Video Article

Lipid Bilayer Vesicle Generation Using Microfluidic Jetting

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Abstract

Bottom-up synthetic biology presents a novel approach for investigating and reconstituting biochemical systems and, potentially, minimal organisms. This emerging field engages engineers, chemists, biologists, and physicists to design and assemble basic biological components into complex, functioning systems from the bottom up. Such bottom-up systems could lead to the development of artificial cells for fundamental biological inquiries and innovative therapies[1,2]. Giant unilamellar vesicles (GUVs) can serve as a model platform for synthetic biology due to their cell-like membrane structure and size. Microfluidic jetting, or microjetting, is a technique that allows for the generation of GUVs with controlled size, membrane composition, transmembrane protein incorporation, and encapsulation[3]. The basic principle of this method is the use of multiple, high-frequency fluid pulses generated by a piezo-actuated inkjet device to deform a suspended lipid bilayer into a GUV. The process is akin to blowing soap bubbles from a soap film. By varying the composition of the jetted solution, the composition of the encompassing solution, and/or the components included in the bilayer, researchers can apply this technique to create customized vesicles. This paper describes the procedure to generate simple vesicles from a droplet interface bilayer by microjetting.

Video Link

The video component of this article can be found at http://www.jove.com/video/51510/

Introduction

It has become increasingly clear that cell biology is a multi-scale problem that involves integrating our understanding from molecules to cells. Consequently, knowing precisely how molecules work individually is not sufficient to understand complex cellular behaviors. This is partly due to the existence of emergent behaviors of multi-component systems, as exemplified by the reconstitution of actin network interaction with lipid bilayer vesicles[4], mitotic spindle assembly in *Xenopus* extract[5], and spatial dynamics of bacterial cell division machineries[6]. One way to complement the reductionist's approach of dissecting the molecular processes of living systems is to take the opposite approach of reconstituting cellular behaviors using a minimal set of biological components. A critical part of this approach involves the reliable encapsulation of biomolecules in a confined volume, a key feature of a cell.

Several strategies exist for encapsulating biomolecules for studying biomimetic systems. The most biologically relevant system is lipid bilayer membranes, which mimic the biochemical and physical constraints imposed by the cell's plasma membrane. Formation of giant unilamellar vesicles (GUVs) by electroformation[7], one of the most widely used techniques for GUV generation[14], typically has a poor encapsulation yield due to its incompatibility with high salt buffer[8]. Electroformation also requires large sample volumes (>100 µl), which could be a problem for working with purified proteins, and inefficiently incorporates large molecules due to difficulty of diffusion between closely spaced lipid layers. Several microfluidic approaches for generating lipid vesicles have been developed. The double emulsion methods, which pass components through two interfaces between layers water-oil-water (W/O/W), relies on the evaporation of a volatile solvent to drive lipid bilayer formation[9]. Others have used a microfluidic assembly line that produces a continuous stream of lipid bilayer vesicles[10] or in two independent steps[11]. We have developed an alternate technique based on rapidly applying fluid pulses against a droplet interface bilayer[12] to produce GUVs of controlled size, composition, and encapsulation. Our approach, known as microfluidic jetting, offers the combined advantages from several existing vesicle generation techniques, providing an approach for creating functional biomolecular systems for investigating a variety of biological problems.

Protocol

1. Infinity Chamber Fabrication

1. Design the infinity chamber (named for its shape) using a computer assisted design (CAD) software, and save the file such that it is compatible with a laser cutter. To create this shape, separate two circles of diameter 0.183 in by a center-to-center distance of 0.15 in. Cut the chamber from 1/8- 3/16 in clear acrylic with the laser cutter. The infinity shape facilitates droplet interface bilayer formation and stability.
2. Drill a 1/16 in hole through the edge of the acrylic chamber to the infinity-shaped well. Repeat on the opposite side. Cut a small rectangle from a 0.2 mm acrylic sheet with scissors or a laser cutter to serve as a bottom to the wells.
3. Apply a thin but complete layer of quick-drying adhesive to one side of the 0.2 mm acrylic and glue it to the bottom of the chamber. Hold the 0.2 mm acrylic tightly in place against the bottom of the chamber and dispense the glue at the interface to allow the glue to create a seal but avoid covering the viewing area. Be sure to align the acrylic so that its edge is flush with the edge of the chamber wall and it completely covers the infinity chamber cutout. This will allow for sufficient jet penetration and prevent leakage of the well.
4. Cut two small pieces of natural rubber to cover the drilled holes. Apply quick-drying adhesive around the hole. Place the rubber over the hole and press on all areas with a pair of forceps to secure. Repeat for both drilled holes. Be sure that all glued connections are complete seals so as to prevent any leakage.
5. Using a 23 G, 1 in needle, poke a hole in the natural rubber on both sides of the chamber to facilitate insertion of the piezoelectric inkjet tip.

