Supporting Information

**Novel application of cellulose paper as a platform for the macromolecular self-assembly of biomimetic giant liposomes**

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**Experimental Section**

*Materials.* We purchased polystyrene well inserts, (CellCrown 24™ ScaffIDex) from Sigma-Aldrich and Whatman No. 1 chromatography paper, Grade 1 Filter paper, 24-well polystyrene multi-well plates (Corning™ Costar™), glass microscope slides (Thermo Scientific™), and glass cover slips (No. 1 thickness, Thermo Scientific™) from Thermo Fisher Scientific (Waltham, MA).

*Chemicals.* We purchased chloroform (purity ≥99.9%), Direct Red 23 (Dye content 30%), sucrose, D-(+)-Glucose, Bovine Serum Albumin–fluorescein isothiocyanate conjugate (FITC-BSA), and α-hemolysin from Sigma-Aldrich. We purchased 20X phosphate buffered saline (PBS) (200 mM sodium phosphate, 3 M sodium chloride, Ultra Pure Grade), 10 X Tris buffered saline (TBS) (1.37 M sodium chloride, 0.027 M potassium chloride, 0.25 M Tris/Tris-HCl, Ultra Pure Grade), HEPES buffer, and sodium fluorescein from Thermo Fisher Scientific. We purchased Annexin V, Alexa Fluor® 488 conjugate from Life Technologies.

*Lipids.* We purchased 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 (Δ9-Cis) PC (DOPC)), egg sphingomyelin (ESM), cholesterol (ovine wool), GM1 Ganglioside (Brain, Ovine-sodium salt), 23-(dipyrrometheneboron difluoride)-24-norcholesterol (TopFluor-Cholesterol), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine (TopFluor PC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhodamine-DPPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (POPG), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) from Avanti Polar Lipids, Inc. (Alabaster, AL).

*Microscopy and Analysis.* Imaging was performed with a confocal laser-scanning microscope mounted on an upright stand (LSM 700, Axio Imager.Z2m, Zeiss, Germany), using a PlanApochromat 20x objective with a numerical aperture (NA) of 0.8 or a PlanApo 63x
water dipping objective with a NA of 1.0. A 405 nm diode laser, a 488 nm diode, or a 555 nm diode laser excited the fluorophores. Electron microscopy was performed with a field emission scanning electron microscope (GeminiSEM 500, Zeiss, Germany), using an accelerating voltage of 1kV.

**Table S1:** Lipid mixtures that we used in our experiments.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Ratio</th>
<th>Phase Identity at 23 ° C</th>
<th>Transition Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC:TopFluor-Cholesterol</td>
<td>99:1</td>
<td>L_d</td>
<td>-20 ° C</td>
</tr>
<tr>
<td>DOPC:Rhodamine-DPPE</td>
<td>99.5:0.5</td>
<td>L_d</td>
<td>-20 ° C</td>
</tr>
<tr>
<td>ESM:DOPC:Chol:TopFluor-Cholesterol:Rhodamine-DPPE</td>
<td>54.8:20:25:0.1:0.1</td>
<td>L_o/L_d</td>
<td>~ 55 ° C</td>
</tr>
<tr>
<td>DPPC:TopFluor PC</td>
<td>99:1</td>
<td>S_o</td>
<td>~ 45 ° C</td>
</tr>
<tr>
<td>DOPS:DOPC: TopFluor-Cholesterol</td>
<td>50:49:1</td>
<td>L_d</td>
<td>-11 ° C</td>
</tr>
<tr>
<td>POPG: TopFluor-Cholesterol</td>
<td>99:1</td>
<td>L_d</td>
<td>-2 ° C</td>
</tr>
<tr>
<td>DOTAP:TopFluor-Cholesterol</td>
<td>99:1</td>
<td>L_d</td>
<td>&lt; 5 ° C</td>
</tr>
</tbody>
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**PAPYRUS in ionic buffers**

