Bulk self-assembly of giant, unilamellar vesicles

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The desire to create cell-like models for fundamental science and applications has spurred extensive effort towards creating giant unilamellar vesicles (GUVs) [1]. However, membrane-forming molecules such as phospholipids, single-tailed surfactants, and block copolymers typically self-assemble into multilamellar, onion-like structures. So although self-assembly processes can form nanoscale unilamellar vesicles [2, 3], scaffolding by droplets [4–7] or surfaces [8–10] is required to create GUVs and route to selectively self-assemble GUVs has remained elusive. Here we show that surprisingly, it is possible to bulk self-assemble cell-sized GUVs with almost 100% yield. The seemingly paradoxical pair of features that enables this appears to be having very dynamic molecules at the nanoscale, that create unusually rigid membranes. The resultant self-assembly pathway enables encapsulation of molecules and colloids, and can also generate model primitive cells that can grow and divide.

The requirements for the bulk-assembly of kinetically-stable giant unilamellar vesicles (GUVs) are seemingly incompatible. First, to obtain unilamellarity, the vesicles must be able to remodel into single bilayers during vesicle formation and escape kinetic traps. As a result, instances of bulk-assembled unilamellar vesicles are typically composed of single-tailed surfactants [2, 3]. However, single-tailed surfactants form bilayer membranes that have a bending modulus on the order of the thermal energy $kT$, and thus the reported bulk-assembled unilamellar vesicles are nanoscale rather than cell-sized, maximising entropy [2]. While vesicles of this size are useful for bulk studies, their utility is restrictive on two fronts: Their imaging requires electron microscopy, which limits the number of timepoints that can be studied during dynamic events such as vesicle division [11], and their small encapsulated interior volume places limitations on vesicle loading. However, molecules that self-assemble into stiffer membranes are typically also kinetically trapped, and require the use of laser heating [12], electric fields [8], surface-adsorption [9, 10], emulsion droplets [4–6], or droplet-based microfluidics [6, 7] to remodel the membranes into a unilamellar form. The likelihood of bulk GUV assembly thus seems low.

We were thus surprised when we found that instead of self-assembling into heterogeneous structures (Fig. 1a), oleic acid could, under certain conditions, selectively self-assemble into giant, apparently unilamellar vesicles (Fig. 1b). Although it is well-known that fatty acids exhibit a rich, pH-dependent variety of self-assembly processes, forming micelles at high pH, neat oil or cubosomes at low pH, and bilayer membranes at a pH near the apparent $pK_a$ [13, 14], we discovered that within the pH range compatible with vesicle formation lies a previously unexplored divergence in self-assembly behaviours.

Figure 1. Fatty acid self-assembly depends on the aqueous solution conditions. a. Confocal microscopy revealed that oleic acid self-assembled into giant vesicles capable of encapsulating and retaining RNA oligomers (green). The membrane was dyed with 10 nM rhodamine B (red). The giant vesicles that self-assembled in 200 mM Na$^+$ bicine, pH 8.43 were very heterogeneous in morphology. b. The giant vesicles that self-assembled in 50 mM Na$^+$ bicine appeared to be unilamellar. c. The pHs at which GUVs assembled for myristoleic acid (14 carbons), palmitoleic acid (16 carbons) and oleic acid (18 carbons) in 50 mM Na$^+$ bicine are shown as open circles (black), and the pHs at which MLVs formed are shown as solid circles. The red circles show the self-assembly of oleic acid MLVs (solid circles) and GUVs (open circles) in 250 mM Na$^+$ bicine. The scale bar represents 10 µm.

We dispersed oleate micelles into more acidic solutions, such as dilute HCl (Video S1), at concentrations designed to yield a final pH in the range of 8.1 to 8.8, and then left the samples overnight on an orbital shaker to allow the self-assembly processes to evolve towards steady-state

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structures [15]. At pH values from 8.1 to 8.4, just below the reported apparent pKₐ of oleic acid, optically transparent solutions formed. Under phase contrast microscopy, however, the solutions were revealed to contain abundant cell-sized spherical vesicles (Fig. S2). By contrast, at final pH values between 8.5 to 8.8, microscopy revealed the presence of very heterogeneous multilamellar vesicles. The same pH-dependence was also found for palmitoleic acid and myristoleic acid vesicles (Fig. 1c). The differences between multilamellar samples and less multilamellar ones were readily apparent by eye, and corresponded well with bulk sample turbidity [16] (Fig. S3). We found that the morphology also depended on salt concentration (Fig. S4), as expected due to the salt-dependence of the pKₐ of oleate in the bilayer membrane phase [13].