2. Experimental Preparation

Store stock lipid solution in chloroform in a -20 °C freezer. For this study, either 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was used. 2 ml of 25 mg/ml lipid solution is typically prepared in this protocol and will last for two months when stored at -20 °C.

1. To resuspend the lipid in *n*-decane, first transfer it from a stock vial to a small glass jar, and gently dry under argon or nitrogen. Hold the jar at an angle while drying to expose more surface area to the gas, allowing the lipid to dry faster.
2. With the cap of the jar only slightly screwed on, allow the dried solution to sit under vacuum in a desiccator for 1-2 hr. Then add *n*-decane to resolubilize the lipid at 25 mg/ml. Vortex the lipid solution briefly and sonicate it in a bath sonicator for 15 min. Store the reconstituted lipid in *n*-decane at -20 °C.
3. Prepare a stock sucrose solution. In a 1.5-ml microcentrifuge tube, add 900 µl of 300 mM sucrose and 100 µl of 1% methylcellulose (MC). Methylcellulose is added to increase viscosity of the solution, which aids in jetting. Optional step: Add an optional 1 µl of dark-colored food dye or fluorescent beads to lend more contrast or fluorescence in imaging, respectively. This solution is a 300 mOsm sucrose solution designed to match cellular osmolarity and to provide contrast during microjetting.
4. Draw the solution into a disposable 1 ml plastic syringe. While holding the syringe with the tip facing upwards, flick the shaft repeatedly to expel any bubbles toward the tip, and push the plunger to eject the trapped air. Be sure to evacuate all air from the syringe before proceeding, as it will interfere with proper piezoelectric contraction responsible for jetting.
5. Install a 0.22 µm filter on the end of the syringe. To prevent air from being trapped in the filter, hold the syringe vertically and push the plunger until a droplet is formed above the tip. Note: A 33 mm diameter syringe filter unit was found to work best, but alternative filters as small as a 3 mm diameter syringe filter can be used to reduce dead volume.
6. Unscrew the female Luer adapter of the inkjet, and securely press it in place over the end of the filter. Again, eject fluid to prevent trapping air. Screw in the top of the inkjet. Fluid should travel to the tip of the inkjet after it is completely attached.

3. Readying the Equipment

1. Using a v-clamp, mount the syringe assembly on the microscope stage. Attach the wire from the inkjet to the inkjet controller. Note: A custom stage was built for this protocol; while the stage design can be determined by the user, it is critical to have independent x-y-z control of the syringe and x-y control of the sample holder.
2. Determine the magnification and necessary lens combination to achieve the desired imaging. Here a 10X objective and 10X eyepiece are used.
3. Use a high-speed camera (≥1,000 fps) to visualize the jetting and vesicle generation. Prior to imaging, perform necessary camera calibration. Utilize image-based auto-trigger within the camera software to initiate image capture.
4. Mount the infinity chamber onto the microscope stage. Secure the chamber by taping it into place on the stage.
5. Carefully align the inkjet tip with the hole punctured in the natural rubber (see **Figure 1b**). To do this, bring the inkjet close to the chamber and adjust the positioning by eye, then make more precise adjustments through the microscope lens. Proceed cautiously, as coarse movements can damage the inkjet tip.
6. Once the inkjet is aligned, back the syringe assembly away from the chamber to prevent any damage to the inkjet during loading of the wells. Be sure that motion of the inkjet is unidirectional so that it remains aligned with the hole in the membrane.
7. Press gently on the plunger of the syringe assembly until a small droplet forms at the inkjet nozzle. This will provide some initial backpressure.
8. Input the jetting parameters. For a trapezoidal bipolar wave, use the following parameters: 20 kHz pulse frequency, 3 µsec rise time, 35 µsec pulse duration, 3 µsec fall time, 65 V applied voltage (pulse amplitude), and 100 jet pulses per trigger (pulse number). However, the applied voltage (pulse amplitude) and pulse number (jet pulses per trigger) can be varied to control vesicle size.

