Electronic Supplementary Information (ESI)

Phospholipid asymmetry in biomimetic vesicles alters membrane permeability

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1. Supplementary Figures

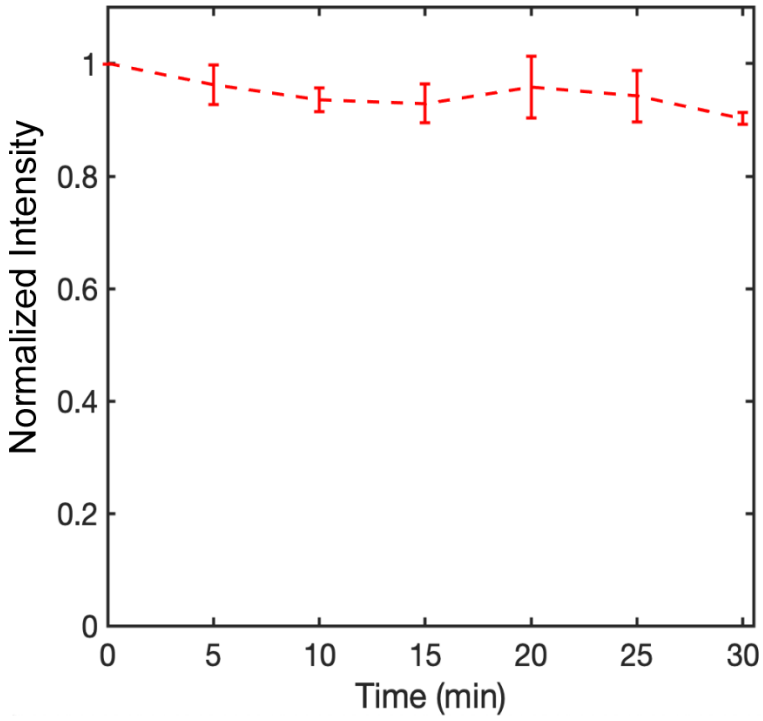

Figure S1: Control experiment showing fluorescence intensity of Texas Red-tagged phospholipids in the inner monolayer. NBD-tagged lipids in the outer monolayer are being quenched by the addition of sodium dithionite. These data show that there is no overall quenching of fluorescence due to other factors. Images were taken every 5 min. The dashed line between data points is to aid visualisation of the data.

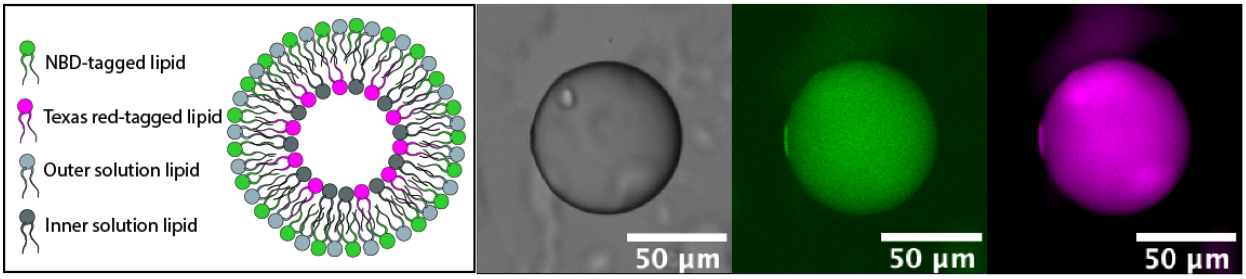

Figure S2: Asymmetric vesicles produced using the microcapillary device. The schematic shows the composition of the vesicles with green denoting NBD-tagged lipids, pink denoting Texas Red-tagged lipids, light grey denoting DOPC lipids in the outer monolayer and dark grey denoting DOPE and DOPC lipids in the inner monolayer in a 75:25 ratio. The microscopy images show the same vesicle in brightfield (left, grey), with a GFP filter to show the NBD-tagged phospholipid fluorescence (middle, green) and with an mCherry filter to show Texas Red-tagged lipid fluorescence (right, pink). These images show that both red and green fluorescent tags are present in the same vesicle to enable measurements of asymmetry. The scale bars are 50 µm.

