ESI- Supplementary Information

SI 1- Materials and Methods

Phospholipids, 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000] (DSPE-DBCO) were obtained from Avanti Polar Lipids (Alabaster, USA). Fluorogenic azide 3-Azido-7-hydroxycoumarin was purchased from Jena Bioscience GmbH (Germany). Squalene (Acros Organics, Antwerp) was used as the oil carrier phase. Alpha-hemolysin from Staphylococcus aureus (lyophilized powder) was obtained from Sigma-Aldrich (Dorset, UK). Polydimethylsiloxane) (PDMS) prepolymer and curing agent kits (Sylgard 184) were obtained from Dow Corning (Midland, MI, USA). Silicon wafers were obtained from IBD Technologies Ltd (Wiltshire, UK). SU-8 negative photoresists and EC development solvent were obtained from Chestech Ltd (Rugby, UK). Poly(dimethylsoloxane-b-ethylene oxide) (PEO) required for the hydrophilic modification of PDMS was obtained from Polysciences Europe GmbH (Eppelheim, Germany). All other chemicals were purchased from Sigma-Aldrich (Dorset, UK).

Device Fabrication

The PDMS microfluidic device was fabricated by means of double-layer photolithography.[1,2] The 'step' was generated by aligning two microchannels and applying different negative photoresists onto the silicon wafer. After development using microdeposit EC solvent, the negative master was exposed to 1,1,2,2-perfluorooctyltrichlorosilane vapour to suppress permanent adhesion to moulded PDMS. PDMS prepolymer and curing agent were then thoroughly mixed in a 10 : 1 ratio, and the mixture was poured onto the master wafer. After curing at 65 °C for 3 hours, PDMS treated with 3% PEO surfactant was mixed; the deeper microchannel [to contain the external aqueous phase] was extricated from the cured PDMS and the PEO-PDMS was applied to this individual channel and cured at 65 °C for a further 3 hours. This was necessary to prevent the wetting of the droplets to the channel surface and release from the oil phase. The PDMS device was bonded via partial curing of PDMS spin-coated across a microscopic glass slide.

Microfluidic generation of giant vesicles

The chip was set up with the three fluid inlets and one vesicle outlet. The internal aqueous phase and oil phase were injected using 1 ml plastic syringes linked to 1.09 mm PTFE tubing (Adtech Polymer Engineering Ltd, Stroud, UK). The external aqueous phase was injected using a 6 ml plastic syringe linked to the same tubing. Three syringe pumps (Chemyx Inc, Stafford, UK) were necessary to pump the reagents into the microfluidic system at controlled flow rates.

Asymmetry characterisation

Vesicles were generated in the same manner as previously outlined and were doped with head-group-labelled alkyne lipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[dibenzocyclooctyl(polyethylene glycol)-2000], (DSPE-DBCO, 5 mol %) on either the inner leaflet and outer leaflet (Fig. 3). This was controlled by adding the DSPE-DBCO (5 mol %) into either the AQ_{IN} phase or AQ_{EX} phase which would determine which membrane leaflet the DBCO would subsequently reside in. Fluorogenic azide (3-Azido-7-hydroxycoumarin dissolved in MeOH, 5mM), was encapsulated internally (AQ_{IN}) or added externally (pipetted into PDMS well containing GUVs) to either 'click' with the DBCO and give a fluorescent signal or afford no signal as a control (as outlined in Fig. 3). Alpha-hemolysin (αHL, 0.5mg mL^{-1}) was added externally to the vesicles in PDMS wells.

Process Visualisation and fluorescence

All microfluidic experiments were imaged with a Leica DM IRB microscope. Fluorescence experiments were visualised with a Nikon Eclipse TE2000-E inverted microscope. The fluorescent species were illuminated using a mercury arc lamp with the appropriate filter sets. Fluorescence images were taken at >100ms exposure time. Images were taken with a QICAM camera (QImaging) and were analysed using ImageJ software. Vesicle fluctuations were imaged using phase contrast on the Nikon Eclipse.

SI 2- Hydrophilic treatment of microfluidic device

The hydrophilic treatment was a key step in the fabrication of the device. As outlined in the *materials and methods* section SI-1, this was necessary to ensure that water-in-oil emulsions were released into the aqueous phase and didn't wet along the side of the channel.

