

## Electronic Supplementary Information (ESI)

### Phospholipid asymmetry in biomimetic vesicles alters membrane permeability

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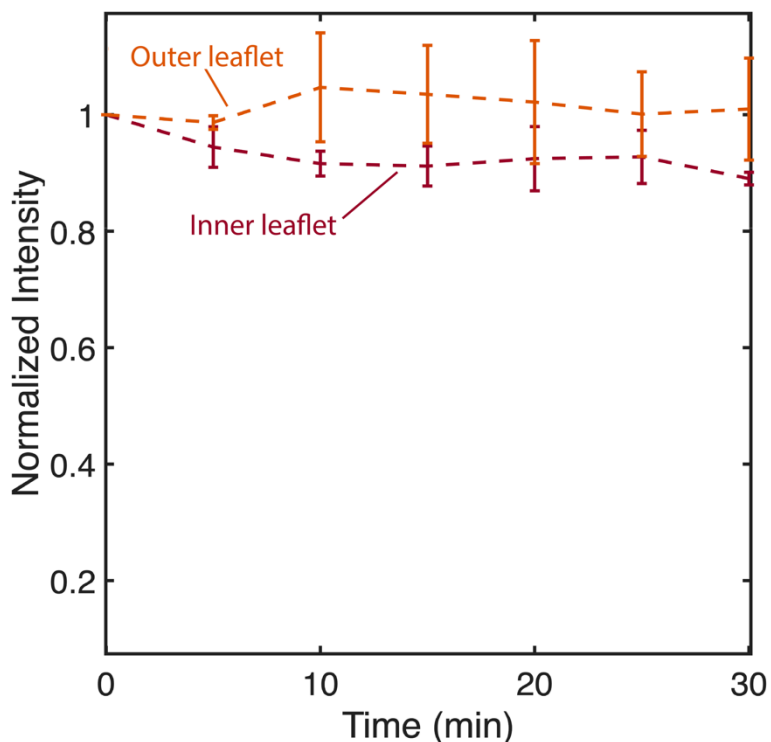
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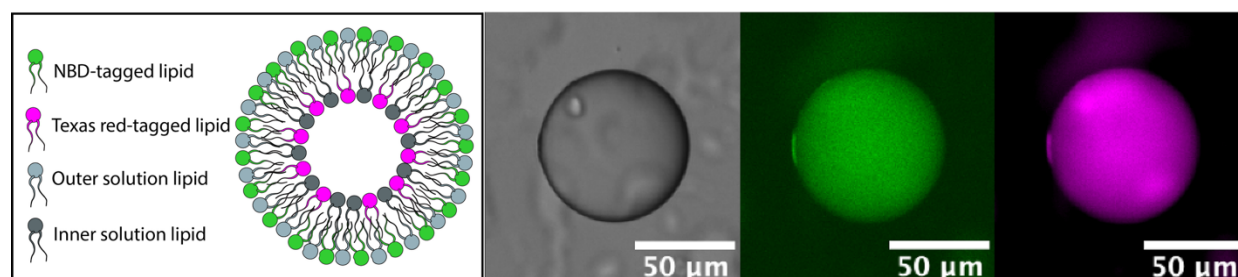
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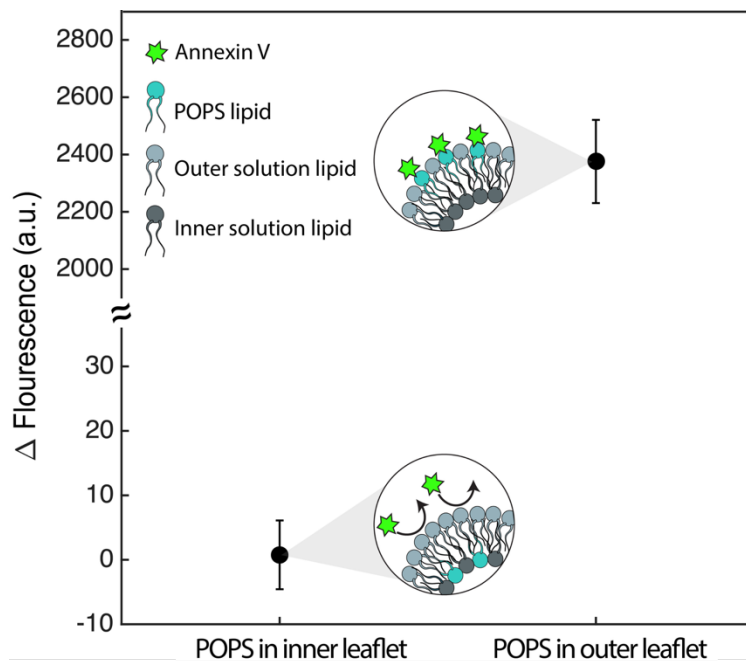
## 1. Supplementary Figures



