SUPPORTING INFORMATION

Liquid-Liquid Phase Separation inside Giant Vesicles Drives Shape Deformations and Induces Lipid Membrane Phase Separation

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S1. Materials and Methods.

S1.1. Materials. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), egg sphingomyelin (egg SM), cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phospho ethanol amine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DOPE-mPEG), GM1 Ganglioside (Brain, Ovine-Sodium Salt) (GM1), lissamine rhodamine B 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (Rho-DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine -N-(7-nitro-2,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) were acquired from Avanti Polar Lipids (Alabaster, AL). Glucose, poly (ethylene glycol) (PEG (8 kg mol^{-1}), MW 8,000 Da), dextran from Leuconostoc mesenteroides (dextran (10 kg mol^{-1}), MW 9,000-11,000 Da) and Dextran from Leuconostoc spp. (dextran (450 kg mol^{-1}), MW 450,000-650,000 Da) were obtained from Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488-Dextran (MW 10 kDa) and Alexa Fluor® 647-Dextran (MW 10 kDa) were purchased from ThermoFisher Scientific (Eugene, OR). Sucrose was obtained from EMD Chemicals (Philadelphia, PA). Chloroform was purchased from Fisher Scientific (Fair Lawn, NJ). Ninety six well glass bottom plates were obtained from MatTek Corporation (Ashland, MA). Indium tin oxide (ITO) coated glass slides (resistance 4-30 Ω) were obtained from Delta Technologies (Loveland, CO). All chemicals were used without further purification.

S1.2. GUV Preparation. Giant unilamellar vesicles (GUV) were prepared following a well-established electroformation protocol from Angelova and Dimitrov. (1) Three separate stock solutions, one consisting of 2:2:1 mol ratio of POPC, Sphingomyelin and Cholesterol doped with 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE and 3 mol% NBD-PE, one consisting of 96.8 mol% POPC doped with 2.2 mol% DOPE-mPEG and 1 mol% Rho-DOPE and the third consisting of 96 mol% POPC doped with 4 mol% GM1 and 1 mol% Rho-DOPE were prepared in chloroform in a concentration of 2 mg/ml. Small droplets (15 µL) of stock solution were spread on the conductive side of each of two ITO-coated slides and dried under vacuum overnight. The dried film on one of the two slides was then directly hydrated either with 14 mM sucrose (control experiments) or with polymer solutions mixtures. The latter included mixtures of (1) 6.0 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}), (2) 4.5 wt% PEG (8 kg mol^{-1}) and 2.02 wt% dextran (10 kg mol^{-1}), (3) 9.5 wt% PEG (8 kg mol^{-1}) and 3.1 wt% dextran (10 kg mol^{-1}), (4) 10 wt% PEG (8 kg mol^{-1}) and 2 wt% dextran (10 kg mol^{-1}), (5) 4 wt% PEG (8 kg mol^{-1}) and 8.3 wt% dextran (10 kg mol^{-1}), and (6) 2.5 wt% PEG (8 kg mol^{-1})

and 15 wt% dextran (10 kg mol^{-1}). The solution droplet was contained using a 1 mm thick rubber 'O' ring (~20 mm diameter) from Ace Hardware (Davis, CA) sealed with high vacuum grease from Dow Corning (Midland, MI). A water-tight chamber was then created by sealing the second ITO slide over the ring, ensuring that no visible air bubbles were trapped inside. Using a function generator, a 4 Vpp AC sine wave was then applied across the two slides at 10 Hz for 3 hr followed by a 4 Vpp AC square wave at 2 Hz for 2 hr. During the formation the ITO sandwich was covered with an aluminium foil to protect from light. Following the formation, the ITO sandwich was disassembled and the solution containing GUVs was collected by a pipet. The GUVs were either used immediately or stored at 4 °C. All vesicles were used within a week of preparation.

