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Rapid Access to Giant Unilamellar Liposomes with Upper Size Control: Membrane-Gated, Gel-Assisted Lipid Hydration

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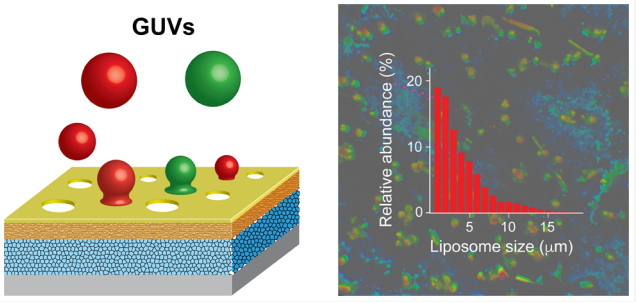
ABSTRACT: Combining gel-assisted lipid hydration with membrane-based lipid extrusion, we demonstrate here a general procedure for rapid preparation of giant unilamellar liposomes with upper size control. Featured in this procedure are planar lipid stacks deposited on poly(vinyl alcohol) gel, which are further laminated atop with microporous polycarbonate membranes. Control of liposome size is thus realized through the uniform-sized pores of the latter, which provide the only access for the underlying lipids to enter the main aqueous phase upon hydration. Production of both single-phased and biphasic (Janus) liposomes using several commonly employed model lipids, including 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and cholesterol, is presented. The size distribution, yield and lamellarity of these liposome products are characterized and analyzed in detail by confocal fluorescence microscopy. This procedure thus offers a simple and fast alternative route to giant unilamellar liposomes with upper size control.



INTRODUCTION

Giant liposomes represent an indispensable class of aqueous-dispersed spherical lipid assemblies, as their micrometer size renders them not only amenable to various microscopic investigations but also suitable as biological cell mimics.[1−3] Several reliable methods have been developed over the years to prepare such liposomes, for example, by hydration/swelling,[4−8] electroformation,[9,10] and emulsion.[11−13] While the amphiphilicity of lipids, and hence their intrinsic tendency to self-assemble in water, almost always guarantee some liposome formation, more challenging is the precise control of lamellarity and size of the final products. To this end, much recent work has been directed toward size-controlled preparation[14−22] of giant liposomes and in particular, single-sized liposome arrays.[23−25] Routine access to reliable size control foreseeably will add new capabilities to our research in membrane biophysics and colloid chemistry, for example, in following size-dependent liposome interactions and the ensemble behavior of liposome populations. Adding to this ongoing effort, we demonstrate here a general and simple procedure for preparation of giant unilamellar liposomes with upper size control.

One proven approach to liposome size control is through apportioning the amount of lipids that assemble into individual liposomes. By limiting and homogenizing the quantity of the constituents, this approach removes a major contributor to liposome size variation, that is, random lipid uptake from a large continuous lipid supply, from the vesiculation process. Depending on how exactly the lipid apportionment is realized, most of the existing work can be fitted into three categories. (1) Microcontact printing,[14,23,24] in which minute amounts of lipids are microarrayed onto a planar substrate via a positive-featured stamp. (2) Lipid backfilling.[17,18,22,25] In this case, the location of lipids on the substrate is defined by a support structure containing, for example, lithographically defined recesses. Once in place, liposome production from these lipid islands can be accomplished following either hydration or electroformation routes, as the case of the microcontact-printed lipid deposits. In both cases, finer control is possible as to whether to keep the liposomes attached to the substrate or release them into the aqueous phase. Quite different from these two is (3) Double emulsion.[15,19−21] In this case, lipid-dispersed water-in-oil-in-water (W/O/W) emulsion droplets are first produced, often through multiphase laminar flows. Subsequent removal of the oil phase from the emulsion then causes the outer lipid monolayer to collapse onto the inner one, producing liposomes with size comparable to the water core.

In the present work, we report a new approach to size-controlled giant liposome production by combining gel-assisted lipid hydration[8] with membrane-based lipid extrusion.[1,26] Featured in this procedure are planar lipid stacks deposited

