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Reconstitution of proteins on electroformed giant unilamellar vesicles

Eva M. Schmid^{*}, David L. Richmond[§], and Daniel A. Fletcher^{*,†,1}

^{*}Department of Bioengineering & Biophysics Program, University of California, Berkeley, CA, USA

[§]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

[†]Physical Biosciences, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Abstract

In vitro reconstitution of simplified biological systems from molecular parts has proven to be a powerful method for investigating the biochemical and biophysical principles underlying cellular processes. In recent years, there has been a growing interest in reconstitution of protein–membrane interactions to understand the critical role played by membranes in organizing molecular-scale events into micron-scale patterns and protrusions. However, while all reconstitution experiments depend on identifying and isolating an essential set of soluble biomolecules, such as proteins, DNA, and RNA, reconstitution of membrane-based processes involves the additional challenge of forming and working with lipid bilayer membranes with composition, fluidity, and mechanical properties appropriate for the process at hand. Here we discuss a selection of methods for forming synthetic lipid bilayer membranes and present a versatile electroformation protocol that our lab uses for reconstituting proteins on giant unilamellar vesicles. This synthetic membrane-based approach to reconstitution offers the ability to study protein organization and activity at membranes under more cell-like conditions, addressing a central challenge to accomplishing the grand goal of “building the cell.”

INTRODUCTION

1. THE POWER OF IN VITRO RECONSTITUTION

Our ability to understand the fundamental mechanisms underlying biological processes is often hindered by the overwhelming complexity, redundancy, and interconnectivity of cellular pathways. A powerful approach to address this challenge is reconstituting simplified cellular processes in vitro from their constituent molecular parts, thereby isolating the pathway or process of interest. Reconstitution provides an important test of the necessity and sufficiency of specific molecules, refines molecular models developed from live cell experiments, and can also reveal unexpected behavior that generates new insights into how complex biological systems operate (Heald et al., 1996; Liu & Fletcher, 2009; Loisel, Boujemaa, Pantaloni, & Carlier, 1999; Loose & Schwille, 2009; Rivas, Vogel, & Schwille,

¹Corresponding author: fletch@berkeley.edu.

SUPPLEMENTARY DATA

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2014; Wollert, Wunder, Lippincott-Schwartz, & Hurley, 2009). Finally, the simplicity of reconstituted systems makes them much more amenable to mathematical modeling and provides an important bridge to the physical sciences (e.g., Alberts & Odell, 2004; Dayel et al., 2009; Pontani et al., 2009; Surrey, Nedelec, Leibler, & Karsenti, 2001). However, a central challenge of reconstitution is to identify and recapitulate the essential physical and biochemical constraints that guide biomolecular processes in live cells (Vahey & Fletcher, 2014).

2. MODEL MEMBRANES FOR RECONSTITUTION

Reconstitution of membrane-based processes presents special challenges due to the difficulty of capturing the compositional complexity and physical properties of biological membranes, while at the same time maintaining a system that is simple enough for routine experimental use. As a result, a wide range of model membrane systems has been developed, each of which recapitulates a subset of the properties of biological membranes (Figure 1 and Table 1), and is thus advantageous for studying particular protein–membrane interactions.

Supported lipid bilayers (SLBs) are simple and robust, and ideal for studying pattern formation, e.g., by reaction–diffusion systems, on a two-dimensional (2-D) surface (Loose, Fischer-Friedrich, Ries, Kruse, & Schwille, 2008). They have the additional advantage of being amenable to total internal reflection fluorescence (TIRF) microscopy, allowing for high-resolution investigation of transient events such as single vesicle fusion (Karatekin et al., 2010). However, the fact that they are supported on a glass slide has significant limitations, such as diffusion kinetics arising from lipid interactions with the underlying glass surface (Przybylo et al., 2006; Scomparin, Lecuyer, Ferreira, Charitat, & Tinland, 2009). For the same reason, the study of transmembrane proteins in SLBs requires sophisticated polymer cushion protocols to allow for functional behavior of the transmembrane domain (Diaz, Albertorio, Daniel, & Cremer, 2008).