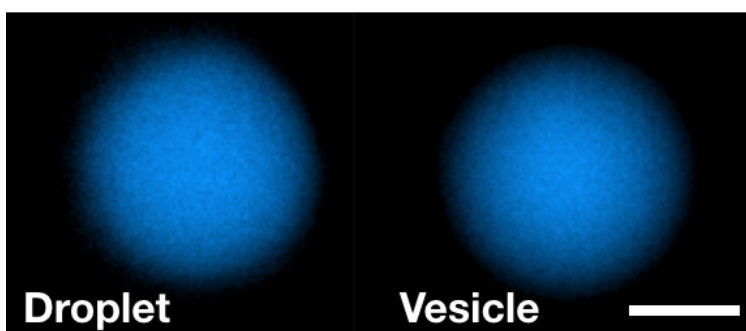
**Figure S1: Control experiment using Texas Red-tagged phospholipids.** Fluorescence intensity of Texas Red-tagged lipids in the outer leaflet (orange data,  $n = 3$ ) and in the inner leaflet (red data,  $n = 3$ ) over time. These data show that there is no overall quenching of fluorescence due to other factors during experiments using NBD-tagged lipids to determine lamellarity and lipid localisation in the bilayer. Images were taken every 5 min. The dashed line between data points is to aid visualisation of the data. Error bars denote standard deviation.



**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 leaflet and dark grey denoting DOPE and DOPC lipids in the inner leaflet in a 75:25 ratio. Representative microscopy images show the same vesicle in brightfield (left, grey), using a GFP filter to show the NBD-tagged phospholipid fluorescence (middle, green) and using 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. Scale bars are 50  $\mu\text{m}$ .



**Figure S3: Annexin V binding assay to assess lipid distribution.** Vesicles were generated with phosphatidylserine (POPS, blue) localized to either the inner (data on the left) or outer (data on the right) leaflet. Annexin V (green stars) was added outside the vesicles. Fluorescence intensity was normalized to pre-Annexin V addition. Vesicles with POPS confined to the inner leaflet exhibited minimal Annexin V binding, whereas vesicles with POPS in the outer leaflet showed a strong binding signal. Note the order of magnitude change in fluorescence intensity between the assays shown by the break in the y-axis. In both cases,  $n = 4$  and error bars denote standard deviation.



**Figure S4: Representative fluorescence 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 hemisulphate. 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.<sup>1</sup> The scale bar is 50  $\mu\text{m}$ .

## 2. Supplementary Table

**Table S1:** Quinine hemisulphate vesicle size and fluorescence intensity data used for encapsulation efficiency calculations.

<b>Droplet area (<math>\mu\text{m}^2</math>)</b>	<b>Droplet intensity</b>	<b>Vesicle area (<math>\mu\text{m}^2</math>)</b>	<b>Vesicle intensity</b>	<b>Encapsulation efficiency (%)</b>
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), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and sphingomyelin (SM, egg, chicken) were purchased from Avanti. 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, Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt and depression slides (75 x 25 x 1.25 mm) were purchased from Thermo Fisher Scientific. Polytetrafluoroethylene (PTFE) tubing (1/16-inch outer diameter, OD, and 0.75 mm or 0.25 mm inner diameter, ID) was purchased from Chromatographic Specialties Inc. Glass gas-tight syringes (1 mL, 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. 20 and 30 gauge blunt stainless-steel needles were purchased from Jensen Global Inc.

#### 3.2 Preparation of lipid solutions

All lipids were purchased suspended in chloroform. Lipids (in the correct ratios, see below) 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 concentration of 10 mg/mL (stock solution). 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 microcapillary device.

To test for lamellarity and asymmetry, lipid vesicles made using synthetic lipids were prepared with a total concentration of 5 mg/mL POPC or DOPC in the outer solution and 1.25 mg/mL POPC or DOPC and 3.75 mg/mL POPE or DOPE in the inner solution (for Figure 2 or Figures S1 and S2, respectively). 1 mol% NBD-PC was added either to the inner or the outer solution for the quenching assays, and 0.4 mol% Texas Red was added to the opposite solution.

