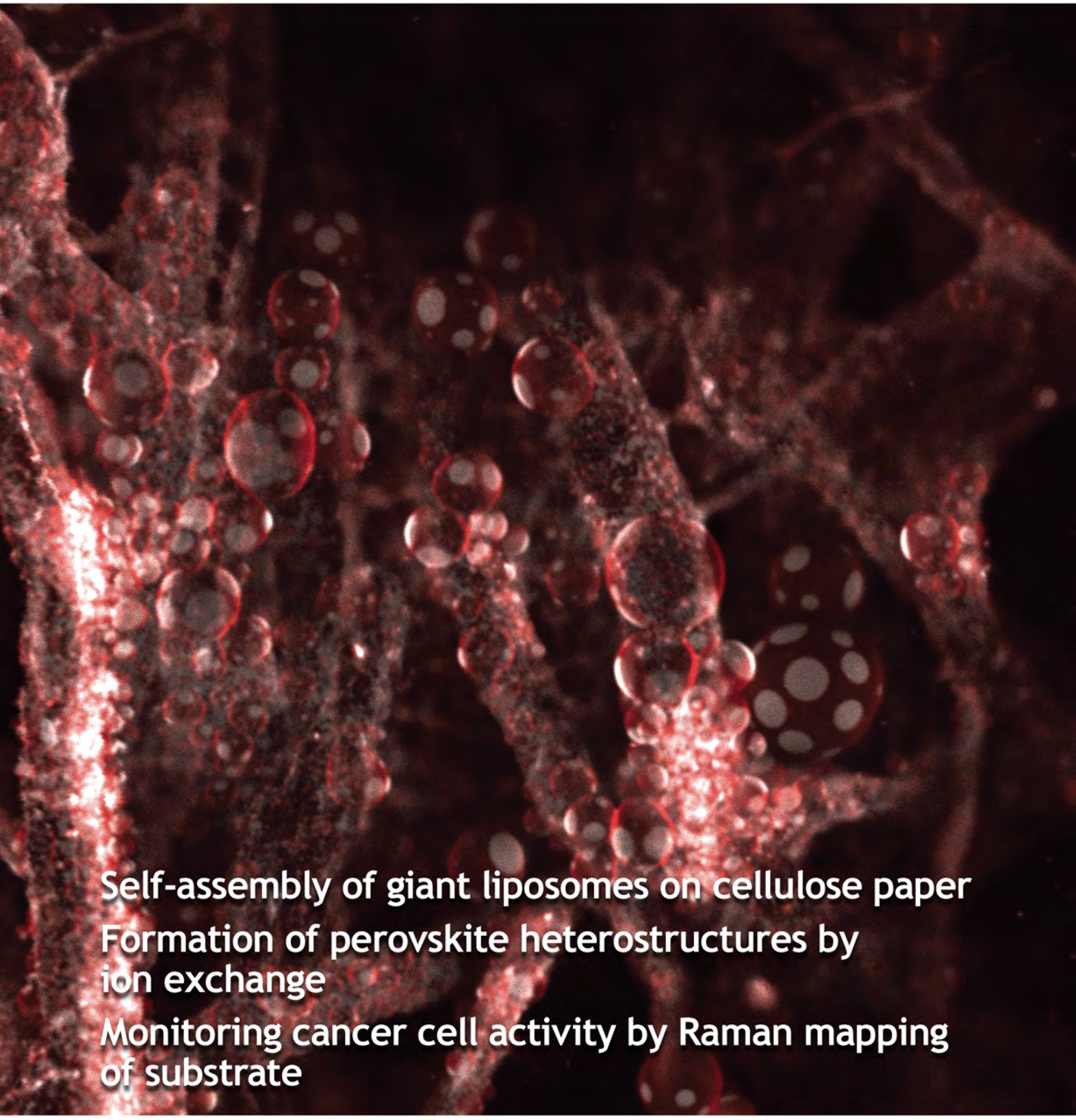
ACS APPLIED MATERIALS & INTERFACES

December 7, 2016
Volume 8
Number 48
pubs.acs.org/acsami



Self-assembly of giant liposomes on cellulose paper

Formation of perovskite heterostructures by ion exchange

Monitoring cancer cell activity by Raman mapping of substrate

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Letter

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Novel Application of Cellulose Paper As a Platform for the Macromolecular Self-Assembly of Biomimetic Giant Liposomes

