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Spherical Nanoparticle Supported Lipid Bilayers: a Tool for Modeling Protein Interactions with Curved Membranes

Erin R. Tyndall, Fang Tian

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, Hershey PA 17033

Abstract

Mechanistic studies of protein-membrane interactions can be complicated by the limitations of the membrane model system chosen. Many of these limitations can be overcome by using a spherical silica nanoparticle to support the membrane. In this chapter we present a detailed protocol for construction of spherical nanoparticle supported lipid bilayers (SSLBs), with methods to improve production are discussed.

Keywords

Spherical nanoparticle supported lipid bilayers; SSLBs; Membrane curvature; Curvature recognition; NMR

1 Introduction

Protein-membrane interactions are key to a variety of essential cellular processes including autophagy, mitosis, endocytosis, and vesicular trafficking [1, 2]. However, the molecular mechanisms of how proteins interact with their membrane environment, and how the membrane environment influences their activities, remain elusive in most cases. This is particularly true for an emerging number of proteins that depend upon membrane geometry for localization or activity [3-7]. Force measurements, structural and mechanistic studies of these proteins strongly benefit from a modeling system where membrane curvature can be stably and precisely defined [8, 9].

Common membrane mimics include micelles, bicelles, nanodisks, small unilamellar vesicles (SUVs), large or giant unilamellar vesicles (LUV/GUV), and planar supported lipid bilayers (Figure 1). There are however some caveats to these systems when they are applied to simulate the native curved membrane environment; micelles are very small, with a diameter of ~5 nm, and are extremely curved, while bicelles and nanodisks are most commonly used to provide a planar membrane surface [10-12]. SUVs and LUVs can be produced with a diameter distribution within a certain range, but their sizes are difficult to adjust and control rigorously [13, 14]. And as the radius increases, as in GUVs, so do stability problems, which

can lead to membrane blebbing, vesicle fusion and other undesirable changes in membrane structure [15].

However, spherical nanoparticle supported lipid bilayers (SSLBs) [16], a unilaminar vesicle supported by a silica bead, overcome many of these limitations [17, 18]. In the prevailing model for SSLBs (supported by studies using an array of techniques including fluorescent microscopy, differential scanning calorimetry, NMR and electron microscopy) the absorbed lipids follow the surface of the silicon beads and form a single bilayer [19-23]. The bilayer is not in direct contact with its support, and is instead supported by a buffer of water molecules (Figure 2). Consequently, this solid but cushioned support allows SSLBs to retain many of the essential properties of cellular membranes, including a well-defined geometry of the bilayer. The mechanical stability provided by the interior support allows the bilayer to be more resistant to potential changes in membrane shape when interacting with the protein while imposing few constraints on the fluidity of the lipids themselves. These unique advantages of SSLBs have been explored to study protein-lipid interaction in the past [24-28]. We have recently applied SSLBs to model different curved membranes and mechanistically and structurally studied their interactions with a membrane curvature-recognizing peptide using fluorescent microscopy and NMR [8, 9]. In this chapter we will describe protocols for the preparation of SSLBs.

2 Materials

2.1 Materials for Lipid Preparation

1. A SpeedVac concentrator with condenser
2. A lyophilizer
3. Glass tubes between 12 and 15 mm in diameter, and compatible with your SpeedVac
4. A laboratory fume hood
5. Either the requisite lipids as powdered lipids, or dissolved in chloroform. See 2.3 for details.
6. Chloroform
7. Chloroform-safe pipette tips such as S8064 from Sigma
8. Ultra-pure water
9. A vortex machine
10. Parafilm
11. Desktop centrifuge and compatible tubes
12. A water bath at 42 °C
13. A dry ice and methanol bath
14. A sonication bath

2.2 Materials for SSLB Preparation

1. Silica beads in aqueous solution or powdered silica beads of the desired diameter. See section 2.3 for details.
2. Ultra-pure water
3. Methanol
4. Desktop centrifuge for beads larger than 50 nm, ultracentrifuge for beads smaller than 50 nm, with compatible tubes.
5. 15 mL centrifuge tubes
6. 1 mM CaCl_2 in ultra-pure water.

2.3 Additional resources

1. Resources for lipids

Avanti Polar Lipids sells a wide range of lipids, including phospholipids and lipid extracts. Lipids can be obtained both in powder form and in chloroform solutions.

A smaller number of lipids may be obtained from Sigma-Aldrich or Anatrace. The latter has a similar range of lipids as Sigma-Aldrich that can be obtained in powder form.
2. Resources for silica beads
 - a. Nissan Chemical: Snowtex product line. This supplier produces a variety of beads that are provided in aqueous solution. Some specially shaped beads are also available if your application requires them.
 - b. Fisher: Size standards. These products are rigorously screened to the National Institute of Standards and Technology's standards for particle size and size distribution. A variety of sizes are available, though some product lines have fewer size options.
 - c. Cospheric Nano: Offers both nanospheres and microspheres; products are shipped as a dry powder and must be rehydrated.