S1.3. Spinning Disk Confocal Fluorescence Microscopy. Spinning disk confocal fluorescence microscopy measurements were performed using an Intelligent Imaging Innovations Marianas Digital Microscopy Workstation (3i Denver, CO) fitted with a CSU-X1 spinning disk head (Yokogawa Musashino-sh, Tokyo, Japan) and a Quantem512SC EMCCD camera (Photometrics Tuscon, AZ). Fluorescence micrographs were obtained using oil immersion objectives (Zeiss Fluor 40x (NA 1.3), Zeiss Plan-Fluor 63x (NA 1.4), and Zeiss Fluor 100X (NA 1.46); Carl Zeiss Oberkochen, Germany). Samples of osmotically balanced GUVs were prepared as described in the previous section. In a typical experiment, the glass bottom 96 well plate containing osmolyte-laden solution was mounted onto the microscope, and once oiled, the objectives were raised to form a meniscus between the cover glass and the objective. To impose osmotic gradients in real-time, GUV suspension is added to the solution in the sample chambers. Rho-DOPE (Ex/Em; 560/583) was exposed with a 50 mW 561 laser line. Alexa Fluor® 488-Dextran (Ex/Em; 495/519), NBD-PE (Ex/Em; 460/535) were exposed with a 50 mW 488 laser line. Alexa Fluor® 647-Dextran (Ex/Em; 650/668) was exposed with a 50 mW 651 laser line. The images are subsequently analyzed using ImageJ (http://rsbweb.nih.gov/ij/), a public-domain software, and Slidebook digital microscopy imaging software (3i Denver, CO).

S1.4. Binodal Line Determination. An aqueous two phase mixture binodal line was constructed using the cloud point method previously described. (2) Briefly, 40 wt% PEG (8 kg mol^{-1}) stock solution and 30 wt% dextran (10 kg mol^{-1})stock solution were first prepared.

A known mass of ~5g 40 wt% PEG (8 kg mol^{-1}) was weighed and place in a glass vial. Small amounts of 30 wt% dextran (10 kg mol^{-1}) solution were added dropwise into the PEG (8 kg mol^{-1}) solution, mixed after each addition, until the solution becomes cloudy indicating a cross of the binodal line to two phase region. Water was subsequently added dropwise into the mixture, mixed after each addition, until the mixture became clear and homogeneous indicating transition back across the binodal curve to homogeneous region. This process was repeated to plot subsequent points which along the binodal line.

S1.5. Phase Composition Determination. The polymer composition of each phase was determined using a combination of refractometry and polarimetry (3). Refractive index measurements were done using an Abbe Auto Refractometer (Leica Geosystems, Norcross, GA). Polarimetry measurements were done using a model No. 343 Polarimeter (PerkinElmer, Billerica, MA). The concentration of dextran (10 kg mol^{-1}) was determined by polarimetry, using a standard curve of known concentrations of dextran. The concentration of PEG was determined using refractometry. Calibration curves of known weight percentages of PEG (8 kg mol^{-1}) and dextran were prepared, and the refractive index the PEG-rich and dextran-rich phases was measured. The contribution of the refractive index from dextran was subtracted from the total refractive index and the remaining refractive index was attributed to PEG. This value was then used to calculate the concentration PEG.

S1.6. Osmotic Pressure Measurement. The osmotic pressure of each phase was determined using osmometry. The osmolality of the PEG (8 kg mol^{-1}) and dextran (10 kg mol^{-1})-rich phases was measured using a Wescor 5100C Vapor Pressure Deficit Osmometer (Wescor Inc., Logan, UT), calibrated with NaCl standard solutions (Wescor Inc). The osmotic pressure, Π (MPa), of each solution was calculated from the osmolality measurements using the Van't Hoff equation: $\Pi = RTc$, where c = osmolality (mol-kg^{-1}) and RT = 2.446 kg-MPa-mol^{-1} at 21°C.

S1.7. Data processing. Images were processed using ImageJ – a public-domain software obtained from http://rsbweb.nih.gov/ij/

S2. Supplementary Figure.