4. Vesicle Generation

1. Add 25-30 µl lipid solution suspended in *n*-decane to the infinity chamber, covering the full surface of both wells.
2. Add 25 µl glucose (of same osmolarity as the sucrose solution) to the outermost edge of one well, pipetting slowly and smoothly. Upon deposition, a drop of glucose should form, because the glucose and lipid solution do not mix. Add another 25 µl of glucose to the opposite well, which will make another drop and form the lipid bilayer membrane in the middle of the chamber within 5-10 min.
3. Insert the inkjet through the natural rubber, and carefully guide it towards the droplet interface bilayer. Approach the bilayer slowly, as the introduced inkjet will displace volume and can rupture the bilayer.
4. When the inkjet is within ~200 µm, apply the jetting with the settings described in step 3.8. The distance from the bilayer may vary primarily depending on the voltage and pulse number, among other parameters. This protocol recommends slowly increasing settings (voltage and pulse number) and observing bilayer deformation.

5. Cleaning the Equipment

1. Detach the syringe assembly from the microscope stage, and dispose of the 1 ml plastic syringe and filter.
2. To clean the inkjet, aspirate the following solutions in order by dipping the tip in the solution 7-10x each: 70% ethanol, 2% Neutrad solution in warm water, 70% ethanol, and ddH_2O. If the inkjet doesn't fit securely on the aspiration pipette, cut a pipette tip to form an adapter.
3. Dry the chamber with tissue. Place the infinity chamber in a 250 ml beaker with 2% v/v Neutrad in warm water, and sonicate for 5-10 min. After sonication, thoroughly dry the wells under compressed air. Any moisture in the wells can compromise the stability of the lipid bilayer membrane, so it is also recommended that the chambers are placed in an oven at 60 °C for 15 min.

Representative Results

We have assembled a microfluidic jetting setup on a conventional inverted fluorescence microscope with a custom stage assembled from machined parts and manual micrometers (**Figure 1**). Characterization of the inkjet provides insight into the vesicle generation process. Varying the distance between the inkjet nozzle and lipid bilayer affects the force applied to cause deformation of the membrane. Close proximity to the bilayer focuses the jet stream and prevents the membrane from dispersing energy away from the point of vesicle generation. The vortex travel increases with the voltage applied to the piezoelectric actuator, consistent with our expectations (**Figure 2**). Vesicle formation and representative jetted vesicles are shown in **Figure 3**. **Figure 3a** shows a representative image sequence for vesicle formation by microjetting. Following formation, vesicle stability tends to vary with vesicle diameter, where smaller vesicles were more stable.

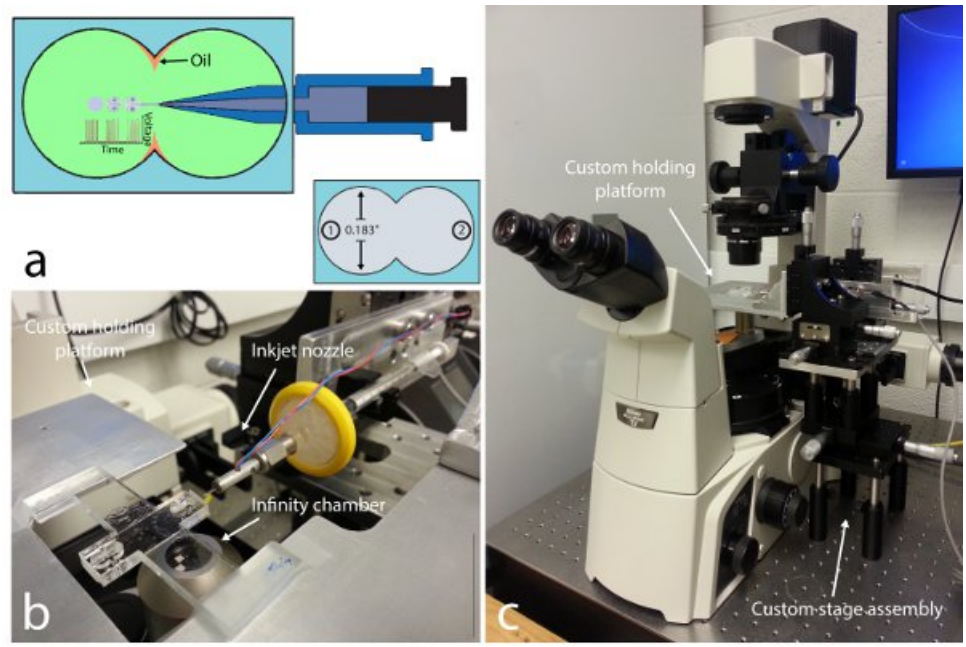

Figure 1. Illustration of the technique and equipment. (**a**) Schematic of piezoelectric-driven jetting process against the droplet interface bilayer. Multiple pulses pushed out in rapid succession form a vortex ring structure that deforms the bilayer to produce GUVs. Bottom right depiction shows the location of glucose deposition and dimension of the well. Once lipid solution has been added to the infinity chamber, 25 µl glucose is added to the outermost edges of the well, first at (1), then at (2). (**b**) The mounted syringe assembly, custom holding platform, and infinity chamber. The chamber is secured in place, and the tip of the inkjet is aligned with the hole in the natural rubber on the side of the well. (**c**) The complete microscope and custom stage assembly. Click here to view larger image.