3 Methods

3.1 Preparation of SUVs

1. Begin by selecting the lipids you wish to use for your SSLBs. If your targeted protein is lipid-makeup insensitive, we recommend 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) as its phase transition temperature allows the resulting SSLBs to be stable at room temperature. If you are using high percentage of charged lipids or lipids of disparate length you will need to take into consideration the various phase transition temperatures and possible interactions with the negatively charged silica beads, see Note 1.

2. Weigh out your lipids in a glass tube and add enough chloroform to dissolve them. If your lipids are already in chloroform, simply add them to the tube. When using chloroform, be sure to not allow it to contact plastic for any length of time. Our laboratory's standard preparation size is 15 mg of DMPC in 200 μ L of chloroform. See Figure 3 B and C for a visual reference, and Note 2 for caveats.
3. Use the SpeedVac to extract the solvent and deposit the lipids on glass. Usually this will require 2-4 hours, longer if you used a larger volume of chloroform. Once the solvent has been removed, the lipids should appear completely dry, as in Figure 3 D. At this point, transfer the glass tube with your lipids to the lyophilizer overnight. This will result in a completely dried lipid film.
4. To re-hydrate the lipids add ultra-pure water to the tube to bring the lipid concentration to 15 mg/mL. Seal with parafilm and allow the lipids to sit at 42 °C for at least an hour with vortexing every 15-20 minutes (Note 3). The lipids appear as a particulate suspension in water, and are pictured in Figure 3 E. At this point the mixture will separate if left to sit.
5. Freeze-thaw the lipid-water mixture. Briefly spin the tube to recover and collect everything at the bottom, and then transfer the suspension to a plastic centrifuge tube. Incubate the tube in the methanol-dry ice bath for 15 minutes, and allow to thaw, while frequently vortexing. Repeat 4 times, to fully freeze-fracture the lipids into unilaminar vesicles. At this point the lipids will appear translucent, and should remain suspended in solution longer, this is exemplified in Figure 3 F.
6. For formation of the SUVs, transfer the lipid mixture into a clean glass tube. Seal the tube with copious amounts of parafilm and insert into a float and add to the sonication bath. Sonicate until the solution becomes clear, or at least translucent; compare Figure 3 G and H (see Note 4). Place your SUVs in the 42 °C water bath until needed; they should remain stably in solution for a few days. We recommend not storing re-hydrated lipids for more than a week before use.

3.2 Preparation of SSLBs

1. Select the desired size of your SSLB; this should correspond to the membrane geometry your protein is known to interact with. If there are a range of sizes

¹-If more than 15% of your lipids are negatively charged you may have to alter the protocol to account for this. A spacer can be used to loosely tether the lipids out further from the bead, such as using avidin as a linker between the lipids and the bead [29].

²-Some lipids and lipid mixtures are not soluble in chloroform. While this will not lead to immediate problems, it is usually indicative of atypical behavior later: including SUVs not being stable at lower temperatures, or incomplete transition to SUV state. You may consider trying to optimize your lipid selection for those of similar lengths and phases transition temperatures. Additionally, be careful using whole lipid extracts, as they often are not pure, and are more difficult to completely make into SUVs.

³-The better hydrated the lipids are, the easier it will be to transition them into SUVs. Leaving the lipids at 42 °C overnight with hour of intermittent vortexing the next day can be helpful for difficult lipid mixtures.

⁴-For optimal sonication, it is necessary to check on the tube every 15-20 minutes. It is best to try and keep the tube "pinned" on one of the nodes using other floats. It is easy to tell when you are in the correct place because the sound will shift slightly, and the surface of your lipid mixture becomes very unstable. However, maintaining position can be difficult, and even if correctly managed, the heat of sonication often melts the parafilm. Thus we recommend checking the position and parafilm integrity every 15 minutes. If after 1 hour you have seen no change in opacity of the solution, try dividing it into two tubes. Figure 3 G shows incompletely sonicated DMPC liposomes, as compared to H. Some lipid mixtures will never become more translucent than G.

applicable to your protein, consider selecting the largest, as it will result in an easier protocol. See Figure 4 A for an example of 50 and 1000 nm beads.

