Size Distributions and Yields of Giant Vesicles Assembled on Cellulose Papers and Cotton Fabric

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Supporting Information

ABSTRACT: Lamellar phospholipid stacks on cellulose paper vesiculate to form cell-like giant unilamellar vesicles (GUVs) in aqueous solutions. The sizes and yields of the GUVs that result and their relationship to the properties of the cellulose fibers are unknown. Here, we report the characteristics of GUVs produced on four different cellulose substrates, three disordered porous media consisting of randomly entangled cellulose fibers (high-purity cellulose filter papers of different effective porosities), and an ordered network of woven cellulose fibers (cotton fabric). Large numbers of GUVs formed on all four substrates. This result demonstrates for the first time that GUVs form on cotton fabric. Despite differences in the effective porosities and the configuration of the cellulose fibers, all four substrates yielded populations of GUVs with similar distribution of diameters. The distribution of diameters of the GUVs had a single well-defined peak and a right tail. Ninety-eight percent of the GUVs had diameters less than the average diameter of the cellulose fibers (∼20 micrometers). Cotton fabric produced the highest yield of GUVs with the lowest sample-to-sample variation. Moreover, cotton fabric is reusable. Fabric used sequentially produced similar crops of GUVs at each cycle. At the end of the sequence, there was no apparent change in the cellulose fibers. Cellulose fibers thus promote the vesiculation of lamellar phospholipid stacks in aqueous solutions.

INTRODUCTION

Giant unilamellar vesicles (GUVs) are closed phospholipid bilayer membranes with diameters greater than one micrometer.1 GUVs are useful in vitro models for biophysical experiments2–5 and for designing biomimetic synthetic cells.6–12 Lamellar stacks of phospholipids on solid surfaces such as glass or roughened Teflon spontaneously vesiculate over the course of 24–36 h in aqueous solutions to form giant vesicles (gentle hydration13–17). Vesiculation is accelerated by applying electric fields orthogonal to the lamellar phospholipid stacks (electroformation18), by supporting the lamellar phospholipid stacks on hydrogel films (gel-assisted hydration19–24), and by supporting the lamellar phospholipid stacks on cellulose paper (PAPYRUS for Paper-Abetted amPhiphile hYdRation in aqUeous Solutions).25,26 Dissolved cargo in the aqueous solution, such as small molecules, proteins, and polysaccharides, encapsulate into the growing GUVs.27–31

With PAPYRUS, vesicle growth and loading can be separated.26 Vesicles that have completed growth remain open to the environment through membrane attachments to the cellulose fibers.26 Cargo can be diffusively loaded into the vesicles after they have formed.26 This temporal decoupling allows differing conditions during the growth stage and the loading stage. For example, temperature and buffer compositions can be first optimized to maximize the growth of vesicles and then modulated to promote the stability of the cargo.26

Cargos that are larger than hundreds of nanometers, such as therapeutic viruses,32,33 vesicular organelle mimics,34,35 or colloidal particles,36 have to be encapsulated into GUVs because these cannot sterically fit into small unilamellar vesicles. Furthermore, macromolecular cargos have been reported to encapsulate at greater efficiencies in large and giant vesicles compared to small vesicles.29,37,38 The larger vesicles, once loaded, can be extruded to smaller sizes for applications.

Using cellulose paper to produce GUVs is advantageous because (i) high-purity cellulose filter paper is readily available, (ii) GUVs free from cellulose contamination25,26 can be obtained within 1 h, (iii) the process is easily scalable because it does not require the use of power or specialized equipment. However, the sizes and yields of GUVs produced on cellulose paper and the dependence on the properties of the paper is unknown. Here, we study the characteristics of GUVs obtained from four different cellulose substrates, three “grades” of high-purity cellulose filter paper and a cotton fabric. We demonstrate for the first time that cotton fabric produces GUVs.