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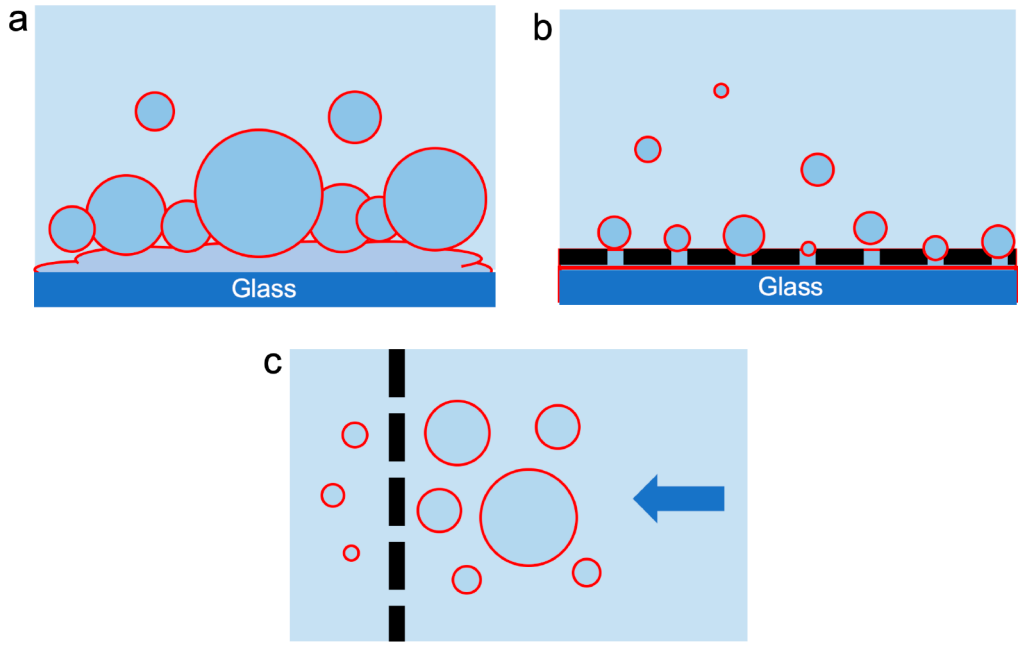

Figure 1. Schematic presentation of liposome preparation methods studied in this work. From a to c: gel-assisted hydration (method 1), membrane-gated, gel-assisted hydration (method 2), and gel-assisted lipid hydration followed by liposome extrusion (method 3). Liposomes and PC membranes are depicted by red circles and broken black lines, respectively.

on poly(vinyl alcohol) gel, which are further laminated atop with microporous polycarbonate membranes. Because these pores provide the only access for the underlying lipids to enter the main aqueous phase upon hydration, they quite conveniently define the upper size limit of liposomes thus produced. Production of both single-phased and biphasic (Janus) liposomes using several commonly employed model lipids, including 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and cholesterol, is presented. The size distribution, yield, and lamellarity of these liposome products are characterized and analyzed in detail by confocal fluorescence microscopy. This procedure thus offers a simple and fast alternative route to giant unilamellar liposomes with upper size control.

EXPERIMENTAL SECTION

Materials. All lipids, including 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (rho-DOPE), 23-(dipyrromethene boron difluoride)-24-norcholesterol (Bodipy-chol), were products of Avanti Polar Lipids (Alabaster, AL). Other chemicals, including poly(vinyl alcohol) (PVA, MW: 145 000), cholesterol, chloroform, were obtained from Sigma-Aldrich. Deionized (DI) water of 18.2 MΩ·cm (Millipore) was used throughout this work.

Track-etched polycarbonate (PC) filter membranes employed in this work were either Whatman Nuclepore (GE Healthcare; thickness: 7−22 μm) or Isopore (Millipore Sigma, thickness: 21−27 μm) membranes with pore sizes of 3, 5, 8, and 10 μm and nominal thickness of 0.25 mm.

Liposome Preparation Procedures. Preparations of giant liposomes via three routes were tested and compared in this work: (1) gel-assisted lipid hydration, (2) membrane-gated, gel-assisted lipid hydration, and (3) gel-assisted lipid hydration followed by liposome extrusion. Their main features are compared schematically in Figure 1.

1. Gel-Assisted Lipid Hydration. This was done by following the gel-assisted hydration method[8] by Marques et al. with minor modifications. Briefly, dry poly(vinyl alcohol) (PVA) films were prepared by first spreading a 5 wt % PVA aqueous solution on precleaned glass slides, followed by drying at 50 °C for 0.5 h. On each of these dry PVA films, a 5 μL drop of a lipid stock solution (1 mM total lipid dissolved in chloroform) was then cast, and subsequently dried under vacuum for overnight at room temperature. Composition wise, these lipid stock solutions contain either a single component (DPPC, DOPC, and POPC), or their binary mixtures with cholesterol at 7:3 mol mixing ratio, or 35/35/30 (mol %) DPPC/DOPC/Chol in the case of Janus liposomes. In the final step, such dried lipid films were hydrated in DI water to yield liposomes. Depending on the lipid composition, the hydration temperature/duration vary: 40 °C for 1 h for low-melting DOPC and POPC (with/without cholesterol), 55 °C for 1 h for DPPC and 60 °C for 2 h for Janus liposomes. At the end of the hydration process, liposomes were harvested with pipettes and stored at 4 °C.