Traditionally, studies of transmembrane proteins such as ion channels and transporters have involved the use of planar “black lipid membranes” suspended between two aqueous volumes (Andersen, 1983; Montal & Mueller, 1972; Mueller, Rudin, Tien, & Wescott, 1962) or the use of small spherical liposomes (small unilamellar vesicles (SUVs) and large unilamellar vesicles; see Table 1) for which detailed protocols for insertion of fusion proteins and other transmembrane proteins are available (e.g., Martens, Kozlov, & McMahon, 2007). These small liposomes are on the order of 50–200 nm in diameter, which means that they are below the diffraction limit of light and must be imaged by electron microscopy, thereby only providing primarily static information of membrane shape and protein function (e.g., Peter et al., 2004).

Visualization of dynamic membrane deformations with optical microscopy can be achieved by using large unsupported membranes such as giant unilamellar vesicles (GUVs) (Liu et al., 2008; Stachowiak et al., 2012). GUV membranes are free to fluctuate, low in curvature, and can be decorated with proteins to study their function and organization. Finally, lipid vesicles made by inverse emulsion and microfluidic jetting enable construction of GUVs with different lipid composition in the inner and outer leaflets and encapsulation of defined contents (e.g., Pautot, Frisken, & Weitz, 2003; Richmond et al., 2011).

3. GIANT UNILAMELLAR VESICLES (GUVs)

Of the available model membrane systems, we find that GUVs are the optimal tool for many protein–membrane reconstitution experiments (for review see Monzel, Fenz, Merkel, & Sengupta, 2009). GUVs (pronunciation note: we prefer G-U-V (jee-you-vee) over Guv (guv)) are easy to produce in any lab and can be made to emulate the fluidity, tension, and deformability of real membranes. In contrast to SUVs, GUVs are also easily investigated by light microscopy, including standard imaging by epifluorescence, confocal, and TIRF microscopy. Furthermore, GUVs are compatible with more advanced techniques, such as fluorescence correlation spectroscopy, fluorescence lifetime imaging, and reflection interference contrast microscopy, which allows for quantitative measurements of dynamic protein assembly and organization on membranes (e.g., Kriegsmann et al., 2009; Monzel et al., 2009). In addition, GUVs are amenable to mechanical perturbations using techniques such as pipette aspiration, atomic force microscopy, and magnetic tweezers (e.g., Roux et al., 2010).

A wide variety of techniques have been developed for making GUVs, such as spontaneous swelling, electroformation, transformation of lipid stabilized water/oil or water/oil/water emulsions into vesicles, and microfluidic jetting. While each of these techniques has certain advantages and disadvantages (for a comprehensive review see Walde, Cosentino, Engel, and Stano (2010)), electroformation, first introduced by Angelova et al. (Angelova & Dimitrov, 1986), is straightforward to implement, leads to high yield of vesicles over a range of sizes, and provides flexibility in lipid composition and therefore also a variety of protein attachment possibilities (Figure 2(A)).

4. PROTEIN ATTACHMENT TO GUVs

There are many strategies for attaching proteins to GUVs. Proteins that have a natural affinity for specific lipids such as phosphoinositides or other charged lipids can be attached to vesicles by incorporating these lipids during vesicle formation. Proteins (or protein domains) without an affinity for specific lipids can be artificially attached to synthetic lipid bilayers via chemical linkage, such as binding of cysteines strategically inserted in proteins to maleimide-functionalized lipid headgroups, or attachment of lysines to succinyl-functionalized headgroups. Biotinylated proteins can also be attached via a streptavidin linkage to biotinylated lipid headgroups. Some membrane-associated proteins contain self-inserting moieties such as hydrophobic helices or lipid tails that attach the proteins to lipid membranes without the need for a synthetic attachment strategy.

A generic strategy for attachment of proteins to lipid membranes is to use Ni-NTA-functionalized lipid headgroups in combination with a multi-His tag cloned either N- or C-terminally onto the protein. This approach is simple, specific, and typically does not interfere with protein function. Protein–lipid interaction via His-tagging is not covalent (in contrast to maleimide linkage, for example) and can be reversed by addition of ethylenediaminetetraacetic acid (EDTA). The affinity of different length His-tags for Ni-NTA moieties has been studied in great detail and provides an efficient attachment system with tunable affinities (Kent et al., 2004; Nye & Groves, 2008).