Asymmetric vesicles designed to mimic red blood cell membranes were prepared with 2.5 mg/mL POPC, 0.5 mg/mL POPE and 2 mg/mL SM in the outer solution. The inner solution consisted of 0.75 mg/mL POPC, 2.25 mg/mL POPE, 0.5 mg/mL SM and 1.25 mg/mL POPS. The outer leaflet solution contained POPC/POPE/SM in a molar ratio of 0.44/0.12/0.44 and the inner leaflet solution contained POPC/POPE/SM/POPS in a molar ratio of 0.14/0.3/0.27/0.14. Symmetric vesicles were formed with POPC/POPE/SM/POPS in a ratio of 0.29/0.3/0.27/0.14 in both leaflets. When using these vesicles to quantify permeability, quinine hemisulphate was added to the inner phase solution at a concentration of 5mg/mL.

Surfactant stock solutions (as described previously by others)<sup>2</sup> were prepared separately and added to the lipid solutions post-extrusion. For the inner aqueous phase, 4 wt% PVA and 16 wt% PEG were added to

deionized water. For the outer aqueous phase, 20 wt% PVA and 1 wt% Pluronic F-68 were added to deionized 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 2 wt% PVA and 8 wt% PEG (inner) and 10 wt% PVA and 0.5 wt% Pluronic F-68 (outer).

### 3.3 Surface treatment of glass microcapillaries

The outer glass capillaries were treated to create a hydrophilic surface. First, the capillaries were cleaned with soapy deionized water, deionized 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 removed 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.<sup>3</sup> In brief, 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 water-in-oil-in-water double emulsions, which were collected in a vial to allow de-wetting, and hence vesicle formation, to occur. To do this, the 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. Blunt syringe tips were inserted into the top protrusions of the junction boxes of the microcapillary device as illustrated in Figure 1a. Using a syringe pump (Cetoni neMESYS), the outer phase flow rate was set to 200  $\mu\text{L}/\text{min}$ , the middle phase flow rate was set to 100  $\mu\text{L}/\text{min}$ , and the inner phase flow rate was set to 50  $\mu\text{L}/\text{min}$ . Double emulsions were carefully collected in a glass vial containing HEPES buffer at 37 °C.

The de-wetting process was imaged for Figure 1c using a Nikon Eclipse Ti2-U inverted microscope and a Phantom VEO 710L high-speed camera. All other vesicles were visualized 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 pipette with approximately 0.5 cm of the tip trimmed off (to reduce shear stress). The vesicle solution was placed in a depression slide 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 (Thorlabs) 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 hemisulphate (Semrock DAPI-3060A). NBD-tagged lipids were imaged at  $\lambda_{\text{ex}} = 460\text{-}500$  nm and  $\lambda_{\text{em}} = 510\text{-}560$  nm and red tagged lipids were imaged at  $\lambda_{\text{ex}} = 550\text{-}590$  nm and  $\lambda_{\text{em}} = 608\text{-}683$  nm. For quinine loaded vesicles and droplets, a Solis-365C light source was used for the UV spectra at  $\lambda_{\text{ex}} = 340\text{-}380$  nm and  $\lambda_{\text{em}} = 435\text{-}485$  nm with a 50 ms exposure time. Fluorescence 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 and vesicle 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.<sup>3</sup> 1 mol % NBD-PC was added to either the outer or inner lipid solution, or to both, as described above, and asymmetric vesicles were formed. 2 h after vesicle formation, ~75  $\mu\text{L}$  of vesicle solution was added to depression slide and 20  $\mu\text{L}$  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 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, as described above. Then, ~75  $\mu\text{L}$  of vesicle solution was added to the depression slide, and images were captured to measure initial fluorescence following the previously described imaging protocols on a Nikon Ti-U2 inverted microscope. Then, 3  $\mu\text{L}$  of annexin V and 4  $\mu\text{L}$  of 50 mM  $\text{CaCl}_2$  were added to the vesicles. Images were taken again 5 min after the addition of annexin V and  $\text{CaCl}_2$ .

### 3.7 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 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  $t$ ,  $F_{\theta}$  is the initial fluorescence, and  $k$  is the rate constant of dye leakage. Both  $F_{\theta}$  and  $k$  were treated as free parameters to account for variability in initial loading and imaging conditions. 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 Figure 1c. 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.

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#### **6. References for the Electronic Supplementary Information**

1. S. Matosevic and B. M. Paegel, *Nat. Chem.*, 2013, **5**, 958–963.
2. N.-N. Deng, M. Yelleswarapu and W. T. S. Huck, *J. Am. Chem. Soc.*, 2016, **138**, 7584–7591.
3. S. Farley, K. Ramsay and K. S. Elvira, *Lab Chip*, 2021, **21**, 2781–2790.
4. J. C. McIntyre and R. G. Sleight, *Biochemistry*, 1991, **30**, 11819–11827.