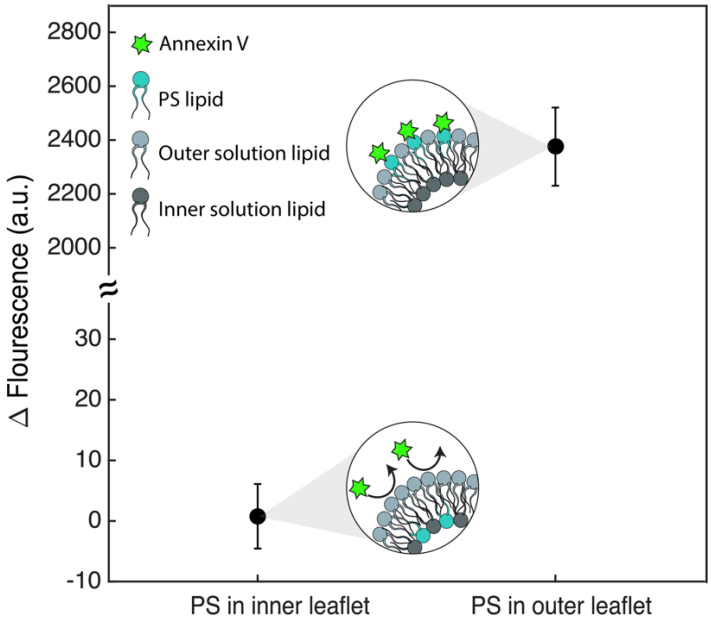

Figure S3: Annexin V binding assay to assess phosphatidylserine (PS) distribution. Vesicles were generated with phosphatidylserine (PS) localized to either the inner (data on the left) or outer (data on the right) leaflet. Fluorescence intensity was normalized to pre-Annexin V addition. Vesicles with PS confined to the inner leaflet exhibited minimal Annexin V binding, whereas vesicles with PS in the outer leaflet showed a strong binding signal. Note the order of magnitude change in fluorescence intensity between both assays shown by the break in the y-axis.

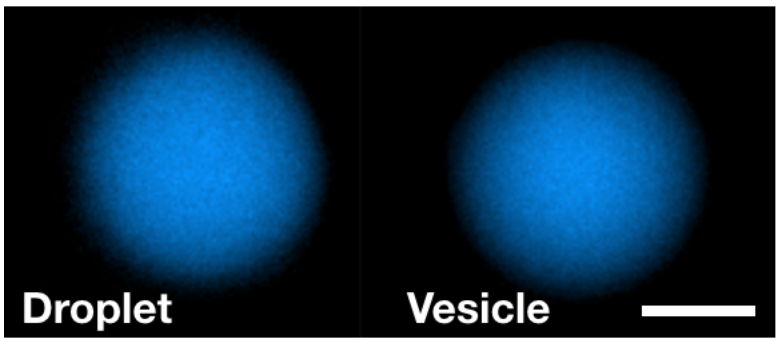

Figure S4: Representative fluorescent images used for the quantification of quinine encapsulation. These images are used to show that the on-chip emulsion creation and de-wetting processes are not removing fluorescence from the vesicles. The image on the left shows a droplet of inner lipid solution (RBC-mimetic phospholipids as shown in Figure 3a in HEPES buffer, 2% w/w PVA and 8% w/w PEG) with 5 mg/mL quinine hemi sulphate. The image on the right shows a vesicle containing the same 5 mg/mL quinine solution having undergone the double emulsion creation and de-wetting processes. The encapsulation yield is calculated by dividing the total fluorescence intensity of the vesicle by the fluorescence intensity of the droplet as described by others.[1] The scale bar is 50 μm.