5. ELECTROFORMATION OF GUVs FOR IN VITRO RECONSTITUTION

Here we present a simple and versatile protocol used by our lab to study a wide range of protein–membrane interactions with GUVs (see examples in Figure 2(B)). We provide a step-by-step protocol and a video taken with Google Glass to document the process of preparing electroformed GUVs (see Video 1: Full GUVelectroformation protocol and Video 2: Short GUVelectroformation protocol). We also show how vesicles emerge from a lipid film within the electroformation chamber by capturing a video of electroformation itself over 3 h with confocal microscopy (see Video 3: GUV production during electroformation). As an example of how electroformed GUVs can be used to address fundamental questions about membrane spatial organization, we introduce a membrane interface system based on synthetic adhesions that can be used to investigate protein sorting at cell–cell junctions and other membrane interfaces.

6. MATERIALS FOR GUV ELECTROFORMATION

The basic materials needed for reconstitution of proteins on electroformed GUVs are given below. The protein and lipid species can easily be adapted for a large variety of individual experiments.

6.1 ELECTROFORMATION CHAMBER SUPPLIES

- Conductive indium tin oxide (ITO)-coated slides (22 × 22) (SPI supplies #06463-AB)
- Silicon gaskets (cut from silicon sheet, McMaster-Carr, Super-Soft Silicone Rubber 1/16" thick) (homemade)
- Glass vials (Thermo Scientific, C4010-1) and caps (Thermo Scientific, C4010-60A)
- Hamilton syringes (10 (Hamilton, #1701), 50 (Hamilton, #1705) and 100 μ L (Hamilton, #1710), a set for “no dye” and “dye”)
- Conductive tape (3M™ EMI Copper Foil Shielding Tape 1181)
- Binder clips (Office Max)
- Clay/putty (e.g., Sculpey).

6.2 IMAGING CHAMBER SUPPLIES

- Glass coverslips 20 × 40 mm, No.1.5 (VWR 48393 230)
- PDMS (polydimethylsiloxane, Sylgard)

6.3 CHEMICAL REAGENTS

- Neutrad Soap (Decon Labs)
- Acetone (Fisher Scientific)
- CHCl_3 (chloroform) (Avantor)
- Sucrose (Sigma Aldrich)

- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Fisher Scientific)
- TCEP (*tris*(2-carboxyethyl)phosphine) (Fisher Scientific)
- KCl (potassium chloride) (Fisher Scientific)
- β -Mercaptoethanol (Fisher Scientific)
- EDTA (Acros Organics)
- Imidazole (Sigma Aldrich)
- KOH (potassium hydroxide) (Sigma)
- mPolyethylene Glycol (PEG)-5000-NHS (JenKem Technology USA)
- Poly-L-lysine (PLL) hydrobromide(Sigma).

6.4 GENERAL LAB EQUIPMENT

- Ohmmeter
- Function generator (Agilent 33120A or equivalent model)
- Osmometer (Osmette II or equivalent model)
- Ultrasonic waterbath (Branson 1510 or equivalent)
- Fluorescence microscope.

6.5 LIPIDS

- DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids)
- DOGS-Ni-NTA (1,2-dioleoyl-*sn*-glycero-3-((*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl), with nickel salt) (Avanti Polar Lipids).

For simplicity, the lipid composition used in the protocol below contains only one highly fluid neutral lipid species (97.5% DOPC) and a single protein attachment lipid species (2.5% DOGS-Ni-NTA). Complexity of the lipid composition can be increased to mimic more physiological lipid compositions (Takamori et al., 2006) by mixing different species, which may be necessary to reconstitute the function of certain proteins. However, it should be noted that high percentages of charged lipids (such as more than 30% Phosphatidylserine (PS)) lead to highly decreased GUV yield (Rodriguez, Pincet, & Cribier, 2005).

6.6 PROTEINS

- Deca-His-tagged mCherry
- Deca-His-tagged GFPuv.

Proteins were expressed and purified using affinity purification and gel filtration on an AKTA system (GE Healthcare).