We used confocal microscopy to confirm that the cell-sized and apparently unilamellar vesicles observed with phase contrast microscopy were indeed unilamellar. We added the lipophilic dye, rhodamine B, to the vesicles after self-assembly, because the membrane fluorescence intensity is expected to reflect the amount of membrane material present. We found that an oligolamellar solution of vesicles showed clear differences in fluorescence intensity between vesicles (Fig. 2a). By contrast, the samples that appeared to have uniform membrane intensity under phase contrast microscopy exhibited a single peak in fluorescence intensity (Fig. 2b). These results are consistent with the last samples being unilamellar.

Intriguingly, the GUVs did not show any membrane fluctuations and appeared extremely spherical (Fig. 1a, Fig. 2a). Although the melting temperature of oleic acid (Tₘ = 14°C) is below room temperature (T ∼ 21°C), the apparent rigidity of the membranes prompted us to verify that the membranes were not in a gel phase. FRAP (Fluorescence Recovery After Photobleaching) experiments on oleic acid GUVs resulted in apparently uniform bleaching across entire GUVs within 1 s. Thus to quantify the fluidity, we instead used supported lipid bilayers with a large bleached area (Fig. 2b), and found that the diffusion constant D = 14.3 ± 4.7 µm²/s (s.d., n = 7) is rapid compared to phospholipid bilayers. It is thus plausible that the bilayers appeared to be rigid not because they are in a gel phase, but because the excess surface area required for surface undulations had somehow been eliminated.

To understand the effect of protonation state on molecular packing in fatty acid bilayers, we carried out molecular dynamics simulations. We chose octanoic acid as the model lipid, because in our experiments the exact nature of the lipid tails did not seem to prevent the formation of GUVs. Linoleic acid, which has two degrees of unsaturation, could also self-assemble into GUVs, as could fatty acid mixtures containing both saturated and unsaturated species (Fig. S5). We simulated octanoic acid bilayer membranes at 25% protonation (3:1 sample, Fig. 3a) and 50% (1:1 sample, Fig. 3b). For both 1:1 and 3:1 bilayers, the majority of protonated octanoic acid headgroups served as hydrogen bond donors to deprotonated octanoates, consistent with existing literature [17]. Much smaller fractions of octanoic acid in both cases were hydrogen-bonded to other octanoic acids or to solvent. While the great majority of molecules that formed the bilayer were hydrogen-bonded to others through their headgroups in the 1:1 bilayer, there were not enough donors for this to occur in the 3:1 bilayer. As a result, the 3:1 bilayer was thinner than the 1:1 bilayer (Fig. 3c), owing to increased repulsion between headgroups, which increased the area per headgroup. By analysing the fluctuations in the membrane using the method of Brown et al. [18], we found that the 3:1 bilayer has a bending modulus 5.5 kT, similar to that of bilayer membranes made from surfactants with a comparable carbon-length to octanoic acid [2]. By contrast, the 1:1 bilayer had a bending modulus Kₑ greater than 20 kT, which is as stiff as phospholipid bilayers (Kₑ ∼ 20 kT) [20]. Thus fatty acid bilayers are surprisingly stiff and moreover, their stiffness can change dramatically with protonation state.