Incubating the lipid-coated paper in ultrapure water for 10 minutes before adding a concentrated stock solution (for example, 50 µL of a 20X PBS stock solution (200 mM sodium phosphate, 3 M sodium chloride) 10 minutes after placing the paper in 950 µL of ultrapure water) produced the best results for forming giant liposomes (**Figure S1a-c**) in ionic
solutions. We observed that the liposomes survived the transient osmotic shock due to the increase in the concentration of osmotically active solutes in the buffer at this early stage of growth. Indeed, the liposomes continued to grow and evolve dynamically (Figure S1d-e). Strikingly however, the shape of the liposomes changed in ionic buffers. The liposomal membranes adhere to one another, and the liposomes in regions in which liposomes formed at high densities deform significantly and take on the appearance of a tightly packed foam with Plateau borders (Figure S1c). Screening of electrostatic charges of the lipids in higher ionic strength buffers may partly explain the apparent change in the appearance of the liposomes.

**Liposomes prepared through PAPYRUS stably encapsulate proteins**

To demonstrate encapsulation of proteins and other fluorescent molecules in liposomes prepared through PAPYRUS, we used 75 nM of FITC-labeled bovine serum albumin in PBS as the growth buffer. The lipid mixture that we used was DOPC:Rhodamine-DPPE 99.5:0.5 mole percent. After a 60-minute incubation, we imaged the liposomes growing on the cellulose fiber surfaces. Figure S5 shows a liposome attached to the fiber. It is clear that the vesicular lumen contained the fluorescent BSA. We bleached the interior of the liposome with a 4.8 s bleach pulse of the 488 nm laser at 100 percent power. Figure S5b shows the liposome immediately after the bleach pulse and Figure S5c shows the liposome after 51 seconds. Figure S5d shows a plot of the fluorescence intensity. It is clear the population of BSA molecules in the liposome was bleached and the intensity did not recover, demonstrating that the BSA molecules in the liposomes were isolated from the BSA molecules in the surrounding solution. When dislodged and transferred into solution that contained no fluorescent BSA, the liposome interiors remained fluorescent Figure S5e). Also, see Figure S9.
Procedure for determining if cellulose incorporated in liposomes prepared through the PAPYRUS technique

Determining if cellulose partitions into liposomes prepared though PAPYRUS.

We employed the dye Direct Red 23 to determine if the cellulose partitions into liposomes prepared through the PAPYRUS method. Reports in the literature indicate that Direct Red 23 binds specifically to cellulose in primary plant cell walls. Upon binding to cellulose, the fluorescence intensity of Direct Red 23 increases dramatically.1

Procedure for preparing Small Unilamellar Liposomes

We evaporated 400 µL of DOPC in chloroform at a concentration of 10 mg/mL in a clean 4 mL glass vial (Wheaton) under a gentle stream of ultrapure nitrogen to obtain 4 mg of phospholipids as a dried film. The vial was left under vacuum for 2 hours to remove all traces of chloroform. Four milliliters of phosphate buffered saline (PBS) was added to the vial and agitated. The resulting turbid suspension, which contained multilamellar liposomes, was sonicated using a QSonic probe sonicator at an amplitude of 5% for 10 minutes. The vial was submerged in an ice bath during sonication to prevent excessive heating of the lipid suspension. The SUV suspension was then centrifuged for 10 minutes at 10,000 rpm to pellet titanium particles that were released from the sonicator tip.

Bulk Binding Assays

We conducted binding assays in a 96-well plate to determine if Direct Red 23 can assay for the presence of cellulose. We measured the fluorescence intensity, in triplicate, of Direct Red 23 at a final concentration of 0.15 mg/mL in i) phosphate buffered saline (PBS), ii) in a
suspension of cellulose pulp obtained by macerating chromatography paper in a blender, and iii) in a suspension of DOPC small unilamellar liposomes (SUVs). Control experiments, also conducted in triplicate, with cellulose pulp and DOPC SUVs without Direct Red 23 measured the background fluorescence of these materials. The plate was ‘read’ by imaging with a Zeiss LSM700 confocal equipped with an EC EpiPlan-Neofluar 2.5 X with NA=0.06 objective. To obtain an image of the relevant wells, we stitched the 10.22 mm x 10.22 mm images together (Figure S6a). Qualitatively, the samples showed similar intensities between Direct Red 23 in PBS and in the presence of lipids, but markedly higher fluorescence intensity in the wells containing cellulose (Figure S6a). The samples were excited with a 5 mW 555 nm diode laser, at 1 % power. A long-pass 585 nm filter was used as an acquisition filter. The photomultiplier (PMT) gain was 750. Figure S6b shows a bar plot of the average intensity of the wells, normalized by the average fluorescence intensity of the Direct Red 23 in PBS.