7. GUV ELECTROFORMATION PROCEDURE

We documented the full GUV electroformation protocol from the perspective of the person making the GUVs using Google Glass (see Video 1: Full GUV electroformation protocol) and condensed the main steps into a shorter overview video (see Video 2: Short GUV electroformation protocol). Still images from the GUV preparation video are shown in Figure 3 and a complete description of each step is provided below. We imaged the process of electroformation by confocal microscopy on an inverted microscope (see Video 3: GUV production during electroformation) and show still images of different phases during the process in Figure 4.

7.1 PREPARING ITO SLIDES

- Clean conductive ITO slides thoroughly with a 2% Neutrad soap solution and rinse in MilliQ water.

Note: ITO-coated slides are sensitive to acids and bases and should be cleaned with only mild detergents.
- Apply the same cleaning procedure to the silicon gaskets that act as spacers later in the chamber assembly.
- Clean ITO slides further with acetone to remove all remaining lipid and soap residue.
- Slides are coated solely on one side with ITO. The conductive side can be determined easily by measuring resistance with an Ohmmeter.

7.2 MIXING LIPIDS

- Mix lipid stock solutions (dissolved in CHCl_3) with Hamilton syringes in desired molar ratios in small glass vials with Teflon caps.

Note: Do not use plastic vials or plastic pipettes to mix lipids dissolved in organic solvent.

Note: Hamilton syringes need to be cleaned thoroughly in clean CHCl_3 between each lipid species to avoid cross contamination. This is especially important when working with fluorescently labeled lipid species. We use dedicated syringes for dye-labeled lipids and nonlabeled lipids to avoid contamination.

Note: Some lipids are not fully soluble in CHCl_3 (for example, phosphatidylinositol-4,5-bisphosphate, PIP_2 , should be dissolved in a $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ mixture (20:9:1)) and should be mixed according to instructions on the webpage of the supplier.

7.3 SPREADING LIPIDS (FIGURE 5(A))

- Use Hamilton syringes to transfer and spread $0.2 \mu\text{mol}$ of lipid onto the conductive side of the ITO slide, leaving a roughly uniform film of lipid covering the entire surface after the solvent evaporates.

- Place lipid-coated ITO slide in a glass desiccator and apply vacuum for 15 min.
 - Note: Lipids dissolved in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ mixtures require longer time for complete solvent evaporation. Therefore the drying time should be extended to 1 h or even over night. When fluorescent lipids are used, shield them from light by covering the desiccator in aluminum foil.

7.4 ASSEMBLING ELECTROFORMATION CHAMBER (FIGURE 5(B))

- Attach a short piece of copper tape to each side of the silicon gasket with a roughly 1 cm overhang. The adhesive side of the tape is nonconductive. The conductive side of tape should be facing away from the gasket.
- Place the silicon gasket with the copper tape on top of a clean ITO slide, so that the conductive side of the tape contacts the conductive side of the slide.
- Remove the lipid-coated ITO slide from the desiccator and invert it onto the other side of the silicon gasket, making sure that the lipid-coated, conductive side of the ITO slide contacts the other piece of copper tape.
- Clamp the chamber shut with two binder clips.
- Slowly fill the chamber with the solution to be encapsulated in the GUVs, avoiding air bubbles. Typically, we use 200–400 mM sucrose. Filling GUVs with sucrose has the advantage of creating contrast upon later dilution into osmotically balanced buffer solutions. Additionally, the density difference between sucrose and surrounding buffer solutions will lead to the GUVs settling at the bottom of the investigation chamber, facilitating imaging.
- Seal the hole in the silicon gasket using a small ball of clay.

7.5 ELECTROFORMATION

- Connect the chamber to a function generator via BNC (Bayonet Neill–Concelman) cables with alligator clips at one end. The alligator clips clamp down on the copper tape that is protruding from the chamber. Multiple batches of GUVs can be made in tandem by using a BNC “splitter” and running multiple sets of cables from the function generator.
- We use a programmable function generator to control electroformation voltage and frequency according to the following program:
 - Phase I: 30 min. Sine wave with frequency $f=10$ Hz. Peak-to-peak amplitude ramps up linearly from 0.05 to 1.41 V.
 - Phase II: 120 min. Sine wave with frequency $f=10$ Hz. Peak-to-peak amplitude is constant at 1.41 V.
 - Phase III: 30 min. Square wave with frequency $f=4.5$ Hz. Peak-to-peak amplitude is constant at 2.12 V.