The dependence of membrane bending modulus on protonation state then led us to contemplate the role of bending modulus in the assembly of fatty acid vesicles. Having rigid membranes penalises the formation of high-curvature, smaller vesicles, and is thus consistent with our finding that we can generate giant vesicles at pHs lower than the apparent pKₐ, despite using an or-
Fatty acids have been prime candidates for constituents of primitive membranes for decades [26], owing to their chemical simplicity and presence on meteorites, which confirms that their synthesis can be abiotic. To examine the potential for fatty acid GUVs to undergo physical-driven growth and division, we encapsulated fluorescently-tagged RNA inside of oleic acid vesicles to create simple protocells. We then added excess oleate micelles to the solution to enable an increase in the surface area to volume ratio as the added oleate became incorporated into pre-existing membranes. We found that rather than growing into tubular vesicles that require shear to divide, as previously reported for MLVs [27], our model

Acid GUV yield decreases to almost negligible (Fig. S9) when orbital shaking at slower speeds, resulting in a maximum shear stress of 0.2 N/m² or less.

According to elastic theory, a piece of membrane under constant strain has a lateral membrane tension $\Sigma$ proportional to the bending modulus $K_c$ (see Methods [1]). From this relation, we can conclude that any tension-driven effects will be more pronounced for vesicles with a higher bending modulus. Because the different bilayers of multimammalian vesicles will experience different amounts of shear [25], if the pH is below the apparent $pK_a$ of the fatty acid, competition between adjacent layers may continue until only one membrane remains. For example, the more relaxed inner membranes may lose fatty acid molecules, which would tend to integrate into the more strained outer bilayers. Furthermore, the driving force for vesicle fusion increases at lower pH, owing to the increase in bending modulus, while the barrier to fusion presumably decreases owing to the decrease in surface charge density. Multiple fusion events between inner and outer membranes could lead to the formation of unilamellar vesicles. Indeed, foam-like intermediates were visible prior to GUV formation (Fig. S10). Finally, after an increase in surface area from membrane remodelling, the requisite increase in volume to compensate for the excess surface area could come from the membrane momentarily rupturing, allowing the influx of suspended materials as large as colloidal particles. The excess surface area could also be lost by budding, as shown below. Consequently, a low pH not only favours the formation of giant vesicles, but also of unilamellar ones.

We anticipate that fatty acid GUVs will be useful for a variety of applications in different fields because of their ease of self-assembly, the ready availability of fatty acids, and their versatility with respect to the encapsulation of contents. Fatty acid GUVs can be loaded with contents ranging from small molecules such as sugars (Fig. 1b), fluorescent dyes (Fig. 4a), and RNA oligomers (Fig. 5), to nanoparticles (Fig. 4b) and colloidal particles (Fig. 4c, see also Video S11). After GUV assembly in the presence of each of these materials, the vesicles can be diluted into a new buffer, or washed to remove unencapsulated material (Fig. 4a–c). While the encapsulation of small solutes using bulk assembly techniques is routine [1], the encapsulation of colloidal particles is usually only possible using droplet-based techniques such as the emulsion transfer method or microfluidics [3]. Our bulk-assembly technique is thus notable for its ability to encapsulate and retain contents across several orders of magnitude in size.
Figure 4. Fatty acid GUVs can encapsulate a wide range of materials. Materials encapsulated include a. pyranine (1 mM), b. 30-nm-diameter Nile Red dyed latex nanoparticles (0.1% w/v), and c. 400-nm-diameter polystyrene latex beads (0.1% w/v; see also Video S11). d–e. Oleic acid vesicles can encapsulate RNA (the same RNA oligomer as in Fig. 1a) and divide upon exposure to oleate micelles pipetted nearby. The time shown represents the number of seconds after injecting oleate micelles. Scale bar represents 10 µm for all images.

Protocells could grow and divide within a few seconds (Fig. 4d,e). The vesicles deformed by undergoing undulations, and higher frame-rate imaging (Video S12, S13) shows that the undulations represent the dynamic formation of lobes, which frequently appear to pinch off, forming smaller daughter vesicles. These intermediate structures are reminiscent of the recent theoretical findings of Ruiz-Herrero, Fai, and Mahadevan [28], whose models show that lobe-like structures can form in vesicles under certain growth conditions. Thus in our system, the intimate coupling of membrane growth with division can be attributed to the buildup of elastic energy when oleate is incorporated into the outer leaflet as follows. Because the membranes are made in the presence of salts with limited permeability, the volume is osmotically constrained at short timescales. Flip-flop across the membrane is known to relax the bending stress, and occurs with a rate of ∼0.5 s⁻¹ [29]. When the rate of membrane growth was potentially faster than the rate at which flip-flop could relieve the accumulated stress, the vesicles were able to undergo a dramatic fission event (Video S12, S13). When the rate of membrane growth was slower, fission did not occur (Video S14). We note that these results also demonstrate the strong propensity of fatty acid GUVs to remain spherical, consistent with large-scale membrane fluctuations being energetically unfavourable due to the high bending modulus.