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Both filter paper and cotton fabric are manufactured from cellulose fibers that are obtained from bolls that form around the seeds of the cotton plant (Gossypium sp).\textsuperscript{39} Cellulose fibers from cotton bolls are the highest-purity natural source of α-cellulose\textsuperscript{39} and is a commodity-scale renewable raw material.\textsuperscript{40} High-purity filter papers consist of cellulose fibers approximately 1.5–6 mm in length obtained from the short fibers attached to the cotton seed (cotton linters). These short fibers entangle during the paper-making process to form a disordered randomly percolated network.\textsuperscript{41} High-purity filter paper is available in grades of different effective porosities (particle retention capacities). Effective porosity in disordered porous media such as paper depend on several parameters such as the number of pores, their sizes, and the tortuosity.\textsuperscript{41,42}

As the number of pores, their sizes, and the tortuosity increase, the porosity of the material decreases. Effective porosity is empirically determined\textsuperscript{43} by the manufacturer by measuring the rate of flow at a constant hydrostatic head apparatus, the Herzberg flow rate is empirically determined\textsuperscript{43} by the manufacturer by measuring the time it takes for water at a constant hydrostatic head of 10 cm to flow through a horizontal disk of the filter paper with an effective area of 10 cm$^2$. The rate of the flow is measured in seconds per 100 mL.\textsuperscript{43} As expected, papers with lower effective porosities have lower liquid flow rates. The properties of the three grades of filter paper that we selected, in the order of descending apparent porosities and liquid flow rates, were (i) Grade 41, effective porosity 20–25 μm, Herzberg flow rate 54 s/100 mL; (ii) Grade 1, effective porosity 11 μm, Herzberg flow rate 150 s/100 mL; and (iii) Grade 42, effective porosity 2.5 μm, Herzberg flow rate 1870 s/100 mL. We refer to these substrates as G41, G1, and G42 paper respectively.

Cotton fabric, also composed of cellulose fibers, has a radically different microstructure than paper. The cellulose fibers used to make cotton fabric are obtained from the staple fibers of the cotton boll.\textsuperscript{39} These fibers have lengths of 20 mm or longer and can reach lengths of up to 40 mm.\textsuperscript{39} Hundreds of these long cellulose fibers are spun into tightly twisted bundles to form yarn.\textsuperscript{39} Then, the yarn is woven to form an interlaced network.\textsuperscript{39} Depending on the weave pattern, the process leaves regular windows of low to no cellulose content between the orthogonal bundles of yarn. Thus, the cellulose fibers in the fabric are highly ordered rather than randomly entangled, such as those in paper.

Our naive expectation was that the differences in the microstructural configuration of the cellulose fibers of the four substrates would lead to differences in the sizes of the GUVs. We performed systematic experiments with three independent samples for each substrate and measured the diameters of the harvested GUVs using single-plane confocal microscopy and digital image analysis. Our sample sizes, $n \sim 40,000–130,000$ GUVs per sample, are several orders of magnitude larger than those reported in the literature.\textsuperscript{21,23,44,46} Larger sample sizes offer greater statistical confidence compared to smaller sample sizes.

We find that the sizes of GUVs obtained from the four different substrates were remarkably similar. The populations of GUVs harvested from the substrates showed a unimodal skewed distribution of diameters, centered around four micrometers, with a prominent right tail. Instead of being affected by the microstructure of the substrate, the GUV sizes were bounded by the average diameter, $d_0$, of the cellulose fibers in the substrates. The microstructure of the substrates affected the yield of GUVs. The average number of GUVs harvested from G42 paper and cotton fabric was about 3 times higher than the average number of GUVs harvested from G1 and G41 papers. Cotton fabric also had the lowest sample-to-sample variation in the number of harvested GUVs.

We also show that a single piece of cotton fabric can support multiple cycles of growth and harvest of GUVs. There was no apparent change in GUV sizes or yields at each cycle. The reusability of fabric provides an economical route for the large-scale production of GUVs.

## EXPERIMENTAL SECTION

**Materials.** We purchased Whatman Grade 41 Filtration paper, Whatman Grade 1 Filtration paper, Whatman Ashless Grade 42 Filtration Paper, glass microscope slides (Thermo Scientific), and glass cover slips (No. 1 thickness, Thermo Scientific) from Thermo Fisher Scientific (Waltham, MA). We purchased unbleached cotton fabric composed of 100% organic cotton from Amazon Inc (Organic Cotton Plus (Lubbock, TX)).