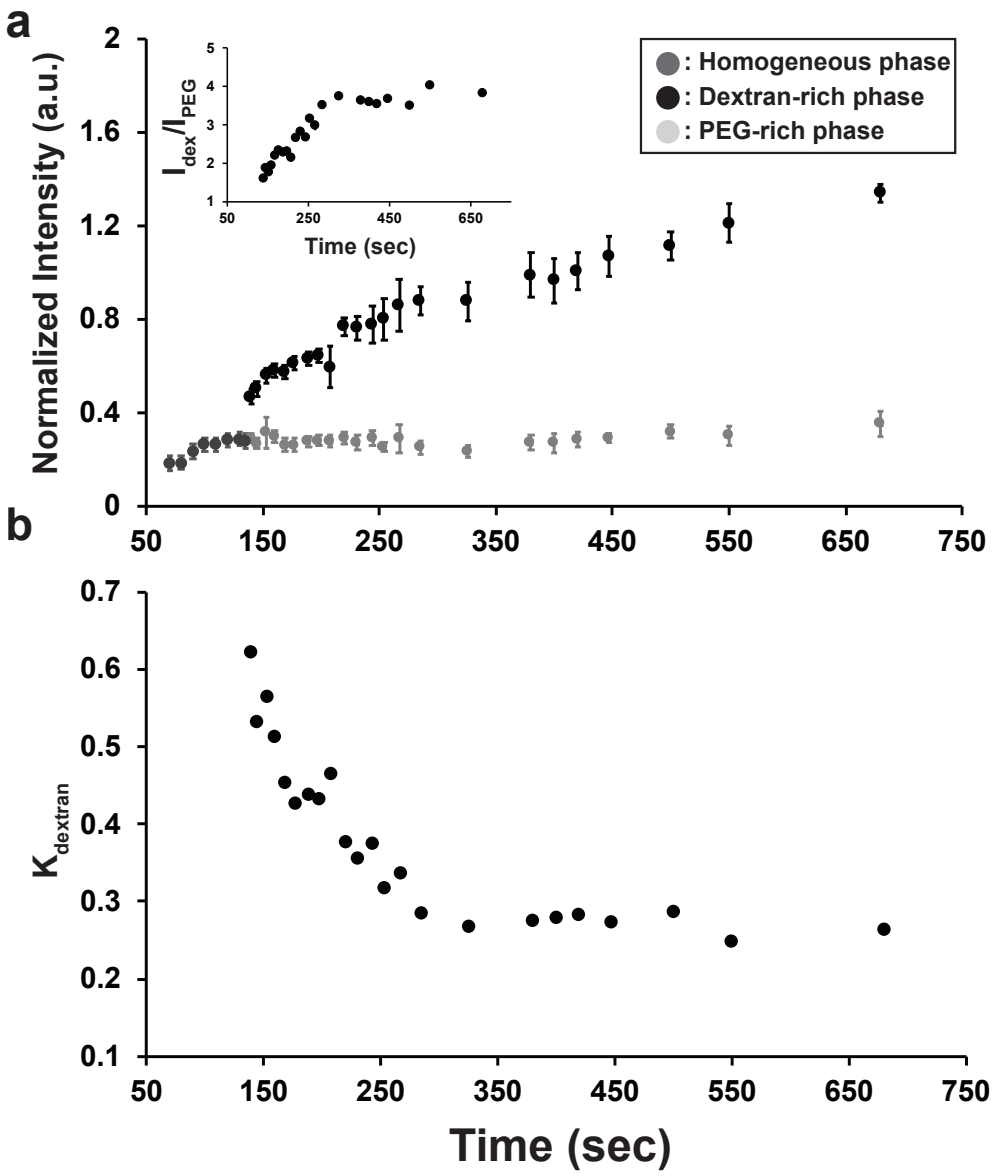

Figure S2.1. Fluorescence intensity measurements of solution encapsulated in an LLPS GUV to determine the partition coefficient (K) of dextran in PEG-rich and dextran-rich phases. (A) Normalized fluorescent intensity profile of dextran (Alexa flour® 488 dextran) in homogeneous phase (●), PEG-rich (●) and dextran-rich (●) phases after phase separation. The inset shows the ratio of fluorescence intensity of dextran-rich phase verses the PEG-rich phase. (b) Measurement of partition coefficient (K) of dextran in LLPS GUV. GUVs contain 96.8 mol% POPC, 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE encapsulate a mixture of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) upon immersion in 143 mM sucrose solution

