Engineering asymmetric vesicles

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Vesicles are bilayers of lipid molecules enclosing a fixed volume of aqueous solution. Ubiquitous in cells, they can be produced *in vitro* to study the physical properties of biological membranes and for use in drug delivery and cosmetics. Biological membranes are, in fact, a fluid mosaic of lipids and other molecules; the richness of their chemical and mechanical properties *in vivo* is often dictated by an asymmetric distribution of these molecules. Techniques for vesicle preparation have been based on the spontaneous assembly of lipid bilayers, precluding the formation of such asymmetric structures. Partial asymmetry has been achieved only with chemical methods greatly restricting the study of the physical and chemical properties of asymmetric vesicles and their use in potential applications for drug delivery. Here we describe the systematic engineering of unilamellar vesicles assembled with two independently prepared monolayers; this process produces asymmetries as high as 95%. We demonstrate the versatility of our method by investigating the stability of the asymmetry. We also use it to engineer hybrid structures comprised of an inner leaflet of diblock copolymer and an independent lipid outer leaflet.

Vesicles are produced in the laboratory by a variety of methods including sonication (1), extrusion (2), swelling (3), electroformation (4), and reverse evaporation (5); all methods rely on self-assembly and lead to a symmetric distribution of lipids on the inner and outer leaflets of the bilayer. Realistic models of biological membranes must incorporate lipid asymmetry (6, 7); moreover, asymmetric vesicles consisting of completely different types of molecules on the inner and outer leaflets would greatly increase the flexibility of vesicle drug delivery systems. Partial asymmetry can be achieved by altering the distribution of specific phospholipids using pH gradients, osmotic pressure, or molecules that promote lipid redistribution (8). However, the chemical constraints of these methods severely limit the applicability of such systems.

In this article, we describe a method for systematically engineering vesicles with asymmetric bilayers where each leaflet is assembled independently. A schematic of the process is shown in Fig. 1. We begin with an inverted emulsion of water droplets dispersed in dodecane and stabilized by the lipids intended for the inner leaflet. This phase is placed over an intermediate phase of the same oil containing the lipids for the outer leaflet. The intermediate phase is placed over the final aqueous phase, and a monolayer of the second lipid forms at the interface. The water droplets in the emulsion are heavier than the oil and thus sediment, pulling the second monolayer from the interface to complete the bilayer, resulting in the formation of asymmetric vesicles in the final aqueous phase. A similar strategy for making vesicles was first demonstrated using a benzene:water:egg-phosphatidylcholine (PC) emulsion (9). The uniqueness of our method lies in the introduction of this distinct intermediate phase that allows us to control the composition of each leaflet independently, thereby engineering asymmetric vesicles.

Here we describe experiments in which we used two phospholipids with the same aliphatic chains but different hydrophilic headgroups to demonstrate the construction of asymmetric vesicles. We used a fluorescence quenching assay (10, 11) to identify the location of the molecules in the bilayers to confirm that we have formed unilamellar asymmetric vesicles. We demonstrate the versatility of our method by investigating the stability of the asymmetry, even after vesicles are produced with lipid distributions that are not favored by molecular shape constraints. We demonstrate the power of our method by using it to engineer hybrid structures comprised of diblock copolymer inner leaflets and lipid outer leaflets.

Methods

Sample Preparation. Asymmetric bilayers were prepared with two monolayers, one consisting of lipids with phosphocholine heads and one consisting of lipids with phosphoserine heads. The lipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS). To determine the distribution of the lipid type we added 0.5 mol% of 1-palmitoyl-2-{6-[(7-nitro2–1,3-benzoxadiazol-4-yl) amino caproyl]-*sn*-glycero-3-phosphocholine} (NBD-PC) or 1-palmitoyl-2-{6-[(7-nitro2–1,3-benzoxadiazol-4-yl) amino] caproyl}-*sn*-glycero-3-(phospho-L-serine) (NBD-PS) to our lipid solutions. These correspond to POPC and POPS molecules for which the oleoyl acyl chain has been truncated and a fluorescent group inserted, preserving the head group and the volume of the hydrophobic tails and retaining the same packing properties. All of the lipids used were purchased from Avanti Polar Lipids as 99% pure chloroform stock solution and were used without further purification.

To prepare the lipid suspension for the inner leaflet, 100 μl of a 25 mg/ml stock solution was placed in a 100-ml glass bottle. The chloroform was evaporated under nitrogen to obtain a dry, thin lipid film. After adding 50 ml of anhydrous dodecane (+99%) to reach a final lipid concentration of 0.05 mg/ml, the suspension was sonicated in a cleaning sonic bath for 30 min and left overnight at 25°C to ensure that the lipid molecules were fully dispersed in oil before emulsifying the aqueous solution.