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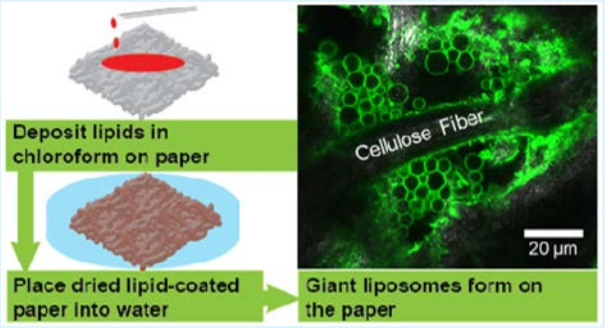
Supporting Information

ABSTRACT: We report a facile and scalable method to fabricate biomimetic giant liposomes by using a cellulose paper-based materials platform. Termed PAPYRUS for Paper-Abetted liPid hYdRation in aqUeous Solutions, the method is general and can produce liposomes in various aqueous media and at elevated temperatures. Encapsulation of macromolecules and production of liposomes with membranes of complex compositions is straightforward. The ease of manipulation of paper makes practical massive parallelization and scale-up of the fabrication of giant liposomes, demonstrating for the first time the surprising usefulness of paper as a platform for macromolecular self-assembly.



KEYWORDS: *cellulose, paper, giant unilamellar vesicles, encapsulation, synthetic cells*

This paper reports the novel application of cellulose paper as a platform to fabricate giant liposomes. Giant liposomes are self-assembled macromolecular structures[1] useful for encapsulating and controlling the release of cargo,[2] synthesizing proteins (cell-free synthesis)[3] and inorganic minerals (biomineralization),[4] constructing artificial cells[5] including red blood cells,[6] building nanoconduits, nanowires, and nanoparticles through bioinspired templating strategies,[7] and elucidating the origins of life through the building of minimal protocells.[8,9] Giant liposomes are also widely used model systems for biochemical and biophysical studies of membrane processes.[10−12] Current methods for producing giant liposomes include, among others,[13] gentle hydration,[14−16] electroformation,[17] gel-assisted formation,[18−20] and microfluidics.[21,22] These methods often require elaborate preparatory steps[13,22] or have limitations that restrict general applicability.[23] For example, electroformation results in lipid peroxidation,[24] works poorly in ionic buffers and with negatively charged lipids,[13] and is challenging and costly to scale-up. Gel-assisted methods suffer from contamination from polymer molecules of the lipid membrane and of the vesicular lumen,[23] and cannot be applied to fabricate liposomes with lipids with high transition temperatures.[18−20] Microfluidic methods, while providing exquisite control of the size and to some extent the composition of the lipids in the membrane of the liposomes,[21,22] often have residual solvents trapped in the membrane.[22]

Cellulose is an abundant biopolymer used in various demanding applications such as composites[25] and in everyday commercial-scale applications such as paper and textiles, and in paper-based biomedical diagnostic[26,27] and energy devices.[28] Cellulose is hygroscopic and hydrophilic, yet essentially insoluble in water and in most common organic solvents, even at elevated temperatures.[29] Although consumer grade papers often have fillers and sizers and are treated chemically to improve print quality, laboratory grade filter and chromatography paper are free of these additives.[30] We show that hydrating dried lipid films on commercially available cellulose chromatography paper or filter paper provides a facile route to preparing giant liposomes (vesicles) that are predominantly unilamellar (GUVs). The swelling of cellulose fibers upon exposure to water[29] likely provides the driving force for separating the lamellae[13] in hydrated multibilayer stacks of lipids into unilamellar liposomes. In contrast, 12−36 h after hydration, electrostatic repulsion between the charged headgroups of hydrated lipids in multibilayer stacks on glass or roughened Teflon produces liposomes in the gentle hydration technique,[14−16] and an oscillating electric field acting on the buffer and the lipids, separates the lamellae in multibilayer stacks to produce liposomes in the electroformation technique.[13,17] The use of cellulose paper as a materials-based platform for the production of liposomes is a significant departure from current methods and greatly simplifies the procedure for fabricating these bioinspired structures.