We have shown that GUVs can be self-assembled in bulk by means distinct from the long-established picture of unilamellar vesicle self-assembly being driven towards unilamellarity by Helfrich fluctuations and spontaneous curvature. Rather, fatty acid GUVs are driven towards a unilamellar architecture because the membrane is able to remodel despite being rigid. Whereas previous reports of unilamellar vesicles made by bulk self-assembly generated nanoscale vesicles, our vesicles are cell-sized, ideal for research using common biophysical tools such as optical microscopy. Because our results can be implemented with ease – requiring minimal infrastructure, cost, chemicals, and skill – we anticipate that these results will facilitate the study of a multitude of research questions in soft-matter science, origins of life, and biophysics.

I. METHODS
   A. Chemicals

Oleic acid, palmitoleic acid, myristoleic acid, and decanoic acid were purchased from Nuchek-Prep (USA). Bicine (99%), HEPES (99.5%), NaOH (solid, 99%), HCl (37%), POPC, pyranine, rhodamine B, sucrose, glucose, (3-aminopropyl)triethoxysilane (97% Sigma-Aldrich) were purchased from Sigma-Aldrich (USA). Ammonium hydroxide (30%), methanol (99.9%), ethanol (99.5%), isopropanol (99.9%), chloroform (99.8%), were obtained from Acros Organics (Fisher Scientific, USA). Other materials used include Hellmanex III (Hellma, Germany), TopFluor PC (Avanti Polar Lipids, USA).
the oligonucleotide r(GGC UCG ACU GAU GAG GCG CG)-AF647 (IDT, USA), NOA 61 epoxy (Norland Products, Inc., USA) 30-nm-diameter Nile Red latex nanoparticles (Spherotech, Inc., USA), 400-nm-diameter latex size standard beads (Malvern, USA). All water used was Millipore (Millipore, USA). All chemicals were used as received.

B. Making vesicles

100 mM fatty acid micelle stock solution were first prepared by adding 100 μmol of fatty acid to 1 mL of 125 mM NaOH in water. Bicine or HEPES buffer stock solutions were adjusted to near the pKₐ of the fatty acid by NaOH addition. The vesicles were then prepared by adding micelle stock solution to a buffered solution, vortexing for 3 s, and then leaving the 0.5 or 1.7 mL microcentrifuge tube (Fisher Scientific, USA) or 20-mL glass scintillation vial (VWR, USA) and evaporated under a stream of nitrogen leaving 10⁻⁸ mPas for water), ρ is the density of the aqueous solution (0.89 mPas for water), a is the amplitude of the rotational stroke (in our case, 13 mm for a 20-mL scintillation vial), and ω is the rotational speed in radians per second.

H. Fluorescence Recovery After Photobleaching experiment

Stock solutions of oleic acid and TopFluor-PC were prepared in chloroform, then deposited in a 20-mL glass scintillation vial (VWR, USA) and evaporated under a stream of nitrogen leaving 10 μmol of oleic acid and 20 nmol of TopFluor PC. 1 mL of 50 mM Na-bicine (pH 8.4) and 150 mM NaCl solution was then added to hydrate the lipid film, and left for 1 hr on a hotplate at 65°C. The solution was cooled to room temperature, vortexed for 5 s, then withdrawn into a syringe (Hamilton Company, USA) and extruded 11 times through polycarbonate membranes with 100-nm-pores (Whatman) on a mini-extruder (Avanti Polar Lipids, USA). Meanwhile, 24×60 mm No. 1.5 glass coverslips (Fisher Scientific, USA).
USA) were prepared by soaking sequentially for at least one day each in 2% Hellamanex III, 70% v/v isopropanol in water, then 2 M NaOH, with water rinses in between. The silanisation solution of 30 mL ethanol, 1 mL (3-aminopropyl)triethoxysilane and 500 µL of 30% ammonium hydroxide solution, was prepared fresh in a 50-mL tube (Falcon, USA). The cleaned glass coverslips were submerged into this solution for 2 mins before washing thoroughly with methanol and dried under nitrogen. The top lip of an open 500 µL microcentrifuge tube was cut off with a razor blade, then glued lip-down onto the cleaned coverslip with NOA 61 epoxy to create a cylindrical well. Curing time was 5 minutes at 365 nm and 4 W with a UVGL-15 Compact UV Lamp (UVP, USA), with the glass placed directly onto the lamp. Then 20 µL of the vesicle solution was deposited into the well, and allowed to spread onto the silanised glass for 60 minutes. Undeposited lipid was then washed three times by removing 90% of the solution and pipetting in fresh buffer. The sample chamber was capped with a 18×18 mm No. 1 glass coverslip (Fisher Scientific, USA) to minimise evaporation during imaging.