The emulsion was prepared by adding 250 μl of an aqueous solution consisting of 100 mM NaCl and 5 mM Tris buffer at pH 7.4 to the lipid suspension and gently stirring the mixture with a magnetic stir bar for 3 hours. This technique produces a bimodal population of small droplets with a mean size of $\approx$0.5 μm and larger droplets with a mean size of a few micrometers. Because emulsion droplets <1 μm maximize the vesicle yield and our control over the size distribution, the largest droplets were separated out by sedimentation, and only the smaller droplets were used for vesicle preparation (12).

The interface and aqueous phases were prepared by placing 2 ml of lipid dispersion over 3 ml of the aqueous solution that would host the final vesicles in a 1-inch-diameter centrifuge tube. The interface phase consisted of the lipid molecules for the outer leaflet dispersed at a concentration of 0.05 mg/ml in a 99:1 dodecane:silicone oil solution. After the interface phase was placed over the aqueous phase, it took 2–3 h for lipids to achieve the coverage required for optimal vesicle formation and for the

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Abbreviations: NBD-PC, 1-palmitoyl-2-{6-[(7-nitro2–1,3-benzoxadiazol-4-yl) amino caproyl]-*sn*-glycero-3-phosphocholine}; NBD-PS, 1-palmitoyl-2-{6-[(7-nitro2–1,3-benzoxadiazol-4-yl) amino] caproyl}-*sn*-glycero-3-(phospho-L-serine); PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine.

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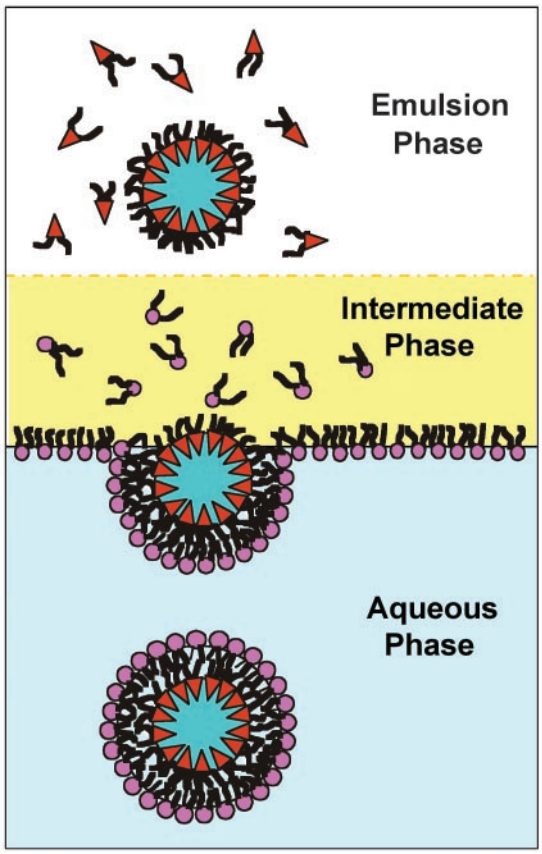

Fig. 1. Schematic illustration of the technique used to engineer asymmetric vesicles. The sample is composed of three parts: an inverted emulsion where water droplets in lipid-saturated oil are stabilized by lipid molecules destined for the inner leaflet; an intermediate phase of lipid-saturated oil heavier than the inverted emulsion phase, and whose lipids form a monolayer at the oil/water interface; and the bottom aqueous phase, which receives the final asymmetric vesicles. The lipids in the intermediate phase are completely different from those in the inverted emulsion and form the outer leaflet of the bilayer; the final structure is an asymmetric vesicle.