The method for producing giant liposomes, shown schematically in Figure 1, is termed PAPYRUS (Paper-Abetted liPid hYdRation in aqUeous Solutions) after the Latin word for paper. The PAPYRUS method involves depositing several tens of microliters of lipids at a concentration of 1−6 mg/mL

Received: September 20, 2016
Accepted: November 14, 2016
Published: November 15, 2016

DOI: 10.1021/acsami.6b11960
ACS Appl. Mater. Interfaces 2016, 8, 32102−32107

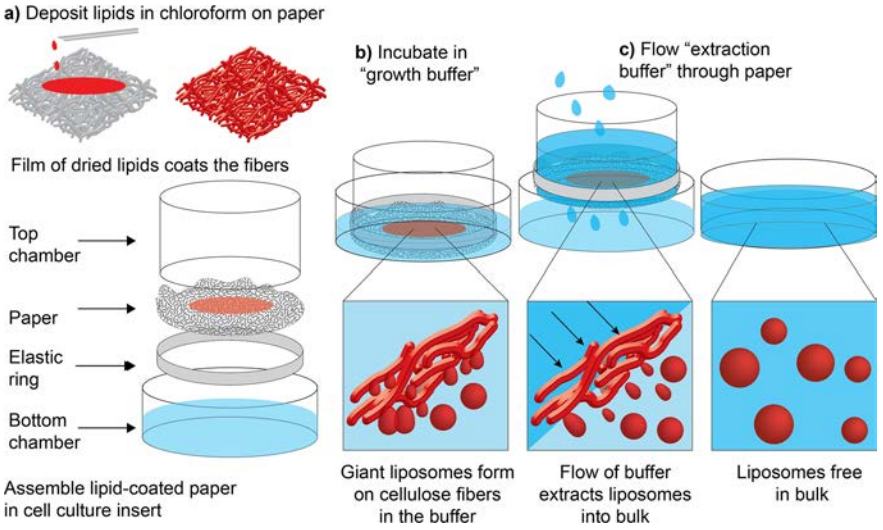


Figure 1. Schematic of the PAPYRUS method. The steps for the method are: (a) Deposit a solution of lipids in chloroform onto the paper, allow the solvent to evaporate and coat the cellulose fibers with a dry lipid film. (b) Place the dry lipid-coated paper into a desired aqueous solvent and incubate for 30−120 min. Sandwiching the paper between polystyrene well inserts and elastic bands offers a convenient means of immobilizing the lipid-coated paper in a buffer-containing chamber. (c) Following a growth period, flow an extraction buffer through the paper to detach the liposomes into the bulk. Alternatively, use a cut pipet tip to aspirate the liposomes from the surface of the paper.

(single component or mixtures, depending on the desired composition of the membrane) dissolved in an organic solvent (typically chloroform) onto small pieces of dry chromatography paper (~1 cm^2). The diameter of the fibers in these papers range from around 15 to 19 μm, with average pore sizes of around 11 μm[30] (Figure 2a). The volatile solvent evaporates rapidly (within tens of seconds), leaving behind a dried lipid film coating the cellulose fibers (Figure 2b). A brief 15 min desiccation under vacuum, to match well established protocols for electroformation,[17] drives residual solvent from the film. The lipid-coated paper is then placed into an aqueous "growth buffer". The choice of growth buffer is dictated by the purpose of the experiment, and can vary from ultrapure water to sugar solutions and to commonly used ionic buffers such as TBS (Tris buffered saline), HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, or PBS (phosphate buffered saline). Numerous giant liposomes, ranging in size from 1 μm to 20 μm or more can be seen coating or floating adjacent to the cellulose fibers after a 1 h incubation in the growth buffer (Figure 2c). A 10 min incubation of the lipid-coated paper in ultrapure water before adding a concentrated stock of salts to obtain the final desired ionic strength, produced the best results for PAPYRUS in ionic buffers (Figure S1). The porous nature of chromatography paper allows easy detachment of the liposomes through gentle flow after the liposomes have formed. For example, aspiration perpendicular to the surface of the paper with a micropipette or gentle gravity driven flow of an "extraction buffer" (which can be of similar composition to or different from that of the growth buffer) through the paper results in liposomes in bulk solution, suitable for further manipulation (Figure 2d).