2. Membrane-Gated, Gel-Assisted Lipid Hydration. In this procedure, PC membranes with specific pore sizes were conformally placed on top of the lipid deposits prior to hydration. To achieve so, the PC membrane was first wetted with DI water briefly and while it was still moist, gently pressed onto the dry lipid stacks similarly formed on PVA gel as before. The glass slide carrying the PC membrane/lipid/PVA assembly was then fitted face-down to a precleaned polypropylene cell (Qorpak polypropylene screw cap, 22−400, unlined). The cap was prefilled with DI water to a level a few mm below its rim, so that the latter was not in direct contact with the lipid stacks upon sealing. To ensure a good seal, the cell is further secured with a low-tension spring clip clamp; to start the lipid hydration, the assembled cell was simply turned upside down. The hydration temperature/duration conditions were identical to those specified in method 1. Thus-produced liposomes are similarly harvested and stored.

3. Gel-Assisted Lipid Hydration Followed by Liposome Extrusion. In this case, the liposomes were produced from a two-step procedure, in which 1 mL POPC or Janus liposome samples obtained by lipid hydration (method 1) were further extruded through PC membranes with 10 μm pores using a plunger-based lipid extruder (Mini-Extruder, Avanti Polar Lipids). The final products were collected after passing the liposome solutions through membranes for 10 rounds.

Confocal Fluorescence Microscopy. Fluorescence images of giant liposomes were acquired on a Nikon A1+/MP confocal scanning laser microscope (Nikon Instruments, Melville, NY) using 10× objective

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Langmuir 2020, 36, 13193−13200

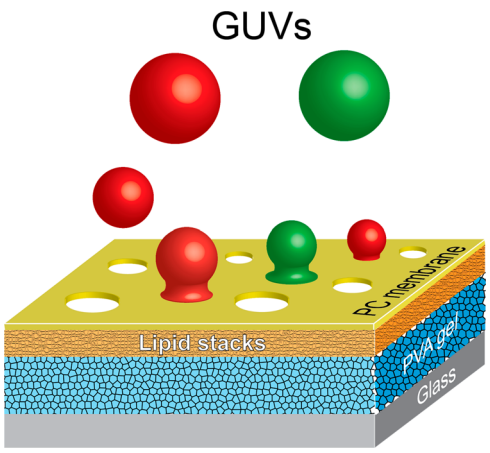

Figure 2. Schematic of the layered design of the membrane-gated, gel-assisted lipid hydration method. For clarity, the water layer atop of the assembly is omitted from the drawing.

and excitation laser lines at 488 and 561 nm. The corresponding green and red emission signals were filtered at 525 ± 25 and 595 ± 25 nm, respectively. The confocal pinhole was typically set at 50 μm. As imaging cells, home-prepared poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) microwells (diameter: 3 mm; depth: 2 mm) fixed on glass slides were employed. All liposome samples were given 1 h to settle in the PDMS cells under ~100% humidity before imaging.

Z-stacked fluorescence images of liposomes as they were forming during hydration (~16 min) were acquired with the same fluorescence microscope by taking *xy*-plane slices along the *z*-axis in 1 μm increment for 50 steps. As samples, lipid stacks of POPC doped with 0.2% rho-DOPE, either deposited on PVA gel or further covered by PC membranes (pore diameter: 10 μm), were hydrated in DI water at room temperature in PDMS chambers (diameter: ~1 cm). Three-dimensional (3D) rendering of these images was performed with Nikon NIS-Elements AR software package.

Fluorescence imaging of liposomes used for lamellarity analysis was similarly acquired with the same confocal microscope, except with a lower dye loading (0.05 mol % rho-DOPE and/or 0.05 mol % Bodipy-chol), in the samples. For each sample, ~200 liposomes were analyzed to obtain the reported yields.

Quantification of Liposome Yields. The relative yields of liposomes prepared from all three methods were determined by fluorescence measurements using a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific). To do so, liposome samples, containing either POPC or POPC/chol (70:30 mol %) doped with 1 mol % rho-DOPE, were first prepared using methods 1 and 2 from comparable amounts of lipid precursors; parts of liposome products obtained from method 1 were further extruded to yield samples of method 3 as described above. All samples were then diluted by a common dilution factor so as to be within the linear fluorescence response window of the spectrometer. The fluorescence emission of these diluted samples was then compared at 590 nm following identical white-light excitation (460–650 nm). For each sample, typically eight replicates were conducted.