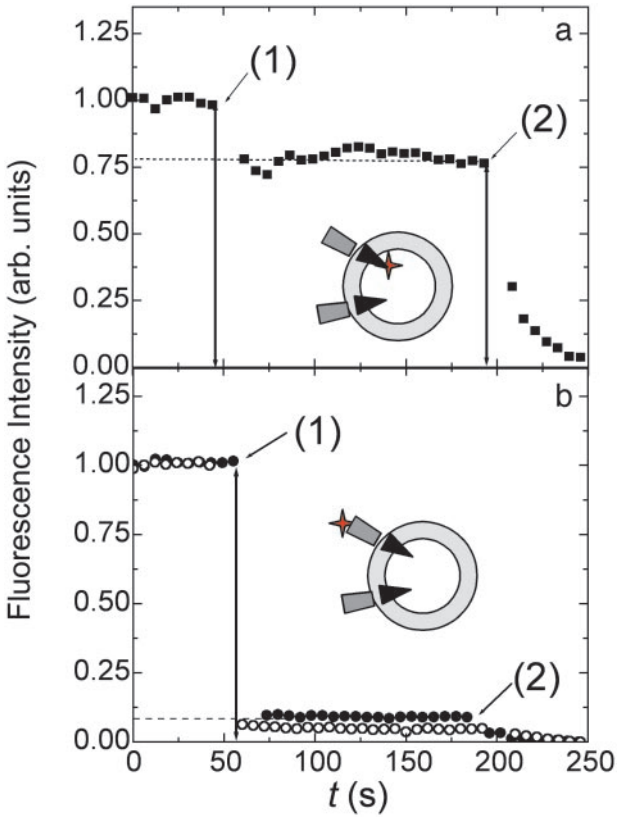

Fig. 2. The fluorescence intensity of vesicles engineered with a POPS inner leaflet and a POPC outer leaflet. The addition of sodium hydrosulfite is signaled by arrow 1, and the addition of Triton X-100 reduced is signaled by arrow 2. (*a*) NBD-PS is added to the inner leaflet and is protected by the bilayer, because >80% of the fluorescence intensity remains on addition of the quencher. (*b*) NBD-PC is added to the outer leaflet, and the fluorescence intensity is reduced by 95% on addition of the quencher. These data confirm the production of asymmetric vesicles. The filled symbols are data taken 1 h after formation of the vesicles; the open symbols are data taken 24 h later; the equivalence of the data reflects the stability of these asymmetric vesicles.

preservation of the outer monolayer composition (12). Once the interface had equilibrated, 100 μl of the inverted emulsion was poured over the interface phase. The silicone oil (specific density 1.05) makes the intermediate phase slightly denser than the emulsion so that it is possible to assemble two distinct oil layers; it also modifies the chemical potential of the intermediate phase, slowing the intermixing of the two lipids to preserve their separation and allowing the engineering of asymmetric vesicles. Then the sample was centrifuged at $120 \times g$ for 10 min to transfer the water droplets through the oil/water interface and into the lower aqueous phase.

After centrifugation, the vesicles were collected using a 5-ml syringe with a long 16-gauge stainless steel needle containing some of the final aqueous solution. With the tip of the needle in the aqueous phase, the buffer contained in the syringe was gently expelled. This has two effects: it allows us to remove oil that might have entered the needle when it crossed the oil phase, and it creates a flow in the aqueous phase that detaches the vesicles from the interface. After recirculating the buffer several times, most of the solution was aspirated and the needle was removed. Finally, the tip of the needle was wiped clean before unloading the vesicle suspension into its final container. After preparation and before using the vesicles for the fluorescence quenching assay, we used optical microscopy to check that the vesicles obtained were not deformed or aggregated.

Hybrid vesicles were prepared using a diblock copolymer, polystyrene-polyacrylic-acid (Rhodia, Cranbury, NJ), composed of a hydrophobic polystyrene block and a hydrophilic polyacrylic-acid block, both of molecular weight 8,000 g/mol for the inner leaflet and egg-PC containing 1 mol% of rhodamine-phosphatidylethanolamine lipids for the outer leaflet. Because dodecane is a poor solvent for the polystyrene block, we used decalin as the continuous phase for the preparation of the inverted emulsion. The polymer was first dissolved in a 100 mM sodium chloride, 5 mM Tris buffer at pH 7.4 and a concentration of 1 wt %. The emulsion was produced by sonicating 0.5% by volume of the aqueous solution in decalin. The emulsion droplets produced were a few micrometers in size and had polydispersities >20%. The rest of the protocol was unchanged.