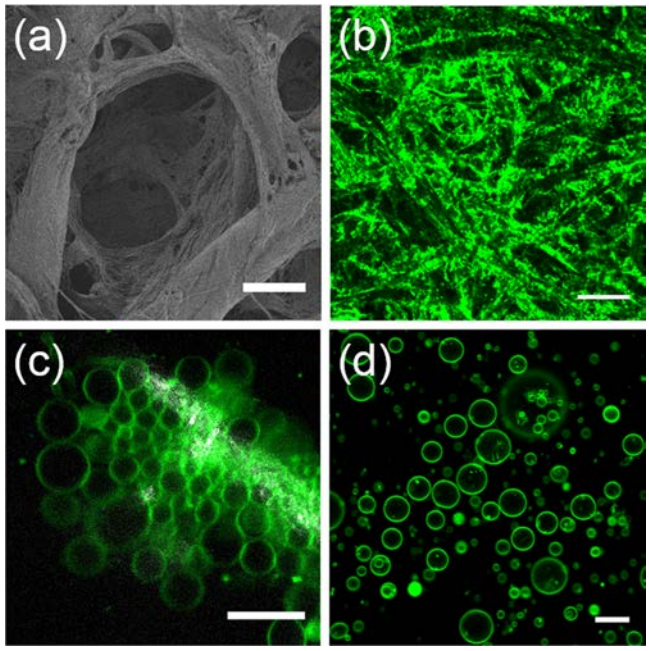


Figure 2. Images demonstrating the progressive steps of the PAPYRUS method. (a) Scanning electron micrograph (SEM) image demonstrating the microstructure of the paper. (b) Fluorescence image of the paper after deposition of lipids demonstrating the dried lipid film coating the cellulose fibers. (c) Region on the same paper (not the same area) after 60 min of incubation in a 250 mM sucrose solution, showing giant liposomes (green circles) coating a cellulose fiber (gray). (d) Giant liposomes in bulk after extraction from the paper. Scale bars: (a) 50 μm, (b−d) 20 μm.

Although we typically allowed the paper to incubate in the growth buffer for 60 min, the process of initial liposome budding was rapid. Within 5 min, the lipid film on the cellulose fibers budded as uni- and multilamellar liposomes (Figure 3a). On average, these small liposomes grew in size during the course of 1 h (Figure 3b,c), with neighboring liposomes occasionally fusing to form larger liposomes (Figure S2a). Over the 1 cm^2 area of the paper, however, the process of growth was highly dynamic. Multiposition imaging revealed that in some regions of the paper, liposomes disappeared over time (Figure S2b) and multilamellar structures evolved to form unilamellar liposomes (Figure S2c). Once extracted from the paper, the liposomes prepared through PAPYRUS do not evolve further, and remain visually unchanged for at least 5 days (Figure S3).