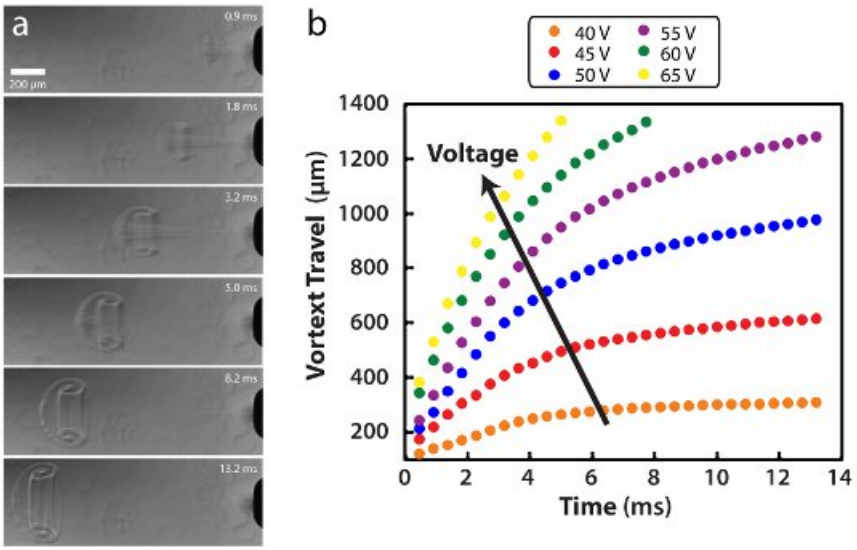

Figure 2. Characterization of the Inkjet. (**a**) Rapid inkjet pulses at 20 kHz (50 pulses at 55 V pulse amplitude) overlap to form a single vortex ring. (**b**) Liquid jet front displacement as a function of time over pulse amplitude range (40-65 V) for fixed pulse number (200 pulses). Click here to view larger image.

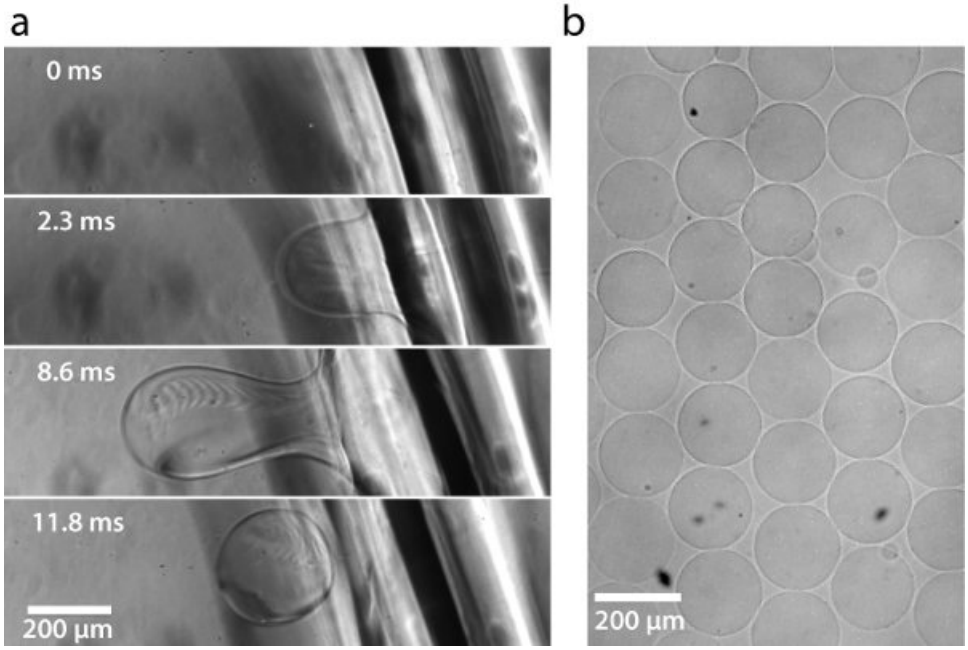

Figure 3. Vesicle Generation. Images of the vesicle generation process and several vesicles produced. (**a**) Deformation of the droplet interface bilayer (DPhPC) produced by rapid pulses of solution cause the membrane to pinch off and form a GUV. (**b**) Many vesicles generated using microfluidic jetting.