**Chemicals.** We purchased sucrose (BioXtra grade, purity ≥ 99.5%), glucose (BioXtra grade, purity ≥ 99.5%), and casein from bovine milk (BioReagent grade) from Sigma-Aldrich. We purchased chloroform with 0.75% ethanol as preservative (ACS grade, purity ≥ 99.8%) from Thermo Fisher Scientific. We obtained 18.2 MΩ ultrapure water from an ELGA Pure-lab Ultra water purification system (Woodridge, IL). We purchased 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 (ω-9-cis) PC (DOPC)) and 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine (TopFluo-PC) from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Preparation of Substrates.** Because the cotton fabric was obtained from a commercial source, we cleaned the fabric with a nonpolar solvent chloroform and a polar solvent ultrapure water to remove nonpolar and polar impurities. Such impurities might have attached to the fabric during the process of manufacture or transport. Cleaning surfaces such as glass, indium tin oxide slides, and platinum wires with a series of solvents before the deposition of lipids is a common step in methods that rely on the vesiculation of lamellar stacks of phospholipid films to produce GUVs.\textsuperscript{21,23,25,44,46} We soaked a 10 cm × 10 cm piece of cotton fabric in 50 mL of neat chloroform for 30 min. After 30 min, we discarded the chloroform and repeated the process. We allowed the chloroform to evaporate and then proceeded to alternately rinse and soak the fabric in an excess of ultrapure water over the course of 3 h. After the final rinse, we dried the fabric at ambient temperature. The manufacturer certifies that the laboratory-grade filter papers are free of impurities. To be consistent with the treatment of the fabric, however, we soaked the fabric in chloroform for 30 min. After 30 min, we discarded the chloroform and repeated the process. Due to the low wet strength of the paper, we soaked it in water for only 30 min with no agitation. We then dried the paper at ambient temperature. To obtain consistently sized substrates, we punched 9.5 mm diameter circular disks from the paper using a paper punch (EK Tools Circle Punch, 3/8 in.) and cut similar sized pieces of fabric with a pair of scissors.

**Growth of GUVs.** We prepared a solution of 99.5±0.5 mol % of DOPC/TopFluo-PC in neat chloroform at a concentration of 2 mg/mL. We standardized the growth conditions by depositing appropriate volumes of the lipid solution to obtain 3 μg of lipid per 1 mg of cellulose (lipid/cellulose mole ratio ~7 × 10$^{-7}$) on the substrates. The small volume of solvent (10–20 μL) evaporated rapidly. We placed the lipid-coated substrates in a standard vacuum desiccator for 1 h to remove traces of solvent. To grow the GUVs, we placed the dry lipid-coated substrates into a 2 mL Eppendorf tube. We then added 500 μL of a solution of sucrose at a concentration of 100 mM and incubated for 60 min.

**Coverslip-Assisted Harvesting of the GUVs.** We placed a 100 μL droplet of 100 mM solution of sucrose on a glass coverslip. We transferred the substrate from the Eppendorf tube into the droplet using forceps. Water remained trapped in the hydrophilic porous cellulose substrates during the transfer, preserving the GUVs. We harvested the GUVs by gently aspirating the solution with a cut
pipette tip, careful to avoid touching the surface of the substrate. We transferred 100 µL of the suspension of harvested GUVs to a clean Eppendorf tube for storage until use. We obtained a high and consistent yield of GUVs in the form of a concentrated suspension. The concentrated suspension can be diluted as needed. This method of harvesting differs from our previously reported method—gravity-induced flow of 1 mL of buffer through filter paper supported on polystyrene well inserts in 24-well plates.25

