as: (i) expanding the current array format into hundreds of membrane corals could be possible, and (ii) a broad range of material compositions could be formed with two different sets of vesicles, simplifying the membrane array formation. Therefore, this method would be generally applicable for use with any molecules that could be incorporated into SLBs, denoting an accurate multiplex-array format for evaluating membrane-associated biomolecules.

To understand membrane–membrane recognition and binding, microfluidics was also employed as a docking unit to tether liposomes on a SLB structure via DNA molecules, hence minimizing the mixing of the vesicles in bulk and generating an array format (Fig. 4b) [29]. Once the tethering occurred, two reacting vesicles colocalized through DNA-mediated docking after collision and their subsequent tandem motion. Computational analyses and imaging by epifluorescence microscopy revealed that copy number and DNA sequence were two major affecting parameters.

Further improvements in terms of ease-of-use and applicability were reported on a 2-D fluidic device (Fig. 4c) [30]. This platform generated circuits of phospholipid films *in situ*, and enabled multiple chemical (functionalization and molecular transport) and physical operations (writing and erasing) according to the user-defined parameters. As depicted in this study, the feasibility of the “lab on a biomembrane” presents highly complex network generation, redesign of diffusional barriers, and hydrodynamic trapping sites. Such a user-defined, software-integrated system could potentially present a variety of experimental designs with high flexibility, facilitating the deployment and manipulation of SLBs in multiple research areas.

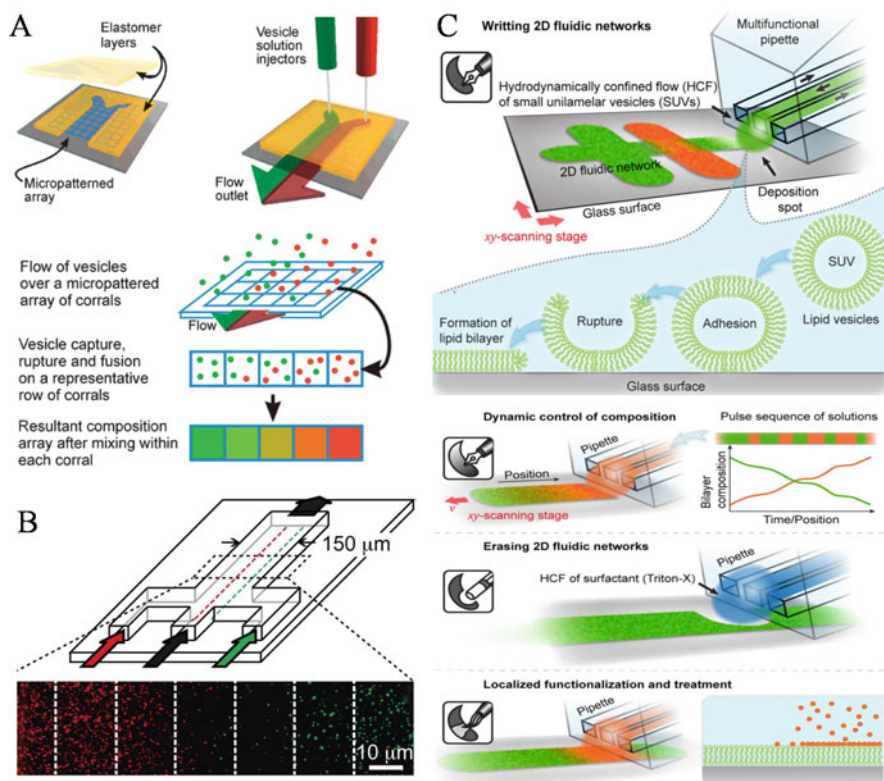


Fig. 4 Array strategies. (a) Schematic illustration represents the converging flow configuration for creating gradient-based array system with two types of vesicles in solution. (Reprinted with permission. Copyright 2000, American Chemical Society [28]). (b) Vesicle deposition on a microfluidic device is demonstrated. Three distinct inputs are introduced into the lanes to create lipid vesicle tethering and bilayer formation in the microchannels. (Reprinted with permission. Copyright 2007, National Academy of Sciences, U.S.A. [29]). (c) The entire process of ‘lab on a biomembrane’ approach is depicted as a schematic. The platform enables multitude processes, such as 2-D writing/erasing, dynamic control of the composition, and functionalization. (Reprinted with permission. Copyright 2013, Springer Nature Group [30])