Note: For different lipid compositions, solution conditions, GUV size and distribution preferences, variations in this program may be more appropriate.

Note: When aiming to achieve phase-separated lipid vesicles (e.g., Veatch & Keller, 2002), the temperature during electroformation needs to be above the highest melting temperature (T_m) to achieve uniform lipid incorporation into the GUVs during the electroformation process. We therefore place the electroformation chambers in a 60 °C oven for mixed lipid species experiments.

7.6 HARVESTING GUVs

- After the electroformation program has run to completion (in our case 3 h), use a large needle (18G) and a 1 mL syringe to remove the sucrose solution (containing GUVs) from the chamber. Extract the solution slowly to avoid shearing of the GUVs as they flow into the needle.

Note: Depending on the lipid composition, GUVs can be stored at room temperature for a few weeks in plastic Eppendorf tubes. However, when using GUVs with unstable lipids such as PIP₂, the GUVs should only be used on the same day of production to ensure robust and reproducible protein binding.

7.7 CHARACTERIZATION

- *Osmolarity:* It is important to know the final osmolarity of the GUV solution to be able to balance surrounding buffer solutions accordingly. We use a freezing point osmometer (Osmette II) to measure osmolarity of the GUV solution and match dilution buffers accordingly.
- *Yield:* To test for GUV yield, dilute GUVs 1:1 into equal osmolarity glucose and image with phase microscopy or Differential Interference Contrast (DIC) microscopy. The GUVs should be easily visible due to the index of refraction mismatch between the sucrose in their lumen, and the surrounding sucrose/glucose solution. If they lose contrast, this may be a sign of bursting and resealing, probably due to mismatched osmolarity.
- *Handling:* GUVs are fragile. Avoid shearing them when pipetting and cut all pipet tips to a larger diameter. They will also rupture on bare glass if there is salt in the medium. PLL, PLL-PEG, casein, or SLBs coating of the cover glass can reduce GUV lysis (for more detail see “Preparing the imaging chamber” below).

8. PROTEIN-MEDIATED MEMBRANE INTERFACE EXPERIMENT

Here we describe one example protein–membrane interaction experiment. We outline the protocol we use to incubate proteins and GUVs in buffers, optimized for confocal microscopy.

8.1 PREPARING THE IMAGING CHAMBER

- We prepare homemade PDMS, (Sylgard) chambers by curing ~5 mm thick PDMS pieces and punching out cylindrical holes of 5 mm diameter (this can be scaled for larger and smaller reaction volumes as needed).
- Preclean coverslips by sonication in 3M KOH for 10 min, rinse thoroughly in ultrapure water, and dry before use.
- Immediately before use, prepare the PDMS pieces for adhesion with a piece of Scotch tape and then simply press them onto a clean coverslip for chamber assembly. If needed, passivate glass bottoms with PLL-PEG to avoid GUV rupture and protein aggregation on the clean glass surface: Fill assembled chamber with 30 μ L PLL-PEG solution (3 mg/mL, synthesized from mPEG-5000-NHS and Poly-L-lysine hydrobromide) and incubate for 10 min on room temperature. Wash chamber thoroughly with MilliQ water, dry, and use immediately.

8.2 PROTEIN-GUV INCUBATION (FIGURE 5(C))—To avoid unnecessary handling of GUVs, we incubate GUVs with proteins directly in the imaging chambers:

- Fill the cylindrical hole in the PDMS chamber with 100 μ L protein solution (typically 25–100 nM, in 25 mM HEPES buffer with 150 mM KCl and 1 mM TCEP, osmotically balanced to match GUV osmolarity).

 Note: Divalent cations (Mg^{2+} , Ca^{2+}) can lead to clustering of negatively charged lipids (such as PIP_2), which in turn may mislead interpretations of protein clustering on lipid membranes. Sufficient amounts of EDTA can overcome this problem. However, EDTA will chelate complexed Ni^{2+} ions necessary for Deca-His-tag/DOGS-NiNTA interactions and can therefore not be used in experiments where this artificial protein attachment method is used.
- Transfer 0.5–1 μ L GUV solution gently with a cut pipette tip to the protein solution.
- Incubate the mixture at room temperature for a few minutes to allow for protein binding to GUV membranes, and for GUVs to settle to the bottom of the imaging chamber.