Confocal Microscopy and Image Analysis of GUVs. We constructed imaging chambers by covalently bonding custom-made PDMS gaskets with a square opening (width × length × height = 5 mm × 5 mm × 1 mm) to glass microscope slides. Before use, we passivated the chamber with a solution of 1 mg/mL casein to prevent rupture of the GUVs on the bare glass.49 We filled the passivated chamber with 25 µL of an isomolar solution of glucose and added a 5 µL aliquot of the suspension of harvested GUVs. We allowed the GUVs to sediment for 3 h before imaging. We captured images using an upright confocal laser-scanning microscope (LSM 880, Axio Imager.Z2m, Zeiss, Germany). We excited the TopFluor dye with a 488 nm argon laser and collected fluorescence using a 10× Plan-Neofluar objective with a numerical aperture of 0.3. We imaged the entire area of the chamber using an automated tile scan routine (49 images [850.19 μm × 850.19 μm (2140 pixels × 2140 pixels)] with a confocal slice thickness of 13 μm). The routine used an autofocus feature that focused at the plane of the glass slide by locating the plane that reflected laser light maximally. We analyzed the confocal images using a custom MATLAB routine (Mathworks Inc., Natick, MA). The routine thresholded the images and then applied a watershed algorithm to segment the fluorescent GUVs from the background. We used the native regionprops function to obtain the equivalent diameters of the segmented objects.

Scanning Electron Microscopy (SEM) of the Dry Substrates and Confocal Microscopy Imaging of the Hydrated Substrates. We obtained SEM images of the dry cellulose substrates using a field emission scanning electron microscope (GeminiSEM 500, Zeiss, Germany). The beam accelerating voltage was 1 kV and we collected secondary electrons from the surface of the substrates using an Everhart–Thornley secondary electron detector. We captured images at a lateral pixel resolution of 1.09 μm/pixel [1120 μm × 840 μm (1024 pixels × 768 pixels)]. For imaging of the hydrated substrates, we rendered the cellulose fibers fluorescent with the cellulose dye Direct Red 2325 and obtained confocal z-stacks at a lateral pixel resolution of 0.4 μm/pixel [640.17 μm × 640.17 μm (1620 pixels × 1620 pixels)] and an axial spacing of 35 μm/slice. We obtained bandpass filtered maximum intensity projections of the z-stacks and measured the diameters of the fibers using ImageJ (NIH, Bethesda, MD).

GUV Growth Cycle Experiments on Cotton Fabric. After harvesting the GUVs at the completion of a cycle of growth, we cleaned the fabric before the next cycle of growth. We rinsed the fabric with 10 mL of ultrapure water and then soaked it in ultrapure water under agitation for 30 min to remove traces of dissolved salts and sugars from the growth buffer. We placed the fabric in a 65 °C oven for 1 h to dry. The dry fabric was soaked in neat chloroform under agitation for 30 min. We removed the fabric from the chloroform, allowed the chloroform to evaporate, and placed the fabric in a vacuum chamber for 1 h to remove traces of chloroform. We deposited fresh lipid on the fabric to start the next cycle of growth.

Results and Discussion

Properties of the Cellulose Substrates. Figure 1a–c shows representative scanning electron microscopy (SEM) images of the papers. The cellulose fibers in the papers appear as twisted flattened cylinders with diameters of approximately 15–21 μm. G41 paper had large pores between the fibers (darker regions in the images). The apparent pores were smaller and fewer in number in the G1 and G42 images. These microstructural differences were consistent with the reported particle retention capacities and flow rates of the filter papers. The SEM images of the cotton fabric showed a highly ordered network of twisted cellulose fibers in the yarn with regions devoid of cellulose fibers between the orthogonal strands of yarn (Figure 1d).

Because the growth of GUVs occurs in water, we characterized the hydrated substrates using confocal microscopy. Figure 1e–h shows the maximum-intensity projection of the three-dimensional confocal z-stacks of the substrates. We applied a bandpass filter to the images to highlight the edges of the fibers. Histograms of the diameters of the cellulose fibers are shown in Figure S1. The average diameter of the fibers, d, was 21 ± 5 μm for G41, 20 ± 6 μm for G1, 16 ± 7 μm for G42, and 16 ± 4 μm for cotton fabric. Thus, G41 and G1 paper have on average fibers with larger diameters, whereas
G42 paper and the cotton fabric have cellulose fibers with smaller diameters.

Sizes of the GUVs are Bounded by the Average Diameter of the Cellulose Fibers. Figure 2a–d shows representative single-plane confocal images of the GUVs harvested from the four different substrates. Qualitatively, the GUVs appeared similar.