8 Conclusions

Microfluidics is a prominent engineering modality, influencing multiple disciplines spanning from medicine, basic science, and pharmaceutical industry. In particular, last decade, liposome and lipid bilayer formation methods have largely benefited from the microfluidic-stemmed strategies since it considerably declines the dimensions of a fluidic process down to the microscale as observed in cellular systems; enables the control of fluids and other control parameters; as well as improves throughput and analytical performance of the platform. As noted in

each subsection, each strategy has the intrinsic pros and cons [3, 4], for instance, hydration, electroformation, and extrusion generate high-quality liposomes, and they, however, have very low encapsulation efficiency. To address this efficiency challenge, pulsed jetting strategy has been developed, but it is difficult to implement. On the other hand, emulsion techniques are also not very compatible with biological processes. Considering the aforementioned critiques, defining the methodology is highly reliant on the intended focus of research and stability of the vesicle produced.

As microfluidics is a versatile and modular modality, multiplexing could be possible via methodologies described here. Integrating an array format into these methods will definitely enhance high-throughput and applicability to other realms such as drug delivery and tissue engineering, as well as bio-inspired materials.

References

1. P. Walde, K. Cosentino, H. Engel, P. Stano, Giant vesicles: Preparations and applications. *Chembiochem* **11**(7), 848 (2010)
2. D.D. Lasic, The mechanism of vesicle formation. *Biochem. J.* **256**(1), 1–11 (Nov. 1988)
3. D. Van Swaay, A. Demello, Microfluidic methods for forming liposomes. *Lab Chip* **13**(5), 752 (2013)
4. D. Carugo, E. Bottaro, J. Owen, E. Stride, C. Nastruzzi, Liposome production by microfluidics: Potential and limiting factors. *Sci. Rep.* **6**, 25876 (2016)
5. P. Taylor, C. Xu, P.D.I. Fletcher, V.N. Paunov, A novel technique for preparation of monodisperse giant liposomes. *Chem. Commun.* **14**, 1732 (2003)
6. P. Taylor, C. Xu, P.D.I. Fletcher, V.N. Paunov, Fabrication of 2D arrays of giant liposomes on solid substrates by microcontact printing. *Phys. Chem. Chem. Phys.* **5**, 4918 (2003)
7. K. Kuribayashi, G. Tresset, P. Coquet, H. Fujita, S. Takeuchi, Electroformation of giant liposomes in microfluidic channels. *Meas. Sci. Technol.* **17**, 3121 (2006)
8. M. Le Berre, A. Yamada, L. Reck, Y. Chen, D. Baigl, Electroformation of giant phospholipid vesicles on a silicon substrate: Advantages of controllable surface properties. *Langmuir* **24**(6), 2643 (2008)
9. S. Aimon, J. Manzi, D. Schmidt, J.A.P. Larrosa, P. Bassereau, G.E.S. Toombes, Functional reconstitution of a voltage-gated potassium channel in giant unilamellar vesicles. *PLoS One* **6**, e25529 (2011)
10. V. Pereno et al., Electroformation of giant unilamellar vesicles on stainless steel electrodes. *ACS Omega* **2**, 994 (2017)
11. Y.C. Lin, K.S. Huang, J.T. Chiang, C.H. Yang, T.H. Lai, Manipulating self-assembled phospholipid microtubes using microfluidic technology. *Sensors Actuators B Chem.* **117**, 464 (2006)
12. F. Liu et al., The exosome total isolation chip. *ACS Nano* **11**, 10712 (2017)
13. L.G. Liang et al., An integrated double-filtration microfluidic device for detection of extracellular vesicles from urine for bladder cancer diagnosis. *Methods Mol. Biol.* **1660**, 355 (2017)
14. L.G. Liang et al., An integrated double-filtration microfluidic device for isolation, enrichment and quantification of urinary extracellular vesicles for detection of bladder cancer. *Sci. Rep.* **7**, 46224 (2017)
15. A. Jahn, W.N. Vreeland, M. Gaitan, L.E. Locascio, Controlled vesicle self-assembly in microfluidic channels with hydrodynamic focusing. *J. Am. Chem. Soc.* **126**, 2674 (2004)
16. K. Funakoshi, H. Suzuki, S. Takeuchi, Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. *Anal. Chem.* **78**, 8169 (2006)