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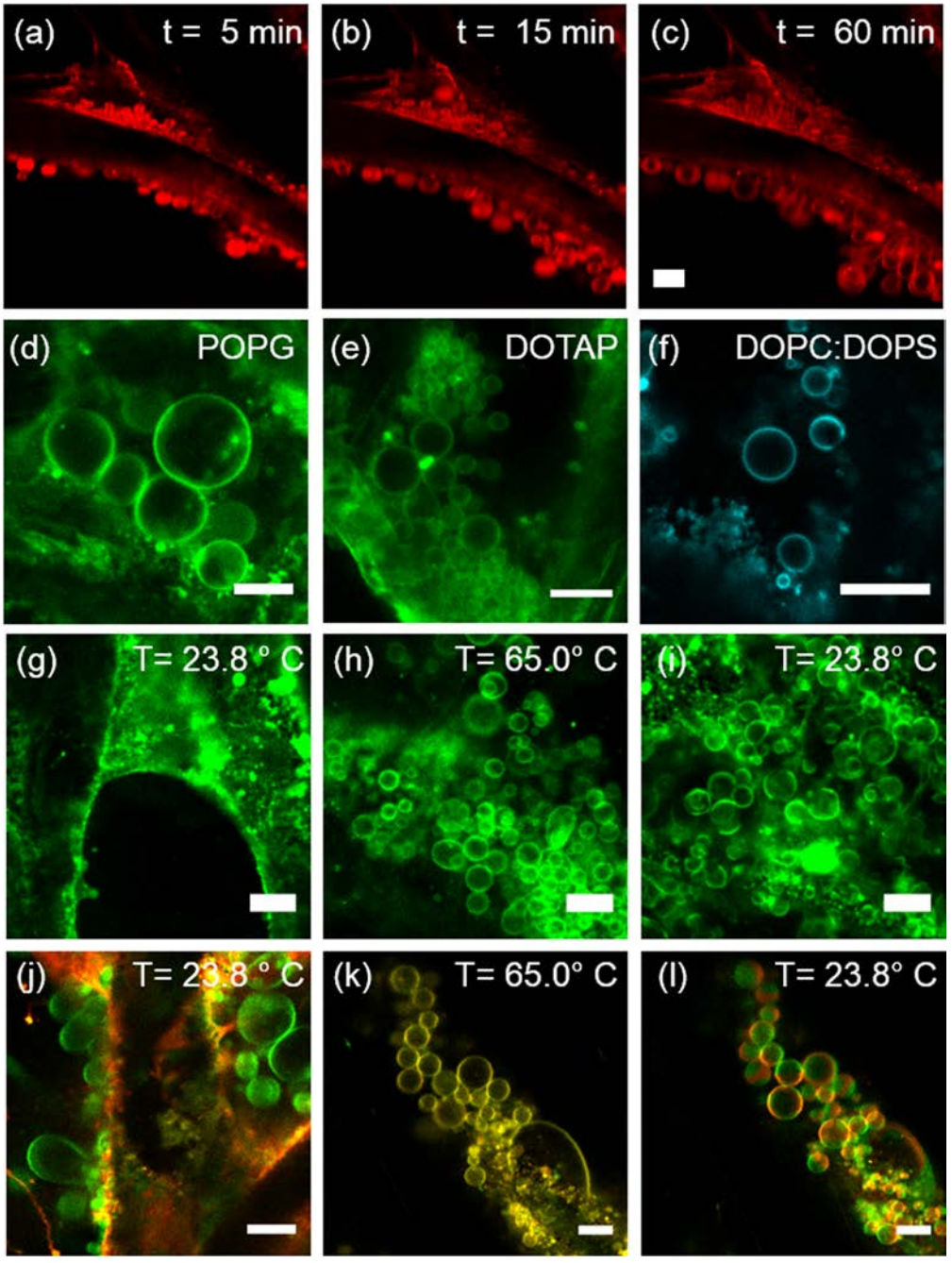

Figure 3. Time-lapse images of liposomes budding off of the fibers at (a) 5, (b) 15, and (c) 60 min. Liposomes prepared from charged lipids, d) POPG, negatively charged, (e) DOTAP, positively charged, (f) a 50:50 mixture of DOPC and DOPS, negatively charged, labeled by the protein Annexin V-AlexaFluor 488 that binds specifically to phosphatidylserine. (g) Fluorescence image shows the absence of DPPC liposomes when grown at room temperature. (h) Numerous spherical DPPC liposomes formed when grown at 65 °C. (i) Upon cooling, the liposomes shrunk and buckled as the membrane transitioned from a liquid state to a gelled state. (j–l) Composite false color confocal images with green (the membrane dye TopFluor-Cholesterol) indicating a liquid ordered (L_o) phase and red (the membrane dye Rhodamine-DPPE) indicating a liquid disordered (L_d) phase, and yellow indicating that both dyes cannot be spatially resolved. The lipid mixture was composed of egg sphingomyelin:DOPC:Cholesterol at a mole percent of 54.8:20:25. j) Image showing the growth of liposomes containing only a single phase from a ternary mixture of lipids when grown at room temperature. (k) At 65 °C, both dyes labeled the liposomes that grew on the fibers, and thus the composite image appeared yellow. (l) Upon cooling, the liposomal membranes phase separated into L_o and L_d domains, demonstrating phase coexistence. Scale bars, 10 μm. Lipid fluorescent labels: (a–c) 0.5 mol percent Rhodamine DPPE, (d, e, g–i) 1 mol percent TopFlour-Cholesterol, (f) Annexin V-AlexaFluor 488, (j–l) 0.1 mol percent Rhodamine-DPPE and 0.1 mol percent TopFlour-Cholesterol.