Estimation of Liposome Size and Size Distribution Analysis. The size of liposomes was determined from representative fluorescence

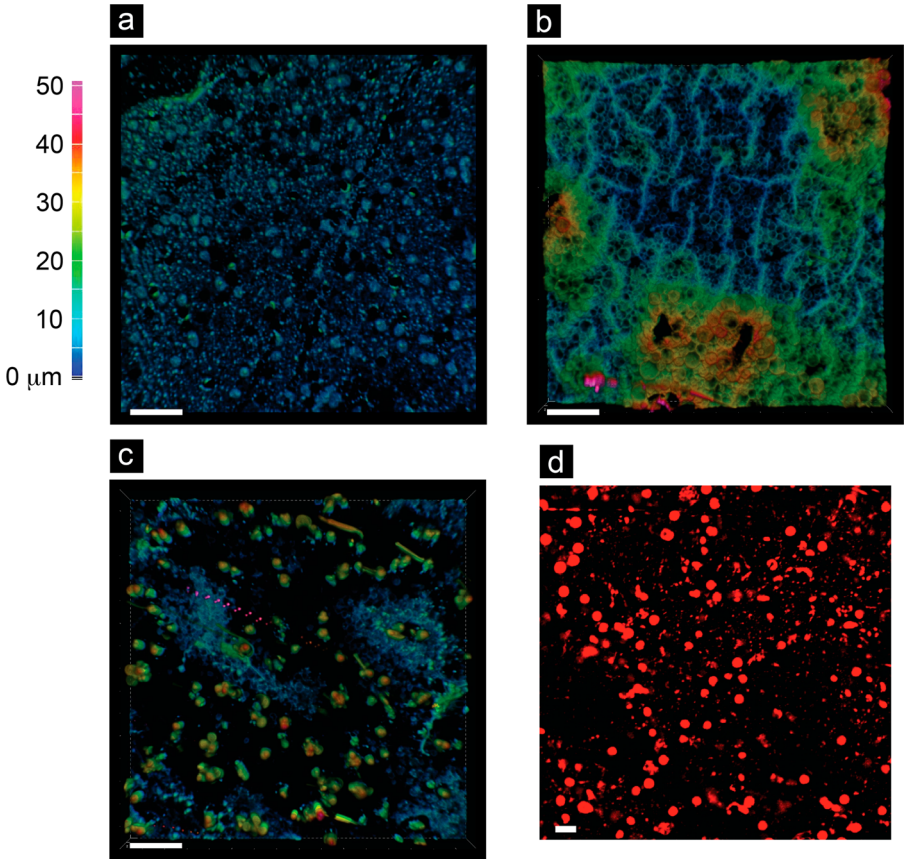

Figure 3. Confocal fluorescence monitoring of liposome formation during gel-assisted hydration (~16 min). Images a–c are *z*-stacked fluorescence micrographs with dimensions (*x*, *y*, *z*) of 318 × 318 × 50 μm, in which the scanned depth in the *z*-axis is indicated by the color-coded scale bar. The scale bars (bottom left) are 50 μm and for the *x* and *y* dimensions. (a) Dry lipid deposits on PVA gel before hydration; (b) Liposome formation on PVA gel alone (method 1); (c) Liposome formation on PVA gel further covered with a PC membrane (method 2; pore diameter: 10 μm. (d) Fluorescence micrograph of a PC membrane (pore diameter: 10 μm) after being used in size-controlled liposome production; scale bar: 20 μm. In all cases, the lipid sample contains POPC doped with 0.2% rho-DOPE.

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Langmuir 2020, 36, 13193–13200