Imaging of the supported lipid bilayer was done on a A1R/Ti confocal microscope (Nikon, Japan). A FRAP routine on NIS Elements was used to acquire the FRAP image data. Lateral diffusion was rapid, so a large bleached region (54 µm) was used to minimise the effect of diffusion during photobleaching. Analysis was performed by following the method of Kang et al. [31].

I. Simulations

Molecular dynamics simulations of 240 total molecules of octanoic acid/sodium octanoate at 1:1 and 1:3 ratios, with over 6000 TIP4P-2005 waters [32] were performed using Gromacs [33]. Force field parameters for alkyl tails used the HH-Alkane model [34], in which intramolecular and tail-tail interactions are taken from the TrAPPE united-atom model [35] and interactions between water oxygen and CH₂ and CH₃ sites adjusted to improve alkane hydration free energies. Following Hess and van der Vegt [36], who used the Kirkwood-Buff formalism to validate ion-ion interactions for Na⁺ with carboxylate against experimental results, we use OPLS [37] values for Lennard-Jones (LJ) parameters and partial charges of these ions and the carboxylate headgroup. OPLS parameters were also used for the carboxylic acid, with the partial charge on the COOH proton adjusted from +0.45 e to +0.5 e. The motivation for this change is that in our preliminary simulations on mixed-protonation state fatty acid bilayers, carboxylate and carboxylic acid headgroups were spatially separated into zones with different degrees of hydration (similarly to one previous simulation study [38]) with very infrequent hydrogen bonding between headgroups. This result runs counter to experimental evidence for H-bond networks at the surface of fatty acid bilayers [17] and to the principle of PA/pKₐ matching [39] (where PA stands for proton affinity), which would place RCOOH above water as a preferred hydrogen-bond donor towards RCOO⁻. The increase was sufficient to bring the gas-phase force field binding energies for trans- and cis-acetic acid to acetate up to 118 and 129 kJ/mol respectively, in line with calculated values from DFT (113-114 and 125 kJ/mol, for formic acid/formate [40, 41]).

A dodecanoic acid bilayer generated using the CHARMM-GUI membrane builder [42, 43] was used as a starting point for the C₈ bilayers. The initial distribution of protonated and deprotonated molecules was selected randomly; diffusion was observed to be fast on the simulation timescale for these FA bilayers (in contrast to phospholipids). Simulations were performed with all bond lengths constrained to fixed distances using the LINCS algorithm [44]. The pressure is maintained at 1 bar and surface tension at zero using semi-isotropic pressure coupling via the Berendsen barostat [35] with τₚ = 2 ps and compressibility of 4.5 × 10⁻⁵ bar⁻¹. The Gromacs default (leap-frog) integrator with a 2 fs time step was used for integration of equations of motion. The Verlet [46] cutoff-scheme was applied for short-range non-bonded interactions with a cutoff of 1.4 nm. Particle-mesh Ewald summation [47] was used to account for Coulomb interactions with a real space cutoff of 1.4 nm. The temperature was maintained at 300 K by velocity rescaling thermostat [48] with τₜ = 2 ps.