2. Supplementary Table

Table S1: Quinine hemi-sulphate vesicle size and fluorescence intensity for encapsulation efficiency measurements.

Droplet diameter (μm)	Droplet intensity	Vesicle diameter (μm)	Vesicle intensity	Encapsulation efficiency (%)
1892.27	3857.83	1892.27	3775.04	97.33
901.93	3802.47	925.27	3655.43	96.13
510.38	2957.54	517.24	2666.91	90.17
225.83	1418.66	261.32	1364.40	96.18

3. Materials and Methods

3.1 Materials

All reagents were used as received unless otherwise stated. 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl-sn-glycero-3-phosphocholine (NBD-PC) and L-α-phosphatidylcholine (egg) were purchased from Avanti. Sphingomyelin (porcine red blood cell), L-phosphatidylserine (porcine brain) and phosphatidylethanolamine (bovine) were purchased from Cayman Chemical. Tris was purchased from Bio Basic Inc. Poly(vinyl alcohol) (PVA, MW = 13 000–23 000 g/mol, 87–88 % hydrolyzed), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, >99.5 %), chloroform, sodium hydrosulphite, quinine hemisulphate monohydrate and squalene were purchased from Sigma Aldrich. Pluronic F-68, potassium chloride and Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt were purchased from Thermo Fisher Scientific. Polytetrafluoroethylene (PTFE) tubing (1/16-inch outer diameter, OD, and 750 μm inner diameter, ID) was purchased from Chromatographic Specialties Inc. Glass gas-tight syringes (1 mL, model 1001 TLL, PTFE Luer Lock) were purchased from Hamilton. Polyethylene glycol (PEG, MW = 6 000 g/mol) and hexanes (mix of isomers) were purchased from VWR. 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane was purchased from Abcr. Polyurethane resin (Vytaflex 30) and release spray were purchased from Smooth-on. Glass capillaries were purchased from Vitrocom. 100 nm polycarbonate filters and the Avanti Mini Extruder were purchased from Avanti Lipids.

3.2 Preparation of lipid solutions

All lipids were purchased suspended in chloroform. Lipids (in the correct ratios) were added to a 10 mL glass round-bottom flask and excess chloroform was removed via evaporation using a steady stream of filtered nitrogen. The flask was then placed in a glass desiccator under vacuum for 1 h. Next, 1 mL of HEPES buffer (10 mM, pH = 7.4) and potassium chloride (140 mM) were added to the flask and the solution was vortexed for 30 s to re-suspend the lipids to a final concentration of 10 mg/mL. The lipid solution was then subjected to 5 freeze-thaw cycles in liquid nitrogen and warm water (50 °C), respectively. Then the lipid solution was heated to 37 °C using a water bath and extruded through a 0.1 μm polycarbonate membrane 19 times. The solutions were kept at 37 °C until they were inserted into the microfluidic device.

Lipid vesicles made using synthetic lipids were prepared with 5 mg/mL DOPC in the outer solution and 1.25 mg/mL DOPC and 3.75 mg/mL DOPE in the inner solution. 0.4 mol% NDB-PC was added either to the inner or the outer solution for the quenching assays, and 0.4 mol% Texas Red PE was added to the opposite solution. Vesicles designed to mimic red blood cell membranes were prepared with 2.5 mg/mL PC, 0.5 mg/mL PE and 2 mg/mL sphingomyelin in the outer solution. The inner solution consisted of 0.75 mg/mL PC, 2.25 mg/mL PE, 0.5 mg/mL sphingomyelin and 1.25 mg/mL PS.