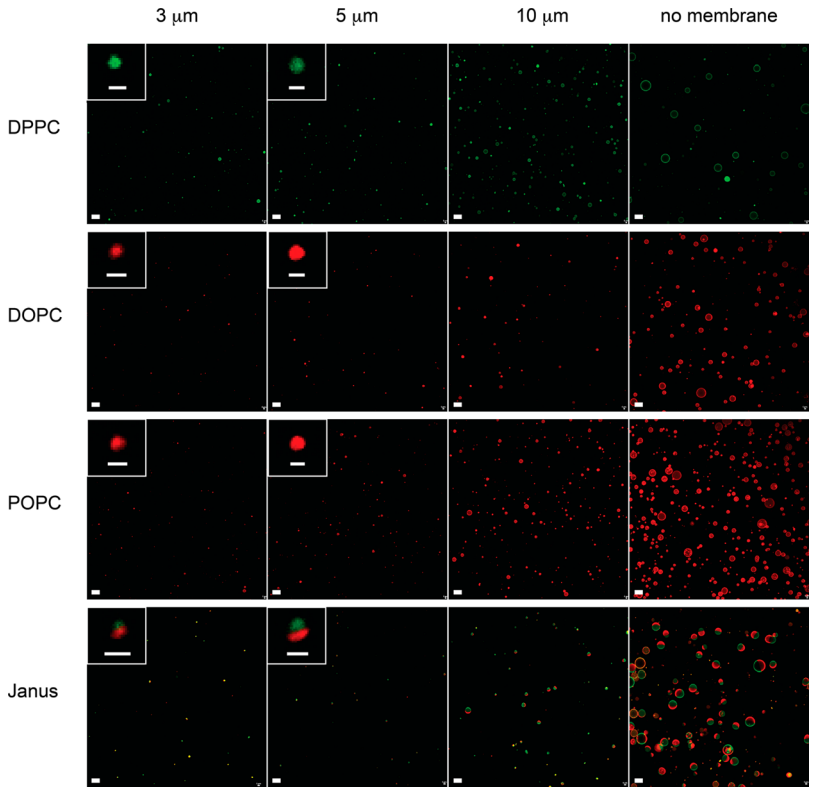

Figure 4. Fluorescence micrographs of four types of giant liposomes (DPPC, DOPC, POPC, and Janus) produced either with or without PC membranes. Nominal pore size of PC membranes used: 3, 5, and 10 μm; scale bar: 25 μm. Insets are close-up images of liposomes prepared with 3 or 5 μm membranes; all scale bars here are 5 μm. See the Experimental Section for more details.

micrographs of liposome samples prepared from multiple batches, which typically involves counting a few thousand liposomes from a dozen micrographs per sample. In the case of homogeneous liposomes, their cross-section area of (A) was first obtained using the "particle analysis" function in ImageJ (version: 2.0.0-rc-69/1.52p). The corresponding liposome diameter (d) was then estimated following the formula $d = 2(A/\pi)^{1/2}$. The size of Janus liposomes was measured using NIS-Elements AR software. The liposome size/count data thus obtained were then compiled in MS Excel spreadsheets, which were used as input files to produce size distribution histograms using Origin data processing package (OriginLab Corp.).

RESULTS AND DISCUSSION

Experimental Design. As shown in Figure 2, our experimental setup features planar lipid stacks sandwiched between a microporous polycarbonate (PC) membrane and a poly(vinyl alcohol) (PVA) gel layer fixed on glass. The latter structure forms the basis of the gel-assisted giant liposome preparation method[8] developed by Marques and co-workers, which we found to be quite reliable for making phase-separated Janus liposomes[27,28] as well. PC membranes, on the other hand, are a standard component in lipid extrusion, a technique broadly employed to produce small (nanosized) unilamellar liposomes.[1,26] To make these disparate methods(/setups) work together, we thus recognized two issues that need to be satisfactorily addressed: (1) Conformal, gap-free lamination of the PC membrane atop of the lipid stacks; and (2) A mechanical driving force strong enough to push the lipid bilayers through the membrane pores, that is, lipid extrusion. As detailed in the Experimental Section, the first issue can be resolved simply by prewetting the PC membrane, whose moist surface tends to cling to the dry lipid stacks by capillarity upon contact with the latter. During the hydration, moreover, water absorption not only drives the swelling of dry lipid/gel layers but also a water influx moving in the opposite direction, further reinforcing the position of the PC membrane sandwiched in between. As to the other issue, we hypothesized that the interior of the membrane pores, hydrophilic and ~20 μm across, poses only a partial and surmountable physical barrier for lipid vesiculation. The opposite scenario, that is, membranes with microsized, water-accessible openings shut off lipid hydration completely when placed atop, would be inconsistent with recent results, for example, on liposome array formation via lipid backfilling.[18,22]

Confocal Fluorescence Characterization of Membrane-Gated Liposome Formation. Using confocal fluorescence microscopy, we first examined (1) Whether liposomes can be produced by this membrane-installed setup and if yes, (2) How the membrane impacts the liposome products (Figure 3). Prior to hydration, the lipid stacks are relatively evenly deposited on the PVA gel surface, taking the latter's contour with an average roughness of a few micrometers (Figure 3a). Minutes into hydration, densely packed liposomes that crowded into several layers could be observed at the gel surface (Figure 3b). With swelling, the gel layer now appears highly corrugated, as previously observed by Marques and co-workers.[8] When the lipid deposits were in addition covered atop with a PC membrane, strikingly, the predominant objects in view were particles of roughly comparable size protruding from the gel surface (Figure 3c). Single z-sliced micrographs collected from the same measurements are included in the Supporting Information (Figure S1). The size and distribution of these objects, furthermore, match with that of the membrane pores; the latter could be visualized and identified readily from the residual rho-DOPE on the membrane after hydration

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Langmuir 2020, 36, 13193−13200