Fluorescence Quenching Assay. We used a fluorescence quenching assay (10, 11) to measure the distribution of tagged lipids between inner and outer monolayers. A suspension of vesicles was prepared with 0.5% of a fluorescently labeled lipid on either the inner or outer monolayer. The fluorescence of the vesicle solution was measured before and after addition of a quenching solution of sodium hydrosulfite (1 M $Na_2S_2O_4$ in 5 mM TES at pH 9, prepared daily). When in the vicinity of the fluorophore, the sodium hydrosulfite extinguishes the fluorescence by reducing the dye. Because this molecule does not diffuse across the

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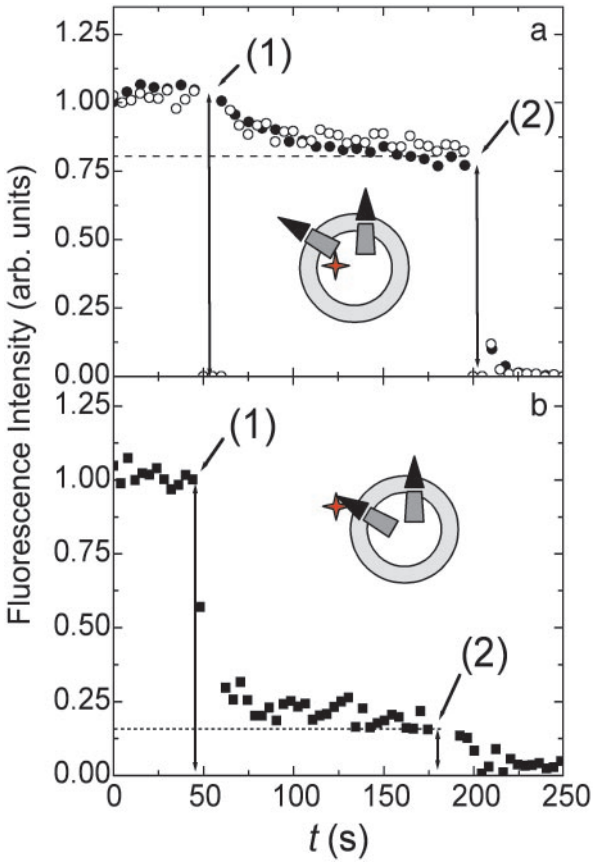

Fig. 3. The fluorescence intensity of vesicles engineered with a POPC inner leaflet and a POPS outer leaflet. This configuration is unfavorable for the spontaneous radii of curvatures of the two lipids. The addition of sodium hydrosulfite is signaled by arrow 1, and the addition of Triton X-100 reduced is signaled by arrow 2. (*a*) NBD-PC is added to the inner leaflet and is protected by the bilayer, as >80% of the fluorescence intensity remains on addition of the quencher. (*b*) NBD-PS is added to the outer leaflet, and the fluorescence intensity is reduced by 80% on addition of the quencher. These data confirm the production of asymmetric vesicles with unfavorable radii of curvatures. The filled symbols are data taken 1 h after formation of the vesicles; the open symbols are data taken 24 h later; the equivalence of the data reflects the stability of these asymmetric vesicles.

lipid bilayer, the addition of the quencher to the vesicle suspension results in the extinction of only the dye located on the outer leaflet of the bilayer. The dye on the inner leaflet was exposed by adding detergent (Triton X-100 reduced) to lyse the bilayer. The excitation wavelength for these measurements was set at $\lambda_{exc} = 470$ nm, and the emission of fluorescence was measured at $\lambda_{em} = 550$ nm.

Results and Discussion

The high degree of asymmetry that is achieved by our technique is illustrated when NBD-PS is incorporated into the inner leaflet of vesicles constructed with a POPS inner leaflet and a POPC outer leaflet; on addition of the quencher, the signal drops only 20% but is then completely extinguished on addition of the detergent, as shown in Fig. 2*a*. These results confirm that 80% of the NBD-PS was on the inner leaflet. Even higher asymmetry is achieved when we use the same lipids, but label only the outer leaflet with NBD-PC; on addition of the quencher, the fluorescence drops dramatically, by 95%, as shown in Fig. 2*b*. These results further confirm the formation of asymmetric bilayers and indicate that the asymmetry is even better on the outer monolayer.

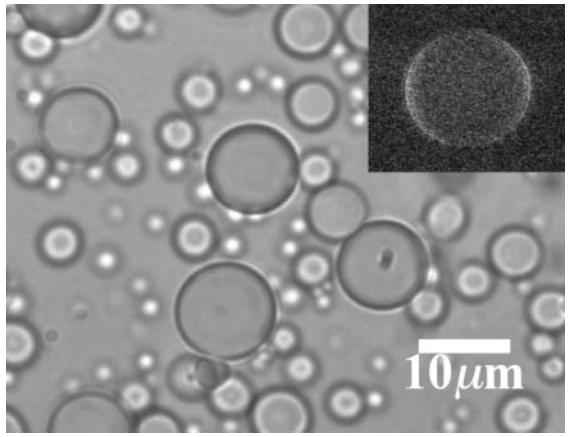

Fig. 4. Phase-contrast-microscope image (×100) of an inverted emulsion stabilized with polystyrene-polyacrylic-acid diblock copolymer. A hybrid vesicle structure was assembled by completing the bilayer with egg-PC containing 1 mol % of rhodamine-phosphatidylethanolamine. (*Inset*) An image of such a vesicle obtained with a scanning laser-fluorescence confocal microscope; the outer lipid layer is visible.