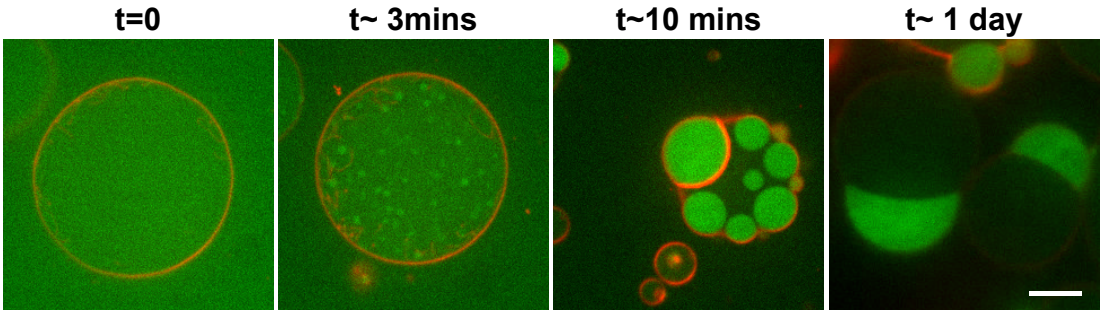

Figure S2.2. Selected frames of confocol fluorescence microscopy images of GUVs encapsulating a mixture of 6.0% (w/w) PEG 8 kg mol^{-1} and 6.4% (w/w) dextran 10 kg mol^{-1} doped with 0.001% AlexaFluor® 488-Dextran upon immersion in 143mM sucrose solution. GUVs contain 96.8 mol% POPC, 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE. Scale bar, 10 µm.

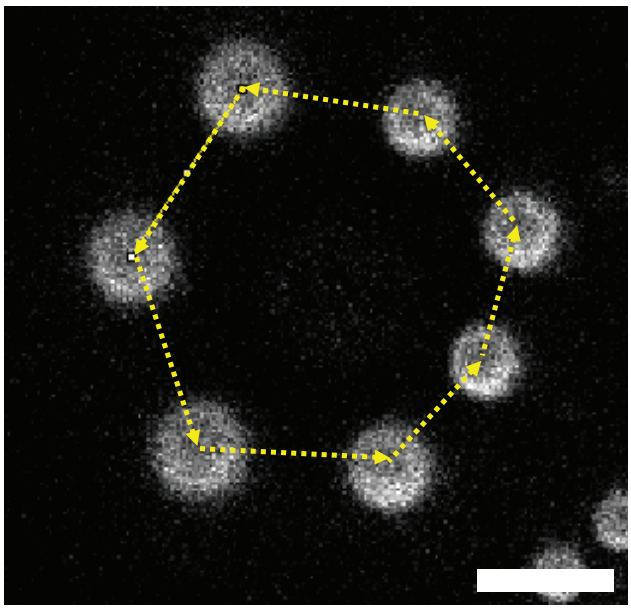

Figure S2.3. Phase ordering of LLPS induced buddings. Center to center distance between each buds is equal to $12.35\ (\pm\ 1.97)\ um$ (n=7). All GUVs imaged consist of a mixture containing 96.8 mol% POPC, 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE. Scale bar, 10 µm.

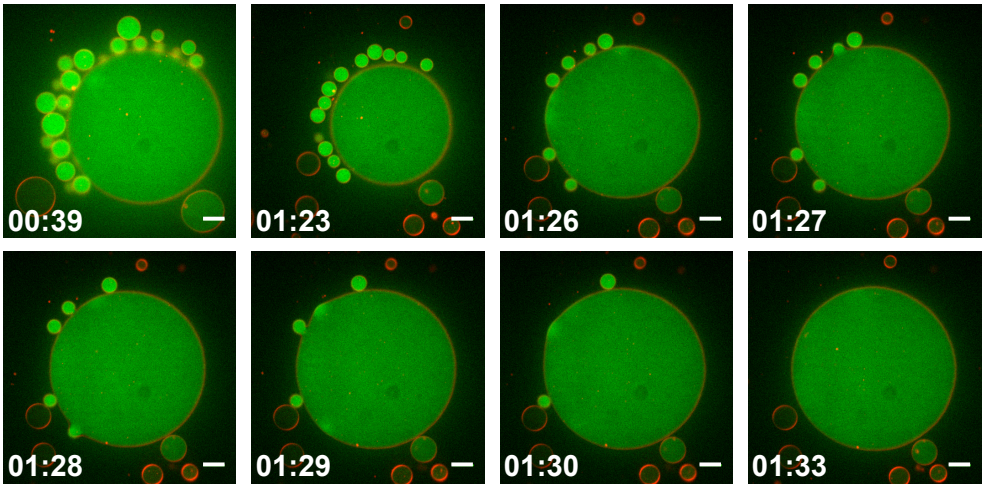

Figure S2.4. Reversibility of osmotic deflation driven liquid-liquid phase separation of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) in GUVs. Confocal fluorescence microscopy image of a single GUV consists of a mixture containing 96.8 mol% POPC, 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE encapsulating 6.0 wt% PEG (8 kg mol^{-1}) and 6.4 wt% Dextran (10 kg mol^{-1}) aqueous phase subjected to pure water (till exterior sucrose concentration equal to 62 mM) after hypertonic trigger deformation in 143mM sucrose solution. Scale bar, 10 µm.