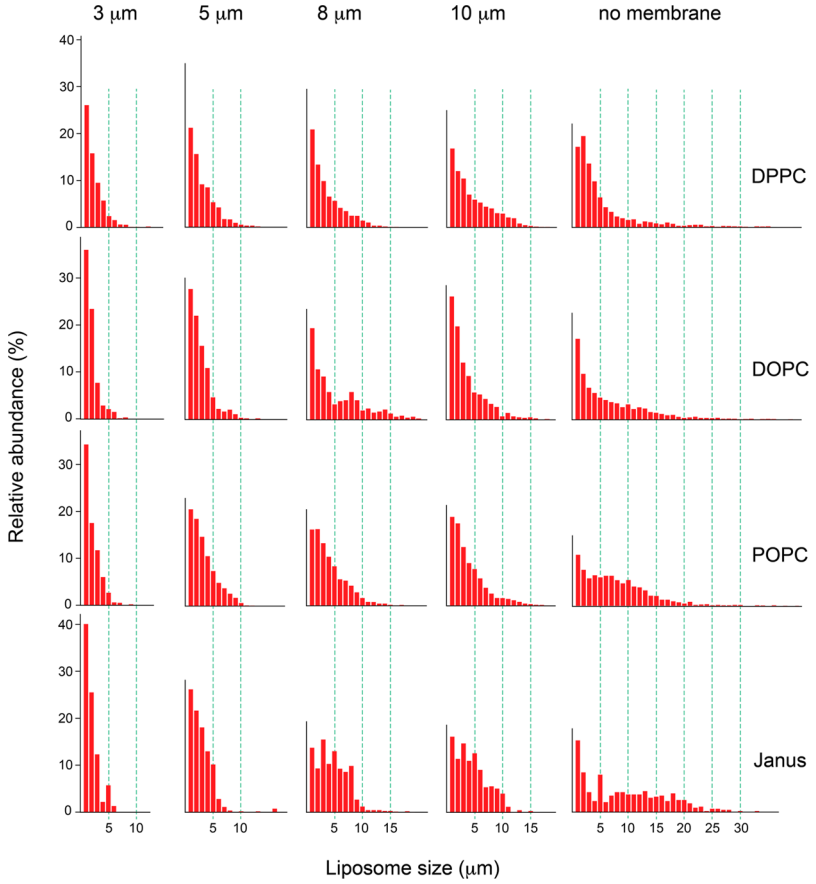

Figure 5. Histograms of liposome size distribution of four types of liposomes (DPPC, DOPC, POPC, and Janus) produced either with or without PC membranes. The PC membranes employed have nominal pore sizes of 3, 5, 8, and 10 μm. In each plot, the red bars represent relative populations of liposomes of certain size in a sample with 1 μm increment, whereas the dashed lines represent the 5−30 μm marks in 5 μm increments. In each series, the relative abundance (y-axis) of liposomes is maintained and comparable across different pore sizes.

(Figure 3d). Thus, it is evident that the particulate feature results from lipid stacks swelling through the pores, likely yielding liposomes on the other side.

Size Distribution of Liposome Products. To further characterize these extruded lipid particles, we next prepared such samples using several types of lipids on PC membranes with different pore sizes. Similar to the membrane-free liposome production, these samples were collected in the aqueous phase and examined by confocal fluorescence microscopy afterward. As shown in Figure 4, good quality liposomes were produced in every series we tested and moreover, there exists a clear dependence of liposome size on the diameter of the membrane pores. This size dependence was further confirmed by our detailed size distribution analysis (Figure 5), and a close inspection of these histograms in addition reveals the following trends. (1) Relatively broad liposome size distribution from (membrane-free) gel-assisted lipid hydration, with diameter ranging from 1 μm (the lower limit of our liposome counting) up to 30−40 μm; (2) Plausible liposome size dependence on lipid type. For the Janus liposome group, specifically, 40% of the population have a diameter larger than 10 μm, giving an overall wider size distribution compared to the other lipid groups. Of the three single-component products, the majority of the liposomes fall within the 10 μm size mark. For DPPC liposomes produced without membrane, moreover, the relative abundance of liposomes decreases sharply as their size becomes larger (Figure 5, top right panel). Assuming such a distribution as the baseline behavior of DPPC, it in effect presents a smaller supply of large liposomes in the corresponding membrane-gated liposome production, which, in turn, causes the resulting size control to appear less effective than other lipid types. This trend should be read with caution, however, considering potential complications due to liposome formation and preparation as well as the finite sampling in our size analysis; (3) Effective upper-limit size control by membrane. With the 10-μm-pore membrane installed, for example, the portion of Janus liposomes with diameter >10 μm went down from 40% to 2%. As a result, ∼30% of liposomes now fall within the size range of 6−10 μm, nearly a doubling from their membrane-free counterpart. When the 5-μm-pore membrane was used instead, <5% liposomes were found to occupy the same size window. And still smaller pores lead to even narrower liposome size distribution. While the membrane-based size control is generally observed in all cases, it is also evident that the membranes do not completely shut off the formation of larger liposomes. Such "leaky" size control is not unexpected, since the continuous, elastic lipid-bilayer sheets can easily squeeze past the pores during the swelling process—in a way similar to lipid vesicles formed by micropipet aspiration.[29,30] Another plausible contributor is the interfusion of liposomes as they come out of adjacent membrane pores at the same time (Figure 3c); (4) Unhindered small-sized liposome production. Not surprisingly, liposomes with size smaller than the membrane pores, which are being produced even when no membrane is used, still form and can pass the pores freely.