2. If your beads are in powder form, you will need to hydrate them before they can be used. Weigh out the powdered beads, and add 2-5 volumes of water. Allow to sit at 42 °C for at least 16 hours, with vortexing. If the beads are not completely hydrated, it will lead to clumping.
3. Shake bead solution to homogenous suspension and add desired volume of bead to a microcentrifuge tube. We usually use a total of 40 mg of beads in each tube.
4. Spin the solution to pellet beads (see Table 1 for spinning times associated with each bead size). Discard the supernatant, and add 1 mL of ultrapure water to resuspend the pellet by aggressive vortexing, see Figure 4 B and C for an example of how the pellet should appear when resuspending (Note 5).
5. Repeat step 4 and wash beads with 1 mL methanol once and 1 mL water 3 times. For final resuspension, target 40 mg/mL in ultra-pure water. Beads should now be stable at room temperature (Note 6).
6. To prepare SSLBs, first ensure all components are at 42 °C. Next, take desired volume of beads and bring volume to 2 mL with ultra-pure water in a 15 mL conical tube. To a second tube, add lipids according value in Table 1, and bring to 2 mL with ultra-pure water. Vigorously pipette the bead mixture into the lipid mixture, and quickly add 8 µL of the 1 mM CaCl₂ solution. Vortex for at least 60 sec.
7. Move the mixture solution to the 42 °C water bath for an hour with vortexing every 15 minutes. An example of how 1000 and 50 nm beads appear during this process is in Figure 5 A and B respectively.
8. Spin the SSLB solution according to Table 1. Once pelleted, discard supernatant and wash pellet with 1 mL ultra-pure water. Vortex to suspend the pellet (Note 7). Repeat 3 times. Figure 5 C and D demonstrate both the pelleted and resuspended SSLBs.
9. Resuspend pellet in 200 µL water. SSLBs are now ready for use as seen in Figure 5 E.

⁵It can be difficult to resuspend the bead pellets. Allowing them to sit in a warm water bath for 10 min, or sonicating them may help loosen the pellet. It is also best to resuspend in an excess of water. For the preparation of a large amount of beads it is best to do it in small lots and combine at the end. The smaller the bead, generally the harder it is to resuspend. Do not pipette up and down; you will lose beads stuck in the tip.

⁶Small beads, such as 20-100 nm, should remain in solution rather than pelleting after resuspension. Larger beads will sink if left to sit on the bench. If your beads are not staying in solution, they may need to be further hydrated before proceeding to the coating stage.

⁷The SSLB pellet should appear similar to the bead pellet, but with a slight increase in opacity and a colored sheen on the smaller sizes. If you have trouble resuspending the SSLB pellet, allow the solution to sit in a water bath for 10 to 15 minutes. We do not recommend sonication once lipids have been added. Using excess SUVs during coating will make resuspension easier. If the pellet has turned milky white or if there are obvious chunks, then the SSLB coating has failed. It is best at this juncture to start over with another set of beads, and consider doubling the amount to CaCl₂ to stabilize the silica-lipid interactions.

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Fig. 1.
Pictorial representations of commonly used membrane mimics.

Fig. 2. Spherical supported lipid bilayers. The size of the SSLB is dictated by the size of the bead. As seen in the lower portion, a layer of water molecules separates the lipids and the supporting silicon bead.

Fig. 3. SUV Preparation. Liposome preparations begin with a glass tube (A). Add lipids (B) and dissolve in chloroform (C). After depositing on glass, the lipids should be completely dried (D and inset). Rehydrate lipids with water (E), and vortex repeatedly to resuspend (F). After a freeze-thaw cycle, return to the glass tube for sonication; lipids should retain appearance. Sonicate until clear (H), (G) shows an example of incompletely sonicated liposomes, which retains opacity.

Fig. 4. Silicon Beads. 1000 and 50 nm beads aliquoted from commercial stock (A). The beads can be difficult to resuspend, and the smaller they are the harder it will be. (B) shows 1000 nm beads immediately after re-addition of water, and the increased opacity can clearly be seen; the inset shows how they appear in suspension. (C) shows the same for 50 nm beads, and it is clear that the beads remain largely in the pellet, and that the pellet is more difficult to see.

Fig. 5. SSLB preparation. (A) and (B) demonstrate how the SSLBs should appear after vortexing during incubation with the CaCl_2 . (C) The pellet for 1000 nm beads appears the same as before coating, and the resuspended mixture is largely opaque. (D) The pellet for 50 nm beads will appear more opaque, and the resuspension should appear iridescent, but not entirely opaque. (E) A side-by-side comparison of completed 1000 and 50 nm SSLBs.

Table 1:

SSLB preparation

Bead Size (nm)	Theoretical lipid required to coat beads (per mg beads)	Experimentally recommended amount	Time and speed needed to pellet
20 – 30	0.4 mg	1.6 mg	15 min at 200,000 x g
50	0.2 mg	0.8 mg	10 min at 16,000 x g
100	0.1 mg	0.4 mg	5 min at 16,000 x g
1000	0.01 mg	0.04 mg	2 min at 16,000 x g