17. P. Jönsson, M.P. Jonsson, F. Höök, Sealing of submicrometer wells by a shear-driven lipid bilayer. *Nano Lett.* **10**, 1900 (2010)
18. N. Malmstadt, M.A. Nash, R.F. Purnell, J.J. Schmidt, Automated formation of lipid-bilayer membranes in a microfluidic device. *Nano Lett.* **6**, 1961 (2006)
19. B. Schlicht, M. Zagnoni, Droplet-interface-bilayer assays in microfluidic passive networks. *Sci. Rep.* **5**, 9951 (2015)
20. S. Ota, H. Suzuki, S. Takeuchi, Microfluidic lipid membrane formation on microchamber arrays. *Lab Chip* **11**, 2485 (2011)
21. T. Osaki, S. Yoshizawa, R. Kawano, H. Sasaki, S. Takeuchi, Lipid-coated microdroplet array for in vitro protein synthesis. *Anal. Chem.* **83**, 3186 (2011)
22. J.C. Stachowiak, D.L. Richmond, T.H. Li, A.P. Liu, S.H. Parekh, D.A. Fletcher, Unilamellar vesicle formation and encapsulation by microfluidic jetting. *Proc. Natl. Acad. Sci.* **105**, 4697 (2008)
23. S.R. Kirchner et al., Membrane composition of jetted lipid vesicles: A Raman spectroscopy study. *J. Biophotonics* **5**, 40 (2012)
24. K. Kamiya, R. Kawano, T. Osaki, K. Akiyoshi, S. Takeuchi, Cell-sized asymmetric lipid vesicles facilitate the investigation of asymmetric membranes. *Nat. Chem.* **8**, 881 (2016)
25. R.K. Shah et al., Designer emulsions using microfluidics. *Mater. Today* **11**, 18 (2008)
26. E. Lorenceau, A.S. Utada, D.R. Link, G. Cristobal, M. Joanicot, D.A. Weitz, Generation of polymersomes from double-emulsions. *Langmuir* **21**, 9183 (2005)
27. Y.C. Tan, K. Hettiarachchi, M. Siu, Y.R. Pan, A.P. Lee, Controlled microfluidic encapsulation of cells, proteins, and microbeads in lipid vesicles. *J. Am. Chem. Soc.* **128**, 5656 (2006)
28. L. Kam, S.G. Boxer, Formation of supported lipid bilayer composition arrays by controlled mixing and surface capture [19]. *J. Am. Chem. Soc.* **122**, 12901 (2000)
29. Y.-H.M. Chan, P. Lenz, S.G. Boxer, Kinetics of DNA-mediated docking reactions between vesicles tethered to supported lipid bilayers. *Proc. Natl. Acad. Sci.* **104**, 18913 (2007)
30. A. Ainla, I. Gözen, B. Hakonen, A. Jesorka, Lab on a Biomembrane: Rapid prototyping and manipulation of 2D fluidic lipid bilayers circuits. *Sci. Rep.* **3**, 2743 (2013)