Note: the ideal protein–GUV incubation time depends on the protein–lipid binding kinetics and the GUV yield. GUVs settle at a rate depending on their size, larger sucrose-filled GUVs settling faster than smaller ones. This can be used to avoid small lipid vesicles in the field of view, which are difficult to resolve by light microscopy and can obscure imaging. See Figure 6 for an example of the changing field of view over time and note also the increasing intensity as the His-tagged mCherry protein binds during incubation. Optimization to image in a time window where protein concentration on the membrane has equilibrated but before too many GUVs have settled, is ideal for analysis.

8.3 IMAGING—We image protein-bound GUVs on a spinning disc confocal microscope AxioObserver ZI (Zeiss AxioObserver Z1, Yokogawa CSU-10) with a cooled EMCCD camera (Cascade II, Photometrics). Images are acquired using a 63× objective (Zeiss, Plan Apochromat 1.4 NA, oil). In Figure 7, we show two example proteins binding to GUVs. The mCherry protein binds via its Deca-His tag to the DOGS-Ni-NTA lipids in the GUVs and labels the membrane. The Deca-His-tagged GFPuv binds to GUVs and in addition to membrane coverage, we observe GUV–GUV pairs and multimers. This phenomenon is due to GFPuv’s *trans*-dimerization capability (dimer Kd: 20–100 μM (CLONTECH Laboratories, Inc., 1998; Phillips, 1997)). GFPuv proteins on the GUV membrane act as adhesion proteins and link two opposing membranes into tight membrane–membrane interfaces. Interface size and shape depend on curvature and tension of the individual GUVs, protein density on the membrane, and *trans*-dimerization affinity of the adhesion proteins. This demonstration of membrane interface formation by GUV–GUV adhesion offers a platform for molecular interactions at cell–cell adhesions and intracellular membrane contact sites in a highly simplified, purely synthetic system without the inherent complexity of a live cell.

DISCUSSION—EMERGING OPPORTUNITIES

We have presented a simple and versatile protocol for reconstituting proteins on GUVs made by electroformation and demonstrated that GUVs can be used as a platform for studying membrane interfaces. This GUV electroformation protocol has been used in our lab for studying membrane organization and shape change associated with the actin cytoskeleton (Liu & Fletcher, 2006; Liu et al., 2008) and endocytosis (Stachowiak et al., 2012), and we expect it will continue to be an important model system that is part of any experimental toolbox for reconstitution of membrane-based processes.

Despite the ease of GUV production and protein attachment offered by electroformation, advanced membrane-based reconstitution will require new capabilities not offered by standard electroformation methods like the one presented here. For example, physiological membranes often include a high percentage of charged lipids (e.g., 14% PS in synaptic vesicles (Takamori et al., 2006)), which are known to reduce GUV yield significantly during electroformation. Moreover, biological membranes have asymmetric lipid composition, a feature that is currently difficult to emulate with electroformation. Furthermore, content control of GUVs is highly inefficient, especially for macromolecules like proteins, DNA, or even cell-free expression systems. The diverse size of GUVs made by electroformation can be viewed as a blessing or a curse depending on the experiment. Finally, one of the most pressing future applications of synthetic cell-like systems is the incorporation of transmembrane proteins into synthetic membranes, ideally with control over their orientation, which is currently difficult to achieve with electroformation.

To address the limitations of electroformed GUVs, variations in the electroformation protocol have been developed to enable the use of high ionic strength buffers (Yamashita, Oka, Tanaka, & Yamazaki, 2002), incorporation of asymmetric lipid composition (Chiantia, Schwille, Klymchenko, & London, 2011), control of internal contents (Limozin, Bärmann, & Sackmann, 2003), control of vesicle size (Kang, Wostein, & Majd, 2013), and insertion of

transmembrane proteins (Dezi, Di Cicco, Bassereau, & Lévy, 2013). In addition, other techniques such as inverse emulsions and microfluidic jetting provide additional control of membrane asymmetry, precise control over GUV contents, and oriented transmembrane protein insertion (Pautot et al., 2003; Richmond et al., 2011; Tan, Hettiarachchi, Siu, Pan, & Lee, 2006), although sometimes with lower yield and more complicated equipment. Improving the ease-of-use, versatility, and availability of advanced techniques for forming controlled GUVs is an important future research goal that will enable the next generation of reconstitution experiments aimed at “building the cell,” potentially even paving the way for the construction of clinically useful cell-like devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Current techniques for making synthetic membranes yield a wide range of different sizes and geometries

GUVs, giant unilamellar vesicle; LUVs, large unilamellar vesicle; SLB, supported lipid bilayer; SUVs, small unilamellar vesicle.