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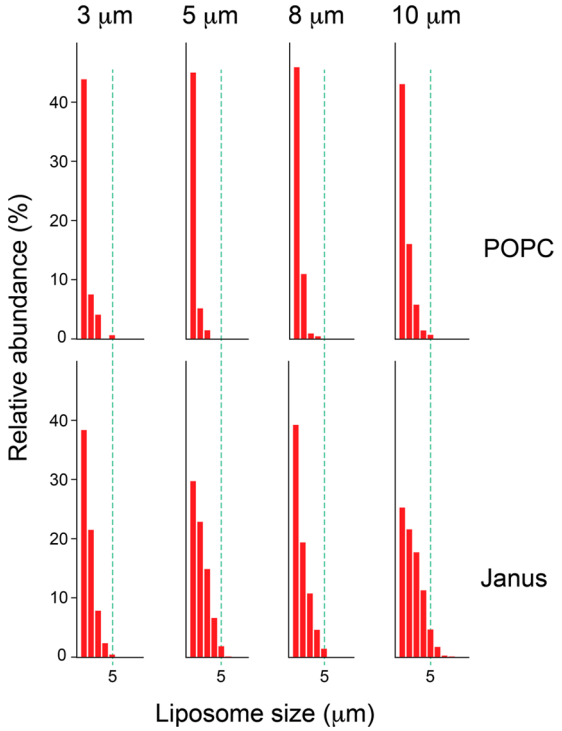

Figure 6. Histograms of liposome size distribution of POPC and Janus liposomes produced by gel-assisted lipid hydration followed by extrusion (method 3). The PC membranes employed have nominal pore sizes of 3, 5, 8, and 10 μm. In each plot, the red bars represent relative populations of liposomes of certain size in a sample with 1 μm increment, whereas the dashed lines represent the 5 μm mark.

As a separate control, we also tested giant liposomes prepared by the conventional extrusion procedure, which was carried out by passing gel-assisted hydration liposome products through PC membranes using an extruder. Here, while narrower size distribution is generally observed, the population of liposomes with targeted size is always low and sometimes missing entirely (Figure 6). Take the extruded POPC liposomes, for example, the majority of the liposomes are just a couple of micrometerss in size, largely independent of the membrane used. For Janus particles extruded with the 10-μm-pore membrane, on the other hand, the relative abundance of liposomes in the size range of 6−10 μm is just 2%, in great contrast to their counterparts produced by the gated procedure, ∼30%. The predominance of small-sized products likely results from liposome rupture and lipid pickup by PC membranes as liposomes pass the pores repeatedly during extrusion, which will be further confirmed in the next section. Similar size discrepancy was also observed previously in preparation of nanosized lipid vesicles,[31,32] where extrusion through >100 nm pores yielded smaller products.

Liposome Yields and Lamellarity. Next, we determined the yield of these membrane-gated liposome products prepared from 10-μm-sized membranes. Using the gel-assisted hydration (method 1) samples as the baseline, we found relative yields of 69% for the membrane-gated (method 2) and 55% for the hydration/extrusion (method 3) POPC liposomes, respectively. Similar yields were also found for POPC/chol (mixing ratio: 7:3) liposomes: 75% for method 2 and 55% for method 3 relative to method 1. Fluorescence spectra obtained from these measurements are included in the Supporting Information (Figure S2). Clearly, the hydrophilic surface and pores of PC membranes