Surfactant stock solutions (as described previously)[2] were prepared separately and added to the lipid solutions post-extrusion. For the inner aqueous phase, 2 wt% PVA and 8 wt% PEG were added to deionised water. For the outer aqueous phase, 10 wt% PVA and 0.5 wt% Pluronic F-68 were added to deionised water. The solutions were placed in glass vials with a magnetic stir bar and mixed for 30 min at 95 °C. These solutions were added to their respective lipid solutions at a 1:1 ratio, resulting in a final concentration of 5 mg/mL inner and outer lipid solutions with surfactants at a final concentration of 1 wt% PVA and 4 wt% PEG (inner) and 5 wt% PVA and 0.25 wt% Pluronic F-68 (outer).

3.3 Surface treatment of glass microcapillaries

The outer glass capillaries were treated to create a more hydrophilic surface. First, the capillaries were cleaned with soapy deionised water, deionised water, isopropanol, ethanol and acetone. They were then

dried with a filtered air gun and placed in a glass petri dish on a hot plate at 95 °C for 30 min. The capillaries were plasma treated at 100 W with air plasma for 1 min (Diener Electronic, Zepto ONE, 0.64 mbar). Capillaries were chemically modified immediately after plasma treatment by submerging them fully in 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane for approximately 15 min. The excess silane was dabbed off with Kim wipes and capillaries were dried using a filtered air gun. The treated capillaries were used within 48 h of surface treatment.

3.4 Vesicle formation and visualization

The microcapillary device was assembled as described previously.[3] In brief, a low-cost Mars Pro 3D Printer (Elegoo), standard glass capillaries, PTFE tubing, and a syringe pump were necessary to assemble and use the device. The device was assembled using a 0.20 mm ID glass microcapillary for the inner phase (heat-ligated to 0.25 mm ID PTFE tubing), 0.75 mm ID PTFE tubing for the middle phase, and a 1.5 mm ID glass microcapillary, surface treated as described above, for the outer phase. The microcapillary device was used to generate double emulsions, which were collected in a vial to allow de-wetting, and hence vesicle formation, to occur. Three solutions (outer aqueous, middle oil, and inner aqueous) were inserted into 1 mL gastight glass syringes. The middle oil phase was a 50:50 v/v mixture of chloroform and hexanes. Syringe tips were inserted into the top protrusions of the junction boxes of the microcapillary device as illustrated in Figure 1a in the paper. Using a syringe pump (Cetoni neMESYS), the outer phase flow rate was set to 200 μL/min, the middle phase flow rate was set to 100 μL/min, and the inner phase flow rate was set to 50 μL/min. Double emulsions were carefully collected in a glass vial containing HEPES buffer at 37 °C.

The de-wetting process was imaged for Figure 1c in the paper using a Nikon Eclipse Ti2-U inverted microscope and a Phantom VEO 710L high-speed camera. All other vesicles were visualised 2 h post formation to allow for full de-wetting and for them to settle on the bottom of vial. Vesicles were collected from the bottom of the vial using a pipet with approximately 0.5 cm of the tip trimmed off (to reduce sheer stress). The vesicle solution was placed in a depression slide (75 x 25 x 1.25 mm, Fisher Scientific) and observed on a Nikon Eclipse Ti-U2 inverted research microscope using a 20X/0.60 objective. Vesicle images were captured with a Hamamatsu ORCA-Flash4.0 V3 camera with a Solis-1C white LED light source (Thor Labs) for lamellarity and asymmetry quenching experiments. The following filter sets were used for acquisition: NBD-tagged lipids (Semrock GFP-4050B), Texas Red-tagged lipids (Semrock mCherry-C) and quinine hemi-sulphate (Semrock DAPI-3060A). NBD-tagged lipids were imaged at λ_{ex} = 460-500 nm and λ_{em} = 510-560 nm and red tagged lipids were imaged at λ_{ex} = 550-590 nm and λ_{em} = 608-683 nm. For quinine loaded vesicles and droplets, a Solic-365C light source was used for the UV spectra at λ_{ex} = 340-380 nm and λ_{em} = 435-485 nm with a 50 ms exposure time. Fluorescent microscopy images were processed manually using the NIS Elements software (Nikon, version 5.11.01). For analysis of the vesicles, a circular region of interest was manually placed over each vesicle (fit to its size) to determine its diameter and mean fluorescence intensity. The same process was used to determine droplet size and intensity for the encapsulation efficiency measurements. For the time series, emulsions were sealed in a vial and stored at 4 °C. Vesicles were collected from the vial and images were taken again after 24 h.