FIGURE 2. Giant unilamellar vesicle (GUV) electroformation

(A) Schematic drawing of the electroformation process. Unilamellar vesicles of nonuniform size are formed from a dried lipid film by swelling under application of an electric field between two conductive slides. (B) Examples of protein–membrane experiments using electroformation in our lab. From left to right: Actin polymerization on membranes changes lipid phase behavior (Liu & Fletcher, 2006); Membranes bundle actin filaments (Liu et al., 2008); Protein crowding tubulates fluid membranes (Stachowiak et al., 2012). ITO, indium tin oxide. (See color plate)

FIGURE 3. Giant unilamellar vesicle (GUV) electroformation protocol

(See color plate)

Still images from **Video 1: Full GUV electroformation protocol** illustrating key steps in the process.

FIGURE 4. Giant unilamellar vesicles (GUVs) produced by electroformation

GUVs imaged during electroformation by confocal microscopy (lipid composition: 99.7% DOPC, 0.3% Lissamine–Rhodamine DOPE (Avanti)).

Still images from Video 3: GUV production during electroformation.

FIGURE 5. Lipid spreading, electroformation chamber assembly, and imaging chamber setup
 (A) Cartoon representation of lipid spreading on a conductive slide with a Hamilton syringe.
 (B) Cartoon representation of electroformation chamber assembly. (C) Cartoon representation of imaging chamber setup. GUV, giant unilamellar vesicle; ITO, indium tin oxide; PDMS, polydimethylsiloxane.

FIGURE 6. Time course of protein binding and giant unilamellar vesicles (GUVs) settling
 (A) Cartoon representation of protein binding to GUVs during settling and optimal experimental setup. (B) Confocal images of Deca-His-mCherry binding to DOPC/DOGS-NiNTA GUVs over time. GUVs settle over time and gain in intensity as Deca-His-mCherry in the surrounding solution binds to the GUVs. Optimal experimental conditions are where protein fluorescence intensity on the GUVs has reached equilibrium and GUVs are not too concentrated (typically around 10 min). FOV, field of view.

FIGURE 7. Formation of membrane interfaces with electroformed giant unilamellar vesicles (GUVs)

Depending on the adhesion potential of the protein used, synthetic GUV membranes form membrane-membrane interfaces. Nonadhesive proteins (Deca-His-mCherry) coat GUV membranes by binding to DOGS-Ni-NTA lipids in the GUVs. Adhesive proteins (dimerizable deca-His-GFPuv) coat the membranes by binding to DOGS-Ni-NTA lipids in the GUVs, and form membrane-membrane interfaces by *trans*-dimerizing across two GUVs. (See color plate)

Table 1**Key Characteristics of Synthetic Membrane Systems for in vitro Reconstitution**

Membrane System	SLB	SUV	LUV	GUV	Jetted Vesicle
Preparation technique	SUV deposition on clean glass	Sonication	Extrusion	Swelling, electroformation, emulsions	Microfluidic jetting
Size	N/A	25–50 nm polydisperse	100–400 nm polydisperse	<100 µm polydisperse	<200 µm monodisperse
Deformability	No	Low	Medium	High	High
Curvature	None	High	Medium	Low	Low
Imaging technique	Light microscopy (incl. TIRF)	EM	EM	Light microscopy	Light microscopy
Control over lipid composition	Yes	Yes	Yes	Yes	Yes
Protein attachment	Yes	Yes	Yes	Yes	Yes
Content control	No	No	No	No (electroformation), yes (emulsions)	Yes
Transmembrane protein insertion	Limited (fluidity)	Yes, detergent assisted	Yes, detergent assisted	Limited, detergent assisted	Yes, oriented

EM, electron microscopy; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; SLB, supported lipid bilayer; SUV, small unilamellar vesicle; TIRF, total internal reflection fluorescence.