The success of our method allows us to explore important physical properties of asymmetric vesicles, including the effect of spontaneous curvature on the ability to form asymmetric vesicles and their stability. The lipids used in these experiments have two different head groups; POPS has a small anionic head group whereas POPC has a larger zwitterionic head group. The spontaneous curvature of lipid monolayers depends on molecular shape resulting from both steric and electrostatic effects, which varies with pH, ionic strength, and solvent (13–18). Although lipid molecules are basically cylindrical in shape, POPS molecules are slightly cone-shaped because of their smaller head group. In these experiments, the aqueous environment includes 100 mM NaCl, making the screening length ≈1 nm. We find that POPS forms an inverted emulsion much more easily than POPC does, suggesting that the steric effect dominates and that the POPS monolayer is characterized by a negative spontaneous radius of curvature, which can even affect structures with macroscopic length scales (13, 14). We can therefore exploit this to investigate the bilayer asymmetry when POPC is on the inner leaflet and POPS is on the outer leaflet, a packing that should result in a stress on the bilayer (18–20). When NBD-PC is added to the inner leaflet, only 20% of the total fluorescence intensity is lost on addition of the quencher, whereas all remaining fluorescence is quenched on addition of the detergent, as shown in Fig. 3*a*. Similarly, when NBD-PS is added to the outer leaflet, 80% of the total fluorescence intensity is lost on addition of the quencher and all remaining fluorescence is lost on addition of the detergent, as shown in Fig. 3*b*. These results confirm that we are able to achieve an 80% asymmetry despite the unfavorable spontaneous curvatures of the bilayer distribution.

We can also investigate the stability of the asymmetry over time for vesicles with favorable and unfavorable spontaneous curvature. When labeled POPC is placed on the outer leaflet, the vesicles remain perfectly stable over 24 h as shown by open circles in Fig. 2*b*. Moreover, even when the labeled POPC is placed on the inner leaflet, the vesicles still remain perfectly stable over 24 h as shown by the open circles in Fig. 3*a*. Significantly, flip-flop is not observed, even in the doubly unfavorable situation of charge asymmetry and poor packing conditions.

The flexibility of our technique extends beyond the assembly of asymmetric bilayers of lipid molecules; it can also be used to engineer novel types of composite bilayers. To demonstrate this,

we combine the toughness of a polymer (21) with the functionality and biocompatibility of lipids to form new hybrid structures. A PSt-polyacrylamide diblock copolymer was used to stabilize the inverted emulsion; a phase-contrast-microscope image is shown in Fig. 4. Egg-PC containing 1 mol% of rhodamine-phosphatidylethanolamine lipid was used to form the outer leaflet. An image of the thin outer layer of labeled lipid of the resulting hybrid structure as seen in a scanning laser-fluorescence confocal microscope is shown in the inset of Fig. 4. In the absence of a means to break these bilayers without the use of an organic solvent, a fluorescence quenching assay cannot be performed on these vesicles. However, it seems likely that the lipids will remain on the outer leaflet because of the long times necessary for polymer rearrangement at an interface (22). These hybrid structures significantly extend the utility and functionality of the vesicles produced; here the polymer provides mechanical support for the bilayer, whereas the lipids provide biocompatibility and the possibility of labeling, which could be useful, for example, in targeting for drug delivery applications (23, 24). Our technique will also work with other diblock copolymers provided the inverted emulsion can be formed.

Our technique provides a method for controlled assembly of highly asymmetric bilayers from amphiphilic molecules of different chemical nature, charge, or spontaneous curvature, without the use of detergents or chemical gradients. Direct engineering of the molecules composing each leaflet leads to many possible applications, including design of immunoliposomes for drug delivery systems with specific binding (23, 24) and a new generation of hybrid vesicles. It also allows us to investigate the effects of asymmetry on the mechanical and chemical resistance of the bilayer, the permeability of the bilayer, and the dynamics of flip-flop in the bilayer; these effects are of critical importance in the complex structures that are biological membranes.

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