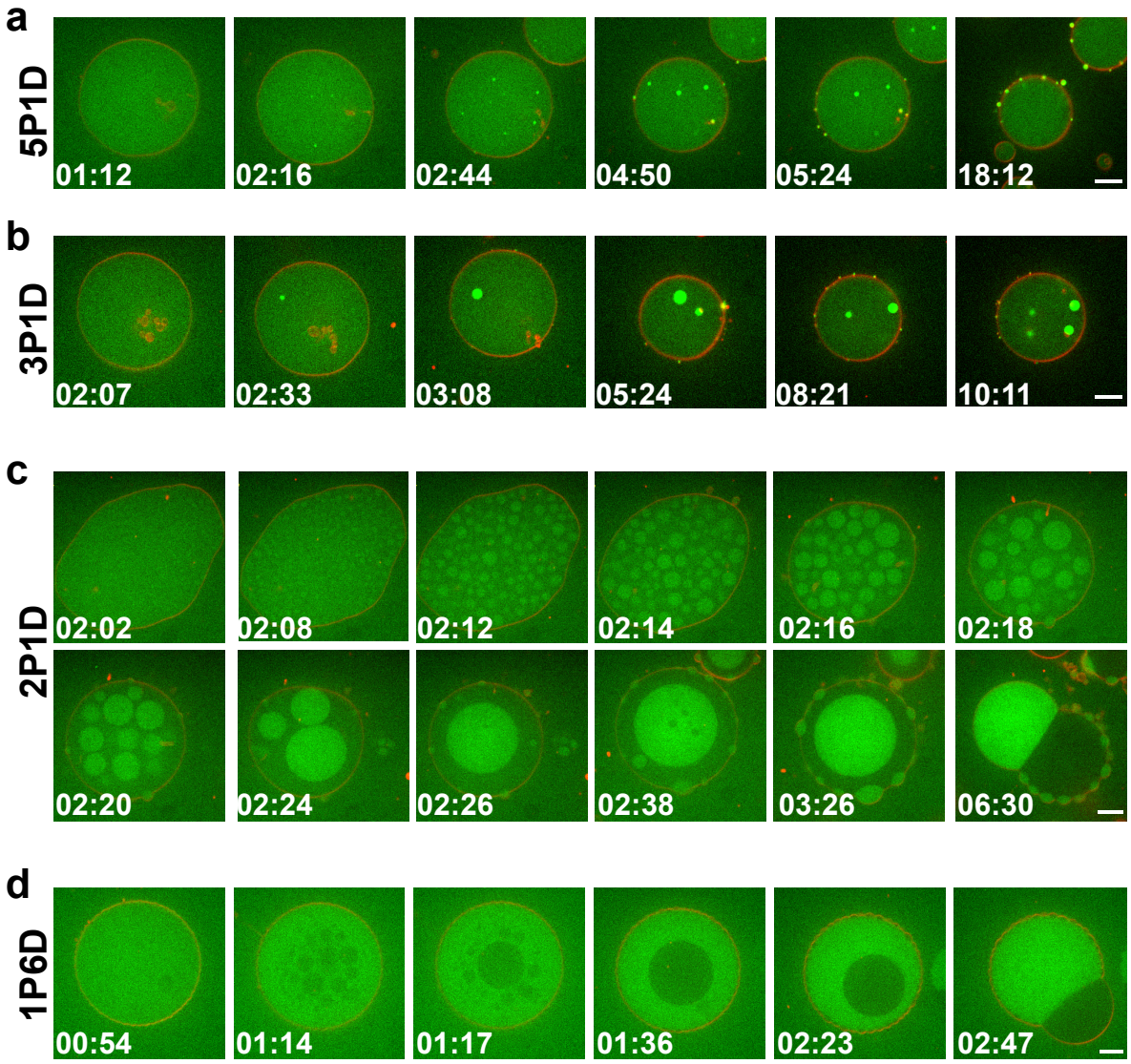

Figure S2.5. Time-lapse confocal fluorescence microscopy image of GUVs encapsulating mixtures with (a) 5:1 (namely 5P1D), (b) 3:1 (namely 3P1D), (c) 1:2 (namely 1P2D) and (d) 1:6 (namely 1P6D) ratios of PEG (8 kg mol^{-1}) and dextran (10 kg mol^{-1}). All GUVs imaged consist of a mixture containing 96.8 mol% POPC, 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE encapsulating PEG and dextran mixtures doped with 0.001 wt% AlexaFluor® 488 dextran upon immersion in (a) 143, (b) 120, (c) 263, and (d) 298 mM sucrose solution. All scale bar, 10 μm.