The PAPYRUS method is general and was successful for all bilayer-forming lipid types that we tested (Table S1). Figure 3d shows the formation of liposomes composed of the negatively charged lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phosphor-rac-(1-glycerol)] (POPG), Figure 3e shows the formation of liposomes from the synthetic positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and Figure 3f shows the binding of the protein Annexin V conjugated with the fluorescent dye Alexa Fluor 488 to liposomes containing 50 mol percent 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine

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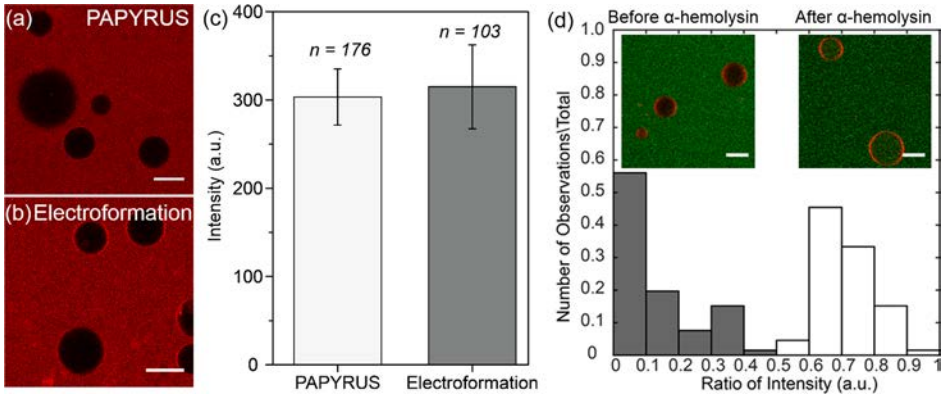

Figure 4. (a, b) Confocal fluorescence images of liposomes incubating in Direct Red 23. The liposome membranes excluded the fluorescent dye, and thus the lumens appeared dark. Scale bars 20 μm. (c) Bar plot of the fluorescence intensity of Direct Red 23 in the membranes of liposomes prepared thorough PAPYRUS (white bar) and electroformation (gray bar). Bars are average intensities from n = 176 liposomes from PAPYRUS, and n = 103 liposomes from electroformation, error bars are standard deviations from the mean. It is clear that the intensity of Direct Red 23 in the membrane was similar for the electroformed liposomes and liposomes prepared through PAPYRUS. d) Histogram of the ratio of lumen intensities to intensity of the continuous phase. Gray bars are intensities prior to addition of 2 μM of α-hemolysin (n = 66), white bars are intensities 30 min post addition (n = 66). Insets are false color multichannel confocal images, with fluorescein colored green and Rhodamine-DPPE colored red. Left inset shows liposomes before exposure to α-hemolysin, the right inset is after exposure. It is clear that the liposomes prepared through PAPYRUS were predominantly unilamellar. Scale bars 10 μm.

(DOPS) on cellulose paper. Annexin V binds specifically to phosphatidylserine, and is a reliable indicator of the presence of phosphatidylserine in membranes.[31] Furthermore, the insolubility of cellulose, even at high temperatures, allowed the following observation with PAPYRUS: Long-chain saturated lipids, and lipid mixtures containing these lipids had to be hydrated at a temperature above the chain transition temperature of the lipids — which could be significantly above ambient — to obtain reproducible growth of giant liposomes.[14,15,32] For example, at room temperature, no liposomes were found on the cellulose fibers with films prepared from the saturated lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) after 90 min of incubation at 23 °C (the transition temperature of DPPC is 45 °C) (Figure 3g). At 65 °C, however, numerous spherical liposomes coated the surfaces of the fibers (Figure 3h). Upon cooling to room temperature, these liposomes shrunk and the membranes crumpled, consistent with membranes undergoing a liquid to solid transition[33] (Figure 3i). Extremely slow recovery of fluorescence intensity in the cross-shaped bleached region, probed through Fluorescence Recovery After Photobleaching (FRAP) confirmed the gelled nature of the membranes (Figure S4a). Liposomes do form on the cellulose fibers at room temperature when PAPYRUS was applied to pseudoternary lipid mixtures containing sterols that show liquid–liquid phase coexistence.[12] To visualize the two liquid phases through confocal fluorescence microscopy, we labeled the lipid mixture with two dyes, TopFluor-Cholesterol and Rhodamine-DPPE. Rhodamine-DPPE is strongly excluded from the liquid ordered (L_o) phase,[11,12] and thus only labels the liquid disordered (L_d) phase, while TopFluor-Cholesterol shows no preference for either phase and thus labels both the L_o and L_d phases.[11] To obtain images that showed the spatial distribution of the dyes, we colored the Rhodamine-DPPE channel red, the TopFluor-Cholesterol green, and overlaid the two channels. Growth at room temperature yielded membranes labeled with a single dye, either only TopFluor-Cholesterol or only Rhodamine-DPPE indicating that only a single phase was present in the liposomes (Figure 3j). In contrast, when grown at 65 °C, the liposomes on the cellulose fibers contained both dyes (Figure 3k). When cooled to room temperature, the dyes separated into spatially distinct domains in the liposomes (Figure 3l). Time-lapse imaging showed the formation of spherical domains that fused and grew with time, further confirming that two liquid phases were present on the liposomes (Figure S4b). Along with ease of obtaining variations in the composition of the membrane, encapsulating macromolecules in the liposomes was also relatively straightforward. When dispersed in the growth buffer, macromolecules such as proteins and polysaccharides spontaneously incorporated into the liposome lumen and demonstrated robust compartmentalization from the surrounding solution (Figures S5 and S8).