Discussion

Many techniques have been developed for vesicle generation, including electroformation, emulsion, and droplet generation[14-16]. However, new experimental techniques are necessary to allow for the design of biological systems with growing similarity to living systems. Microfluidic methods in particular have offered an increased level of control governing membrane unilamellarity, monodispersity of size, and internal

contents[17,18], bringing vesicle models closer to biology. Furthermore, characterization and experimentation using microfluidic jetting has shown effective incorporation of oriented membrane proteins, membrane asymmetry, and encapsulation[3,13].

This technique is straightforward, but includes some steps where caution should be taken. Both the acrylic sheet and natural rubber must form complete seals with the infinity chamber during fabrication. Otherwise, the infinity-shaped wells will leak and risk bilayer stability. During experimental preparation, the researcher must be sure to entirely evacuate the syringe assembly of air, initially and after attaching each component. Bubbles within this assembly create a compressible volume within the jet and mitigate or negate the jetting effect of the piezoelectric actuator. While readying the equipment, the main concern is damaging the fragile inkjet nozzle. Finally, the deposition of glucose requires the most attention during steps preceding vesicle generation, as this establishes the lipid bilayer for subsequent jetting.

Vesicle generation by microfluidic jetting is reliable and repeatable; however, discrepancies amongst inkjets require some familiarity and parameterization. In our experience, the introduction of the inkjet nozzle into the infinity chamber prior to jetting may displace up to several microliters of glucose depending on the nozzle dimensions, producing a slight bend in the bilayer away from the inkjet. By disproportionally dispersing the glucose solution when originally establishing the bilayer, this effect can be offset and a planar bilayer will result. This not only enhances bilayer stability but also allows for better control over vesicle formation. A 20 µm orifice diameter of the inkjet was used to obtain results shown in this paper. An orifice diameter of 10-20 µm is recommended. Minimization of vibrations is also recommended; simple rubber cutouts were used to support the microscope table and dampen laboratory vibrations.

Although this protocol is applicable to many lipids, DPhPC was chosen for its particular chemistry and high bilayer stability. Other primary lipids tested were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). Comparatively, DPhPC had a stronger tendency to form a consistent droplet interface bilayer and to produce unilamellar vesicles. Upon initial formation, the bilayer in this protocol had an apparent thickness due to a nonplanar interface and the incorporation of oil. The shape of the aqueous droplets formed a curved bilayer, and this appeared as a thickness in a given field of view. DPhPC allowed the initial contact zone on the interface between the two droplets to expand, driving oil away from the bilayer. Due to this energetically favorable process, more and more of the contact zone between the two droplets became a bilayer, and the observable thickness decreased over time. This growth of the bilayer region was tested under variability of the length of the bilayer; the initial chamber design was adjusted slightly (the second generation design consisted of two circles of diameter 0.15 in separated by a center-to-center distance of 0.13 in) to necessitate smaller volumes and resulted in a smaller bilayer interface. Both the new and initial design allowed for accurate vesicle generation, yet neither design showed a dominant advantage in bilayer thinning. Another tested optimization was computer-controlled backpressure applied to the piezoelectric inkjet. While this gave a more quantitative control over the flow rate while jetting, it was not used throughout the majority of experimentation.

This method offers the combined advantages of several existing techniques. Multiple GUVs can be generated at high frequency (~200 Hz) due to the high concentration of lipid molecules, although rapid vesicle generation was not the focus of this work. Since this technique jets against a single lipid bilayer, membrane unilamellarity is expected and has been observed. Additionally, a wide range of solutions can be encapsulated independent of specific solute properties such as molecular weight or charge, thus enabling more potential applications[17]. Also, due to the size of vesicles formed (a range is possible of >10 µm to <400 µm), observation by conventional microscopy techniques is adequate[13].

Microfluidic jetting can be applied to a variety of biological problems. One specific example is cellular biomechanics; the deformability of GUVs renders them an ideal tool to study the force generation and self assembly of encapsulated actin networks that showed interesting effects when assembled on the surface of a GUV[4,19]. Additional applications include drug delivery systems, cell-size bioreactors, and modular systems for synthetic biology, biophysics, and a variety of other fields in basic science, industry, and medicine where compartmentalized biomolecules are desired.

Disclosures

No conflicts of interest declared.

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