We quantified the diameters of the GUVs using a custom MATLAB image analysis routine. A histogram reveals that the distribution of GUV diameters was skewed, with a single well-defined peak and a right tail (Figure 2e). In this histogram, \( n = 131 \, 296 \), and the bin widths are 1 \( \mu \)m. The inset shows a zoomed view of a section of the right tail. Skewed distribution of vesicle sizes obtained through gentle hydration of lamellar phospholipid stacks on glass was first noted by Reeves and Dowben in 1968.\(^{13}\) We posit that skewed distribution of sizes is an intrinsic property of GUVs produced through methods that rely on the vesiculation of lamellar phospholipid stacks (i.e., electroformation, gel-assisted hydration, and cellulose-abetted hydration). Inspection of published histograms of GUV sizes obtained through electroformation\(^{14−47}\) and gel-assisted hydration\(^{11,23,24,48}\) reveals the likely generality of the right-tailed distribution. Unlike a normal distribution, which is symmetric, the mean, median, and mode of a skewed distribution do not coincide. Thus, representing the GUV sizes with arithmetic moments, such as the mean and standard deviation, without further qualification, likely misses the true statistical distribution of the population.

Right-tailed distributions of sizes occur regularly in dispersed particulate systems. Such distributions have been reported for the sizes of grains in nanocomposites,\(^{40}\) of nanocrystals,\(^{51}\) of draining foam bubbles,\(^{52}\) of sonicated small vesicles,\(^{53}\) of dividing bacteria,\(^{54}\) of raindrops,\(^{55}\) and even of animal flocks.\(^{56}\) Probability distribution functions that give rise to a right-tailed distribution include, among others, the log-normal distribution,\(^{45,57,58}\) exponentially truncated power law,\(^{56}\) and the Weibull distribution.\(^{53}\) Theoretical models that consider fundamental processes such as the rate of nucleation and rate of growth, and/or the rate of multiplicative processes such as the rate of fragmentation or coalescence, have been developed to fit these empirically determined particle size distributions.\(^{50−56}\) Such models are currently unavailable for GUVs obtained from the vesiculation of lamellar phospholipid stacks. We are currently working on a model for the formation of GUVs on cellulose substrates using our data.

We plot the size distributions as a box-and-whisker plot to compare the different populations (Figure 2f). The three different boxes per sample are the three independent experiments performed for each substrate. The lower whisker indicates the minimum size (1 \( \mu \)m to consider only GUVs). The bottom half of the box represents the 25th percentile of the distribution. The red line indicates the median of the distribution. The top half of the box is the 75\(^{th}\) percentile. The top whisker indicates the 98th percentile of the GUVs. The red diamonds are the remaining 2% of the GUVs.

Sample-to-sample variation in the GUV sizes was minimal, and the distribution of the GUV sizes was remarkably similar between the three grades of paper and the cotton fabric. The median diameter of the GUVs was between 5 and 6 \( \mu \)m for all the substrates. The mode was 4 \( \mu \)m. Fifty percent of the GUVs were between 4 and 9 \( \mu \)m in diameter. We conclude that the microstructure of the cellulose substrates (which varied...
significantly, see Figure 1) does not control the sizes of the GUVs.

We plotted \(d_f\) of the substrates as blue dashed lines in the box plot (Figure 2f). It is apparent that 98% of the GUVs had diameters smaller than \(d_f\). The median diameter of the GUVs in the substrates is about 25 percent of the average fiber diameter. Although the formation of GUVs > \(d_f\) was statistically rare, due to the high yield of GUVs, we obtained \(\sim100\,000\) vesicles with diameters that ranged from 20 to 60 \(\mu\)m in a 100 \(\mu\)L suspension. The distribution of the diameters of GUVs on the cellulose fibers before harvesting was similar to the distribution of diameters of the harvested GUVs (Figure S2). Longer incubation times, including overnight incubation, did not change the size distribution of the GUVs (data not shown). We conclude that the diameters of the GUVs are limited by the diameter of the cellulose fibers.