S3. Supplementary Video.

S3.1. Supplementary Video S1. Osmotic deflation driven liquid-liquid phase separation of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) in GUVs. Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 143mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.2. Supplementary Video S2. Reversibility of osmotic deflation driven liquid-liquid phase separation of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) in GUVs. Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 143 mM sucrose for 1 hr and then subject to 62 mM sucrose solution by adding certain amount of H_2O to dilute 143 mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.3. Supplementary Video S3. Osmotic deflation driven liquid-liquid phase separation of 10 wt% PEG (8 kg mol^{-1}) and 2 wt% dextran (10 kg mol^{-1}) in GUVs (5P1D, PEG: dextran=5:1). Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 10 wt% PEG (8 kg mol^{-1}) and 2 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 143mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.4. Supplementary Video S4. Osmotic deflation driven liquid-liquid phase separation of 9.5 wt% PEG (8 kg mol^{-1}) and 3.1 wt% dextran (10 kg mol^{-1}) in GUVs (3P1D, PEG: dextran=3:1). Time-lapse movies of confocal fluorescence microscopy

images of single GUVs containing a mixture of 10 wt% PEG (8 kg mol^{-1}) and 2 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 120mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.5. Supplementary Video S5. Osmotic deflation driven liquid-liquid phase separation of 4 wt% PEG (8 kg mol^{-1}) and 8.3 wt% dextran (10 kg mol^{-1}) in GUVs (2P1D, PEG: dextran=2:1). Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 4 wt% PEG (8 kg mol^{-1}) and 8.3 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 263mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.6. Supplementary Video S6. Osmotic deflation driven liquid-liquid phase separation of 2.5 wt% PEG (8 kg mol^{-1}) and 15 wt% dextran (10 kg mol^{-1}) in GUVs (1P6D, PEG: dextran=1:6). Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 2.5 wt% PEG (8 kg mol^{-1}) and 15 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 298mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.7. Supplementary Video S7. Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 4.5 wt% PEG (8 kg mol^{-1}) and 2.02 wt% dextran (450 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 (labeled as green) upon immersion in the external dispersion medium containing 143mM sucrose. The GUVs are composed of 96.8 mol% POPC and doped with 2.2 mol% DOPE-mPEG, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.8. Supplementary Video S8. Osmotic deflation driven liquid-liquid phase separation of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) in GUVs

with 4 mol% GM1 in membrane. Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 488 dextran (labeled as green) upon immersion in the external dispersion medium containing 143mM sucrose. The GUVs are composed of 95 mol% POPC and doped with 4 mol% GM1, and 1 mol% Rho-DOPE (labeled as red). Scale bar: 10 μm

S3.9. Supplementary Video S9. Osmotic deflation driven liquid-liquid phase separation of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) colocalizing membrane molecules in GUVs with ternary lipid composition. Time-lapse movies of confocal fluorescence microscopy images of single GUVs containing a mixture of 6 wt% PEG (8 kg mol^{-1}) and 6.4 wt% dextran (10 kg mol^{-1}) doped with 0.001 wt% Alexa Flour® 647 dextran (labeled as blue) upon immersion in the external dispersion medium containing 157mM sucrose. The GUVs are composed of POPC: Egg-SM: Cholesterol in a molar ratio of 2:2:1 doped with 2.2 mol% DOPE-mPEG, 1 mol% Rho-DOPE (labeled as red) and 3 mol% NBD-PE (labeled as green). Scale bar: 10 μm

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2. Hatti-Kaul, R., *Aqueous Two-Phase Systems: Methods and Protocols*. Humana Press: New Jersey, 2000; Vol. 11.

3. Albertsson, P.-Å.; Tjerneld, F., [1] Phase diagrams. In *Methods in Enzymology*, Academic Press: 1994; Vol. 228, pp 3-13.