The background fluorescence of lipids and cellulose (black bars) was negligible under the imaging parameters we employed. The fluorescence intensity of Direct Red 23 increased by ~ 100 percent in the presence of cellulose (gray bar). The intensity of Direct Red 23 increased by ~13 percent in the presence of lipids.

**Electroformation**

We used established protocols to prepare giant liposomes using the electroformation technique.\(^2\)\(^-\)\(^4\) We deposited 10 µL of DOPC at a concentration of 1 mg/mL onto a clean indium tin oxide (ITO) coated glass slide using a glass syringe (Hamilton). The slide was placed in a vacuum desiccator for 30 minutes to remove all traces of chloroform. A PDMS gasket was used to construct a barrier around the lipid film, 600 µL of 250 mM sucrose solution was used to hydrate the lipid film, and a second ITO slide was used to close the chamber. The slides were connected to a function generator (Agilent) with conductive copper
tape. A sinusoidal AC field was applied at a field strength of 0.5 V/mm and frequency of 10 Hz for 1 hour. The giant liposomes were removed from the chamber and suspended in a 250 mM solution of glucose.

**Giant liposomes binding assays**

We added Direct Red 23 to a final concentration of 0.15 mg/mL to samples containing liposomes prepared through the electroformation method and the PAPYRUS method. We imaged the liposomes with a Plan-Apochromat 20X objective with a NA of 0.8. The 555 nm diode laser was set at 10%, and a LP 585 filter was used as an acquisition filter. As shown in Figure 4 of the main paper the vesicular membranes do not show significant accumulation of the dye. To obtain a quantitative measure of the intensity, we wrote a custom routine in MatLab to measure the mean intensity of a ten-pixel ring coincident with the position of the vesicular membrane. Figure S7a shows a representative false-colored raw image, Figure S7b shows the representative image of a binary mask generated by the routine, and Figure S7c shows the overlay, demonstrating the general accuracy of the routine. We measured the average intensity of the membrane on 103 liposomes prepared through electroformation and 176 liposomes prepared though PAPYRUS. It is clear that liposomes prepared through PAPYRUS were indistinguishable from liposomes prepared through electroformation (main paper).

**Procedure for determining the unilamellarity of the liposomes**

We prepared liposomes using PAPYRUS with a membrane composition of DOPC: Rhod- DPPE 99.5:0.5 mole percent. The liposomes were extracted from the surface of the paper and sodium fluorescein was added to the external solution to a final concentration of 40 µM. A 40 µL aliquot was placed onto a coverslip and 20 images were captured with a Plan-Apochromat
20X objective. Two-channels were collected, the rhodamine channel imaged the membranes, while the FITC channels imaged the fluorescein. α-hemolysin was added to a concentration of 2 µM to a second 40 µL aliquot and incubated for 30 minutes, before capturing 20 images using the same imaging parameters. Images were analyzed with Matlab. The images were analyzed by obtaining the location of the liposomes using the rhodamine channel. The ratio of the fluorescence intensity within the liposomes with the external solution was used as a measure of the permeability of the liposomes. To account for background intensity, we subtracted the minimum intensity value from all values. In the absence of α-hemolysin, the liposomes were impermeable to fluorescein (Figure S8). Upon addition of the α-hemolysin, the liposomes became permeable and the FITC leaked out into the continuous phase (Figure S9). FITC that was present in the continuous phase can also diffuse into the liposomes, as demonstrated by FRAP experiments (Figure S10).