**Cotton Fabric and G42 Paper Yielded a Larger Number of GUVs.** We also quantified the number of GUVs that we obtained from each substrate. To allow comparison between the substrates, we normalized the GUV counts by the mass of lipid deposited, i.e., we report the number of GUVs per \(\mu\)g of lipid. The average number of GUVs per \(\mu\)g was similar between G41 and G1 papers (Figure 3). The inset table shows the coefficient of variance (%) of the number of GUVs for each of the substrates.

![Figure 3. Comparison of GUV yields among the substrates.](image)

The inset table shows the coefficient of variance (%) of the number of GUVs for each of the substrates. The average number of GUVs obtained from G42 paper and cotton fabric was about 3 times higher than the average number of GUVs obtained from G41 and G1 papers. The average number of GUVs per \(\mu\)g of lipid was similar between the cotton fabric and G42 paper. Sample-to-sample variation in the number of GUVs harvested from cotton fabric was lower than the sample-to-sample variation in the paper samples (Figure 3, inset table). We attribute this observation to the ordered arrangement of cellulose fibers in the fabric. We note that even the lowest yielding papers appear to exceed the GUV yields reported for electroformation.45

Compared to G41 and G1 papers, cotton fabric and G42 paper have lower \(d_f\). Additionally, G42 paper has a higher particle retention capacity and a lower liquid flow rate, reflective of the denser packing of cellulose fibers and smaller pores. Although the cotton fabric had large windows of no cellulose between the orthogonal strands of yarn (Figure 1d), the cellulose fibers in the yarn were densely packed. Thus, our data suggest that substrates with smaller average fiber diameters and denser packing of cellulose fibers yield a larger number of GUVs per unit mass of lipid.

**Cotton Fabric Supports Multiple Cycles of GUV Growth.** Paper is an attractive substrate for fabricating GUVs at the laboratory benchtop due to its low cost and disposability. At larger scales, the cost of raw materials, the cost of disposal, and general sustainability favor reusable substrates. Paper has low wet strength. In contrast, cotton fabric can withstand repeated mechanical insults such as abrasion and mechanical washing.

We performed experiments to determine if fabric can support sequential cycles of GUV growth. One cycle of growth consisted of deposition of lipids, growth for 60 min, and then harvest of the GUVs. Cleaning and drying the fabric after the harvest and depositing fresh lipids repeated the cycle. We performed five cycles on a single piece of fabric. Figure 4a,b shows representative images of the GUVs obtained from pristine fabric in the first cycle and the GUVs obtained from the fabric after five cycles. The vesicles appeared indistinguishable. Quantitative comparisons confirmed that the GUV sizes (Figure 4c) and yields (Figure 4d) were similar in each of the five cycles. Other than a slight shrinkage of the yarn, the fabric after the fifth cycle of growth was indistinguishable from the fabric imaged after the first cycle of growth. Thus, it is likely that the fabric can support additional cycles of GUV growth. These experiments suggest that the process of GUV growth does not alter the cellulose fibers. The cellulose fibers accelerate the rate of vesiculation of lamellar stacks of phospholipids compared to the rates of vesiculation on glass \(^{3,4}\) and remain unchanged at the end of the process.

**CONCLUSIONS**

All the cellulose substrates that we tested produced populations of GUVs with right-skewed unimodal distributions in diameters. The distribution in sizes was similar among the
different substrates and bounded by the average diameter of the cellulose fibers. The GUV yields, however, were different between the substrates. The yields were highest from G42 filter paper and cotton fabric, with sample-to-sample variation lowest for the cotton fabric. Thus, G42 filter paper is optimal for the PAPYRUS method. Cotton fabric supports multiple cycles of growth and harvest without any apparent change in the properties of the GUVs. Our results demonstrate that cotton fabric is a superior substrate for fabricating GUVs due to high yields and minimal sample-to-sample variation. Finally, although we focus on phospholipids in this paper, these results are likely general to vesicles grown from other lamellar phase forming amphiphiles.\(^6\)

**ASSOCIATED CONTENT**

Supporting Information
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Histograms of the diameters of the cellulose fibers in the four substrates; representative images of GUVs on cellulose fibers and a histogram of GUV diameters measured while still attached to the cellulose fibers (PDF)

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Notes
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