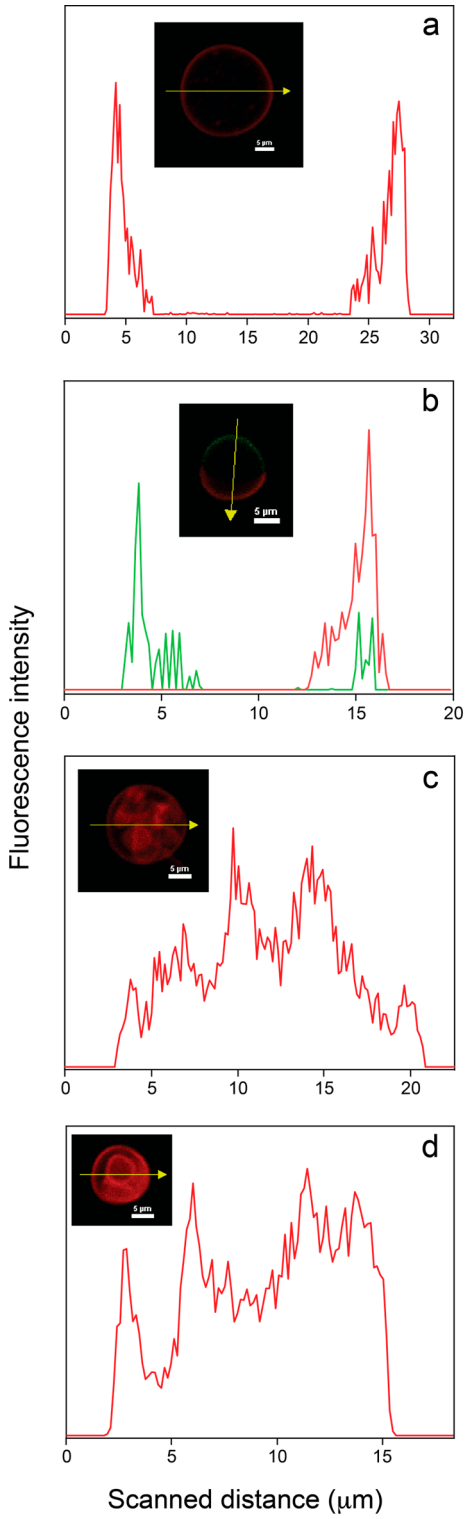

Figure 7. Fluorescence microscopic characterization of liposome lamellarity. Lipid composition: (a, c) DOPC; (b) DPPC/DOPC/chol 35/35/30 (mol %), (d) POPC, all additionally containing 0.05% rho-DOPE. Insets: fluorescence images of the liposomes analyzed; the arrowed lines indicate the position/direction of the fluorescence intensity scan. Scale bar: 5 μm.

are suitable sites for lipid deposition. Many microporous materials,[33−36] including PC membranes, have been employed previously as solid supports to afford stable formation of lipid bilayers. On the other hand, it is interesting to note that, with

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Langmuir 2020, 36, 13193−13200

its <10% porosity (estimated from fluorescence analysis of Figure 3d), the 10-μm-pore membrane laminated atop of the lipid stacks only causes a moderate, ~30%, decrease in liposome yield. This result highlights the dynamic and continuous nature of the lipid hydration/swelling process, by which the bulk of the lipid deposits can move about and exit successfully from the membrane openings that account for <10% of the total area.

Finally, we assessed the lamellarity of membrane-gated liposome products based on their cross-section fluorescence intensity profiles. As demonstrated by other workers previously,[5,37,38] fluorescence intensity scan across the equator of lightly labeled, homogeneous giant unilamellar liposomes typically yields a symmetrical profile featuring two sharp maxima at the boundary together with a flat baseline region in the middle, which correspond respectively to the rim and the lumen of liposomes. Two of such cases are shown in Figure 7a,b, whose profiles can be easily differentiated from multicompartment (Figure 7c) or multilamellar (Figure 7d) liposomes. Using this method and based on counting about 200 particles in each case, we found the following relative yields of unilamellar liposomes: 78% for POPC and 89% for Janus liposomes. These yields are at least comparable to those obtained from method 1 (no membrane): 64% for POPC and 92% for Janus liposomes, suggesting that the placement of microporous hydrophilic membrane on top of the lipid/gel layers does not detrimentally alter the lipid hydration/swelling process.

CONCLUSIONS

Combining gel-assisted lipid hydration with membrane-based lipid extrusion in a single step, we have demonstrated above a new procedure for rapid production of giant liposomes with upper size control. This procedure maintains many of the same benefits of gel-assisted lipid hydration technique, such as generality, flexibility and speed, and adds upper size control to the latter with a moderate decrease in liposome yield. Mechanically, the successful coupling of gel hydration with PC membranes demonstrates an interesting alternative driving force to lipid extrusion, which typically relies on hydraulic pumping. While the level of size control achieved here is not on par with some other recently reported approaches, this procedure enables freestanding, largely unilamellar liposome batches to be produced in one straightforward hydration step with no extra preparation or setup, such as micropatterning or microfluidics, required. Combined, these attributes should enable a range of new applications, for example, when the ensemble behavior of liposomes of different lipid types needs to be cross-examined or correlated. One interesting possibility lies in the study of motional characteristics of giant liposomes, which depend on not only the particle size, but also multiple other factors, such as the lipid/water interface and lipid phase(s). To this end, the method presented here provides us quick access to multiple liposome populations with a common size cutoff. Experiments are underway in this laboratory to explore some of such research possibilities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.0c01496.

Additional fluorescence images of liposomes produced either with or without PC membranes and fluorescence spectra on liposome yields (PDF)

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Author Contributions

†Equal contribution.

Notes

The authors declare no competing financial interest.

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