J. Bending modulus

To estimate bending modulus Kᵥ from the simulation data we followed the method of Brown et al. [18, 19] by analysing the fluctuations of fatty acid tail tilt vectors in Fourier space. In the long wavelength/low-q limit, the following relation should hold:

$$\langle |\hat{n}_q|^2 \rangle = \frac{k_BT}{K_q q^2}$$  \hspace{1cm} (1)

where $\hat{n}_q$ is the longitudinal Fourier mode along wavevector q of the molecular tilt. To obtain this quantity, we took snapshots at 5 ps intervals starting at 15 ns and analysed the positions of C1 and C8 sites on all molecules, irrespective of protonation state. The midplane of the bilayer was taken as the mean z coordinate of all C1 sites. Molecules whose terminal methyl (C8) site was farther than 1 standard deviation from the midplane were excluded from the analysis; these included both molecules that escaped into the solvent and those with very nonstandard configurations, and constituted fewer than 5% of all molecules at any time. Molecules were assigned to a 12 × 12 grid based on the lateral position of their C1 site and a leaflet based on whether the z coordinate of the C1 site was above or below the midplane. A unit vector for each molecule i was calculated as:
\[ \mathbf{n}_i = \frac{(\mathbf{r}_{C8,i} - \mathbf{r}_{C1,i})}{|\mathbf{r}_{C8,i} - \mathbf{r}_{C1,i}|} \]  

(2)

A mean vector for each grid square was then calculated by subtracting the sum of the unit vectors in the lower leaflet from the sum of unit vectors in the upper leaflet in that grid square, and dividing by the total number of molecules in that square. If a grid square was empty, the interpolation scheme described in Reference [19] was used to assign its mean vector. A two-dimensional fast Fourier transform of the \(x\)- and \(y\)-coordinates of the grid square vectors was taken for each frame, and the longitudinal component of this tilt vector fluctuations are found by projecting the \(x\)- and \(y\)-components along the \(q\)-vector. The average of the resulting square amplitudes, taken over all frames and over wavevectors with the same magnitude, is then taken. Rearranging Eq. 2 yields

\[ \frac{K_c}{k_B T} = q^{-2} \langle \hat{n}_q^2 \rangle^{-1} \]  

(3)

K. Elastic theory

A piece of bilayer membrane that occupies an area \(A\) under stress (and an area \(A_0\) under no external stress) has a dimensionless strain \(u = A/A_0 - 1\). The lateral tension of the membrane \(\Sigma\) is related to the membrane’s stretching modulus \(K_{\text{stretch}}\) by \(\Sigma = K_{\text{stretch}}u\). Assuming the bilayers are two uncoupled elastic sheets [20, 49], the stretching modulus is related to the bending modulus \(K_c\) by \(K_{\text{stretch}} \propto K_c/h^2\) where \(h\) is the bilayer thickness. This leads us to the relation between membrane tension and bending modulus \(\Sigma \propto K_c u/h^2\).

ACKNOWLEDGMENTS

A.W. would like to acknowledge the support of the NASA Postdoctoral Program Fellowship in Astrobiology and the UNSW Sydney Scientia Fellowship. J.T.K. acknowledges computational resources of the Extreme Science and Engineering Discovery Environment (XSEDE) Comet cluster at the San Diego Supercomputer Center, which is supported by the National Science Foundation Grant No. ACI-1548562, Allocation No. TG-MCB110144. J.W.S. is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by a grant (290363) from the Simons Foundation to J.W.S.

Author Contributions

All authors contributed to the writing of the manuscript and interpretation of the data. J.T.K. conceived of and did the molecular dynamics simulations and subsequent analyses. J.W.S. conceived of the vesicle division experiments. A.W. conceived of, did, and analysed the experiments.

Competing Interests Statement

The authors declare no competing interests.

Data availability statement

The data supporting the findings of this study are available within the paper and its supplementary information files. Additional data that support the findings of this study are available from the corresponding authors upon reasonable request.

Code availability

Code used to analyse the membrane intensity, as well as the diffusion constant from FRAP data, are available from the corresponding authors upon reasonable request.

Materials and Correspondence

Supplementary Information is available for this paper. Correspondence and requests for materials should be addressed to anna.wang@unsw.edu.au.

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