3.5 Fluorescence quenching assays

Asymmetry was determined using a previously described assay.[3] 1 mol % NBD-PC was added to either the outer or inner lipid solution, or to both, to form asymmetric vesicles. 2 h after vesicle formation, ~75 μL of vesicle solution was added to depression slides and 20 μL of 100 mM sodium hydrosulphite in 1 M Tris (pH 10) were added to the sample slowly. Images were taken every 10 s for 150 s following the previously described imaging protocols on a Nikon Ti-U2 inverted microscope.

3.6 Annexin V assay

Leaflet localization of phosphatidylserine (PS) was determined by fabricating vesicles containing lipid solutions of pure POPC and POPC/POPS in a 1:1 ratio in either the inner or outer solution. ~75 μL of vesicle solution was added to depression slides, images were captured to measure initial fluorescence following the previously described imaging protocols on a Nikon Ti-U2 inverted microscope. Then, 3 μL of annexin V and 4 μL of 50 mM $CaCl_2$ were added to the vesicles. Images were taken again 5 min after the addition of annexin V and $CaCl_2$.

3.7 Red-blood cell mimicking vesicle generation

Asymmetric liposomes were formed with the same lipid preparation procedure outlined above. The outer leaflet solution contained POPC/POPE/SM in a molar ratio of 0.44/0.12/0.44 to make a total of 10 mg of lipid solution and the inner leaflet solution contained POPC/POPE/SM/POPS in a molar ratio of 0.14/0.3/0.27/0.14 to a total of 10 mg of lipid. Symmetric vesicles were formed with an outer leaflet solution of POPC/POPE/SM/POPS in a ratio of 0.29/0.3/0.27/0.14.

3.8 Fluorescence-Based Permeability Analysis

Fluorescence decay from dye-loaded vesicles was used to quantify membrane permeability. Fluorescence intensity was recorded over time for individual symmetric and asymmetric vesicles. To estimate the apparent permeability, the fluorescence decay curve per vesicle fluorescence was fitted using a nonlinear least-squares approach in MATLAB. The decay was modeled as a first-order exponential function:

$$F(t) = F_\theta \cdot e^{-kt}$$

where $F(t)$ is the fluorescence at time *t,* F_θ is the initial fluorescence, and k is the rate constant of dye leakage. Both F_θ and k were treated as free parameters to account for variability in initial loading and imaging conditions. Fitting was performed in MATLAB, the apparent permeability coefficient, P_{app}, was then calculated using the fitted k value and the measured vesicle radius, r, to ensure we take into account differences in vesicle sizes, according to:

$$P_{app} = \frac{k \cdot r}{3}$$

4. Author Contributions

PA gathered the data presented in this paper and performed the data analysis. ARM performed the preliminary experiments and gathered the data for Figures 1c and S4. KR conceived the initial project idea, and designed and developed the microfluidic platform. KSE supervised KR, ARM and PA. PA wrote the first draft of the manuscript. PA and KSE wrote and edited the final manuscript. We would also like to thank Sean Farley for valuable discussions and Dr. Alejandro Forigua for helping edit the final manuscript.

5. Acknowledgments

KSE's position is funded through the Canada Research Chair program and the Michael Smith Health Research BC Scholar program in partnership with the Canadians for Leading Edge Alzheimer Research Foundation. Her laboratory was equipped using funding from the Canada Foundation for Innovation John R. Evans Leaders Fund (CFI-JELF) and the British Columbia Knowledge Development Fund (BCKDF). PA, ARM and KR were funded through KSE's Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant.

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