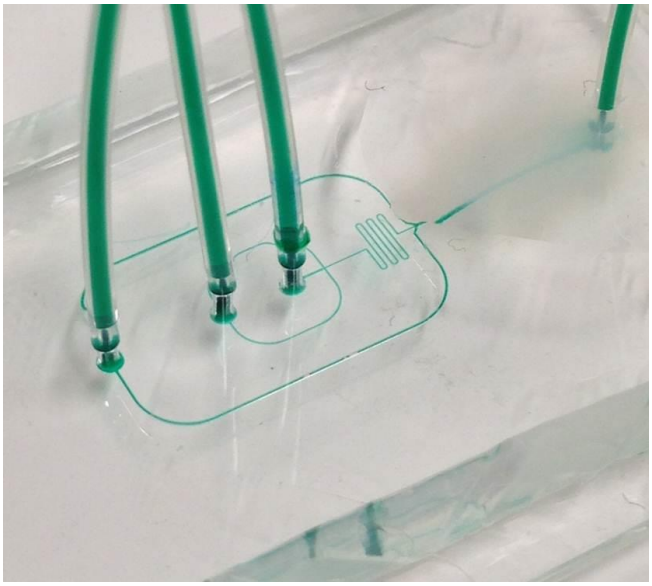

Figure S2: Image of the PDMS microfluidic vesicle production device with dye flowed through the channels. The cloudy region is treated with PEO-PDMS (3%) to afford a hydrophilic aqueous channel where vesicles are formed and collected from.

SI 3- Fluctuation analysis of symmetric and asymmetric vesicles

Bending rigidity measurements

There are various methods reported in the literature for determining the bending rigidity of vesicles, namely micropipette aspiration or scattering techniques.[3–5] Fluctuation analysis involves analysing the thermal fluctuations of a GUV membrane. Experimentally, this involves imaging vesicles at a high speed (~120 frames per second) using optical microscopy. By collecting these time sequences we were able to analyse up to 5000 frames of a vesicles contour at a time.

The resulting videos were analysed using custom-built software which maps the changes in the fluctuations around an average shape of the vesicle.[6] The contour fluctuation is broken down into equatorial normal modes using a discrete Fourier transform. We investigated modes 5-20 for each system as lower modes are dominated by tension and vesicle displacement factors.[6] Modes >20 cannot be detected reliably due to their fast relaxation rate.

The vesicles were prepared as previously described with the internal aqueous phase composed of sucrose solution (400mM) and the external aqueous phase a glucose extruded lipid solution (450mM). Vesicles were generated, collected and viewed in homemade PDMS wells. The vesicles were generated and visualised at T=21˚C using phase contrast microscopy at a frame rate of ~120 per second. 60 second recordings of the vesicles fluctuating were taken. The contours of the symmetric binary mixtures and asymmetric GUV populations were analysed.
The fitting regime for the fluctuation analysis method is explained in further detail by Yoon *et al.*[7]

The data obtained from the fluctuation analysis of microfluidic GUV populations was fit according to **Eqn. E1**.

$$\langle h(q_x, y=0)^2 \rangle = \frac{1}{L}\frac{k_B T}{2\sigma}\left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{k_c} + q_x^2}}\right) \qquad \text{E1}$$

From the power spectrum generated from the vesicle contour analysis we were able to fit the raw data according to two parameter E1 to obtain both bending rigidity and tension values.

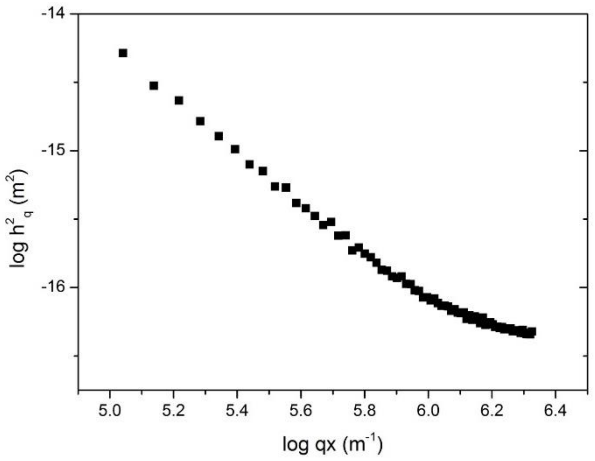

Figure S3: A power spectrum of the thermal fluctuations of a single GUV, which shows how the amplitudes, h, of modes q_x, were fitted according to Eqn. E1. The data illustrates the range of modes that were analysed during the contour analysis. Only modes 5-20 were fit according to E1 to obtain bending rigidities. This is due to the lower modes (<5) being affected by membrane tension as well as the membrane overall topology. The higher modes (>20) are impossible to detect reliably due to limited optical resolution, their fast relaxation rate and the camera noise. This power spectrum was generated for asymmetric POPC inner, DOPC outer vesicles.

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