**Reproducibility of liposome extraction through PAPYRUS**

Giant liposomes grow reliably when lipid-coated paper is incubated in aqueous buffers. To determine the reproducibility of the extraction of lipids from the paper, we deposited respectively 10 µL of a solution of DOPC:TopFluor-Cholesterol 99:1 mole percent in chloroform at a concentration of 6.5 mg/mL onto five pieces of paper. Each paper had 65 µg of lipids. We incubated the pieces of lipid-coated paper in 1.5 mL of sucrose at a concentration of 250 mM for 1 hour. We then extracted the liposomes by flowing, through gravity-driven flow, 1.5 mL of a solution of glucose at a concentration of 250 mM. The liposomes that were extracted from the paper were thus suspended in a total of 3 mL of sugar solution. We took 600 µL aliquots from each of the five wells respectively, placed it into three wells in a 96-well plate (200 µL in each well), and measured the fluorescence intensity in the
wells with a SpectraMax M2e Multimode plate reader. The excitation wavelength was set at 485 nm and emitted fluorescence was collected at 538 nm. It is clear that the fluorescence intensity of solution from the 5 samples were comparable (Figure S12a), demonstrating the reproducibility of the method. We also took 40 µL aliquots from the bottom of the wells after a 24-hour period (to allow the liposomes that were more dense than the surrounding solution to sediment and concentrate) and imaged the liposomes with a confocal microscope. Our images confirm the presence of liposomes in the solution (Figure S12b). To obtain an estimate of the amount of lipid that was extracted from the paper, we built a standard curve of the fluorescence intensity as a function of the known concentration of small unilamellar vesicles (SUVs) prepared with the same lipid composition as those for the giant liposomes. We find that approximately 80% of the lipids that were on the paper was extracted into the bulk through this method.

Supporting Information References


Supporting Figures:

Figure S1. Images of liposomes formed in ionic buffers. The lipid mixture used here was DOPC:TopFluor-Cholesterol 99:1 mole percent. a) Within 10 minutes, nascent liposomes budded from the cellulose fibers in ultrapure water. The liposomes generally appeared spherical, with membranes separated from one another. b-c) At 10 minutes, we added a concentrated solution of PBS stock (20X) to make a 1X PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.5) solution in the growth chamber. The liposomes clearly survived the osmotic shock. In the ionic buffer, the membranes of the liposomes appeared to be bound together (the white arrows highlight some pertinent regions). This observation is indicative of reduced electrostatic repulsion between the membranes. c) Liposomes in regions in which liposomes formed at high densities deformed significantly and took on the appearance of a tightly packed foam with Plateau borders (gray is the reflected light image of the cellulose fibers). d-f) Time lapse images of liposomes showing that the liposomes continued to grow and increase in size in the ionic buffer. All scale bars 10 µm.
**Figure S2.** a) Fusion event between two liposomes (red arrows). Forty-eight minutes before, both liposomes were smaller. b) Images showing a region where liposomes reduced in size over time. c) Images showing a multilamellar liposome converting to a unilamellar liposome. Liposomes labeled with TopFluor-Cholesterol (green) or Rhodamine-DPPE (red). Scale bars 5 µm.
**Figure S3.** Stability of liposomes prepared through PAPYRUS. a) Representative confocal fluorescence micrograph of liposomes (DOPC:TopFluor-Cholesterol 99:1 mole percent) after extraction from the cellulose paper. b) Representative image of the sample 5 days later. The liposomes are stable and remain largely unchanged during this period. Scale bars 20 µm.
Figure S4. Demonstration of the various phase behaviors obtained from liposomes prepared through PAPYRUS. Phases are indicated by different colors; green labels a L₀ phase and red labels a L₅ phase. a) A DPPC liposome at room temperature appears crumpled. The intensity of a cross-shaped photobleached region on the membrane does not recover over the course of 78 seconds demonstrating that the membrane is in a gelled state. b) A liposome with a composition that favors liquid-liquid phase coexistence, when quenched to room temperature exhibits the formation of many spherical domains that coarsen with time. Scale bars 5 µm.
Figure S5. a-c) Stills from the time series of a liposome that was grown in a solution containing 75 nM FITC labeled BSA in HEPES buffer. The membrane composition was 99.9 mole percent DOPC and 0.1 mole percent Rhodamine-DPPE. The right images are from the FITC channel. The left images are from the Rhodamine channel. The interior of the liposome was bleached by applying a laser pulse at 100 percent power with the 488 nm laser for 4.8 seconds. It is apparent that the FITC-BSA molecules in the interior of the liposomes were bleached. The intensity did not recover, demonstrating that the population of BSA molecules encapsulated in the liposome was isolated from those in the outside of the liposomes. d) Plot of the intensity vs time of the liposome lumen demonstrating that the bleach pulse altered the intensity of the BSA in the liposome permanently. e) Liposomes extracted from the paper, and transferred into a solution devoid of fluorescent molecules demonstrating the robust encapsulation of the proteins.
Figure S6. Bulk binding assays of Direct Red 23. a) Confocal fluorescence image of a 3 X 4 array from a 96-well plate. From left to right, the wells contained 0.15 mg/mL Direct Red 23 in PBS, cellulose pulp in PBS, cellulose pulp in PBS + 0.15 mg/mL Direct Red 23, 1 mg/mL of DOPC small unilamellar liposomes (SUVs) in PBS, DOPC SUVs in PBS + 0.15 mg/mL Direct Red 23. b) Bar graph of the fluorescence intensity of the wells normalized to the fluorescence intensity of Direct Red 23 in PBS buffer (gray). The black bar is the fluorescence intensity of the lipids and the cellulose in the absence of Direct Red 23. It is clear that autofluorescence is negligible under these acquisition settings. The intensity of Direct Red 23 bound to cellulose is twice that of the intensity of Direct Red 23 in buffer. Bars are means of the intensity of the three wells, and error bars are standard deviations. Scale bar 10 mm.
Figure S7. (a) Image of liposomes prepared through PAPYRUS in a solution containing 0.15 mg/mL of Direct Red 23. (b) Image of the mask showing the 10 pixel ring-shaped region identified by the automated routine, and (c) an overlay of the identified regions and the image of the liposomes showing that they coincide. Scale bar 20 µm.
Figure S8. a-c) Encapsulation of fluorescein in liposomes prepared through PAPYRUS. Fluorescein is indicated in green and Rhodamine-DPPE is indicated in red. When the region was bleached, the fluorescence in the interior of the liposomes did not recover, while the intensity in the continuous phase recovered. This demonstrates that the liposome membranes were intact and served as barriers to transport of the hydrophilic charged dye into the liposomes. d) Intensity plot of the experiment. Scale bar 10 µm.
Figure S9. a) Schematic of the mechanism of action of α-hemolysin. Multilamellar and unilamellar liposomes encapsulate fluorescein. When the liposomes are unilamellar, the addition of α-hemolysin to the continuous phase causes the membrane to become permeable to the dye. If more than one membrane is present (multilamellar), the protein is only able to porate the outer membrane. The dye remains encapsulated. b-d) A collection of liposomes exposed to 2 μM of α-hemolysin. The dye leaks out of the liposome over time demonstrating that they are unilamellar. e) Plot of the normalized intensity of the liposome interior shown in (f-i). The process of leakage is relatively fast. The intensity in the interior of the liposome decreased to match the intensity of the continuous phase within 20 seconds of exposure to α-hemolysin. Scale bar 10 μm.
Figure S10. a-c) Once porated by α-hemolysin, there is free exchange between the dye in the continuous phase and the vesicular lumen. In these experiments, the vesicular lumen was photobleached by applying a 4.8 s pulse with the 488 nm laser at 100 percent power. Over the course of 60 seconds, the intensity of the dye in the liposome interior increased, as nonphotobleached dye diffused into the liposome. d) FRAP curve showing recovery of the intensity of the dye in the lumen of the liposome. Scale bar 10 μm.
Figure S11. Photograph of the items used to perform PAPYRUS conveniently.
**Figure S12.** Reproducibility of the extraction of lipids from paper. a) Fluorescence intensity of the solution extracted from 5 different papers treated identically. It is clear that there were no significant variations in the intensities between the 5 samples. The bar is a mean of the intensities of the three wells in the 96-well plate for each sample, and the error bars are standard deviations from the mean. b) Representative image of giant liposomes that were present in the solution in the wells. Scale bar 20 µm.