To determine if cellulose associated with the liposomal membranes prepared through PAPYRUS, we conducted a fluorescence-based assay using Direct Red 23. Direct Red 23 fluoresces brightly upon binding to cellulose in the primary cell wall of plants[34] (Figurs S6 and S7). Liposomes prepared both through PAPYRUS (Figure 4a) and through electroformation (Figure 4b) in a solution containing Direct Red 23 did not show an enrichment of Direct Red 23 in the membranes. Quantitative analysis of the fluorescence of Direct Red 23 of liposomes prepared through PAPYRUS and electroformation showed that the liposomes were indistinguishable from each other (Figure 4c), confirming that cellulose does not incorporate into the membranes of liposomes prepared through PAPYRUS. Such a result is not surprising, considering the insolubility of cellulose in both water and chloroform.[29]

We probed the unilamellarity of giant liposomes prepared through PAPYRUS by conducting a liposome permeation assay with the membrane-porating protein α-hemolysin.[21] When present in solution, α-hemolysin is able to permeabilize unilamellar liposomes by creating pores in the outer membrane. Multilamellar liposomes retain their barrier characteristics since the protein does not translocate through the pores created in the outer membrane to access the interior membranes[21] (Figures S9 and S10). We prepared liposomes through PAPYRUS and transferred the liposomes into a solution containing 40 μM of sodium fluorescein. The bilayer excluded

DOI: 10.1021/acsami.6b11960
ACS Appl. Mater. Interfaces 2016, 8, 32102−32107

the dye from the interior of the liposomes, which thus appeared black against a bright background (Figure 4d, left inset). A histogram of the distribution of the ratio of the lumen intensities to the intensity in the continuous phase confirmed that the liposomes excluded the dye (Figure 4d). When exposed to 2 μM of α-hemolysin, the liposomes became permeable to fluorescein (Figure 4d, right inset), and consequently the distribution of intensities shifted to higher values, demonstrating that the liposomes were unilamellar.

A significant advantage of PAPYRUS over existing techniques is the extreme simplicity of the method and its compatibility with many different compositions of lipids and aqueous buffers. Unlike electroformation—the most widely used technique currently—a method that requires a power source, specialized apparatus, platinum wires, or indium tin oxide (ITO) coated slides that degrade,[35] PAPYRUS relies on readily available, disposable, and economical paper. The method also eliminates the potential for contamination by soluble polymers and changes in lipid properties due to electric field-induced oxidation. Finally, the ease of manipulation of paper makes practical massive and economical parallelization and scale-up of the fabrication of giant liposomes by employing readily available fluid receptacles such as Eppendorf tubes or multiwell plates (Figure S11). PAPYRUS thus further broadens the use of giant liposomes, and introduces the many benefits of paper, apparent in other fields,[26−28] to the field of lipid-based materials and technologies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b11960.

Details of experimental methods; details on the characterization of the liposomes including encapsulation, unilamellarity, repeatability, use of ionic buffers, and phase behavior; and Figures S1−S12 (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by NSF Award CBET-1512686. SEM imaging and characterization was performed on a scanning electron microscope purchased through NASA Grant NNX15AQ01A. We thank Dr. David Gravano for helpful conversations.

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