Biophysical Journal, Volume *110*

Supplemental Information

Solution Asymmetry and Salt Expand Fluid-Fluid Coexistence Regions of Charged Membranes

Bastian Kubsch, Tom Robinson, Reinhard Lipowsky, and Rumiana Dimova

Biophysical Journal, Volume 110

Supplemental Information

Solution Asymmetry and Salt Expand Fluid-Fluid Coexistence Regions of Charged Membranes

Bastian Kubsch, Tom Robinson, Reinhard Lipowsky, and Rumiana Dimova*
Max Planck Institute of Colloids and Interfaces, Science Park Golm, 14424 Potsdam, Germany

* Address reprint requests and inquiries to dimova@mpikg.mpg.de

Materials

1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), sodium salt (DOPG), chicken egg sphingomyelin (eSM), and cholesterol (Chol, ovine wool, > 98 %) were purchased from Avanti Polar Lipids (Alabaster, IL). The fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{18}$) was acquired from Molecular Probes (Eugene, Oregon). Chloroform obtained from Merck (Darmstadt, Germany) was of HPLC grade (≥ 99.8 %). Polydimethylsiloxan (PDMS) and curing agent (Sylgard 184) were purchased from Dow Corning (Midland, Michigan). NaCl (> 99.8 %), HCl (37 %), and Tris (≥ 99.9 %) were from Roth (Karlsruhe, Germany). Sucrose (≥ 99.5 %) was obtained from Sigma-Aldrich (St. Louis, Missouri). Ultrapure water was used for all buffer preparations.

Preparation of GUVs

GUVs were prepared from a 4 mM lipid stock in chloroform containing different ratios of charged DOPG, eSM and Chol. As a fluorescent marker, the mixtures additionally contained 0.1 mol% of $DiIC_{18}$. Vesicles were grown by spontaneous swelling according to Ref. (1). Briefly, 10 – 15 µl of lipid stock were deposited on a clean ~1 cm² polytetrafluoroethylene (PTFE, known as Teflon) plate and desiccated under vacuum at 60 °C for 2 h. The dried lipid film was pre-swollen in a water-saturated atmosphere at the same temperature for 4 h. Finally, the film was hydrated with the growing solution, here, either 210 mM sucrose (210 mOsm/kg) or high-saline buffer composed of 100 mM NaCl, 10 mM Tris at pH 7.5 (210 mOsm/kg), and incubated at 60 °C overnight. After swelling, GUVs were cooled down to room temperature (~ 23 °C) within one hour. The vesicles accumulated in a red clump visible by eye during swelling which was harvested and re-suspended in 1 mL of the hydration solution. The sample was then ready for analysis of symmetric solution conditions. To create asymmetric solution conditions, GUVs were grown in the internal solution and diluted by 20 times in the desired external solution. Both solutions were osmotically matched (Osmomat 030, Berlin, Germany) before dilution. The vesicles were left for equilibration for at least 1 h before observation. Phase state observations of all solution conditions were performed on the day of GUV harvest.

GUV visualization and phase state assessment

For phase diagram mapping, epifluorescence microscopy was used. Per batch, 30 – 70 GUVs with no or minimal defects and a diameter larger than 5 µm were analyzed for their phase state. The whole batch was assigned the dominating majority phase state among the GUVs analyzed. In order to describe the phase state of vesicles, certain criteria were followed. The single-liquid state (Lo or Ld) was characterized by a homogeneous distribution of $DiIC_{18}$ throughout the entire vesicle which appeared spherical and smooth. The liquid-liquid coexistence state (Lo/Ld) was marked by the appearance of circular domains with smooth boundaries, which were free to diffuse. Solid-liquid coexistence states (S/Lo, S/Ld, S/Lo/Ld) featured angular, sometimes finger-like domains with static boundaries. If the solid domains dominate, vesicles showed an overall angular appearance. GUVs assigned to the Lo/Ld coexistence region were analyzed with a 40x/0.6 NA objective (Axio Observer.D1, Zeiss, Oberkochen, Germany), while those assigned to a solid-liquid coexistence state were observed via a 63x/1.2 NA water objective (TCS SP5, Leica, Wetzlar, Germany) due to their fine structures. GUVs of identical compositions but exposed to different solution conditions were always observed with the same objective to allow direct comparison. The visual assessment of phase states based on these criteria ensured correct comparisons across different solutions. Yet, for compositions yielding very small vesicles and/or small domains, judgments of phase states may vary among individual observers. Batch observations where ~50 % of vesicles were phase-separated (if composition close to the border between single-liquid and liquid/liquid regions) were repeated several times in independent experiments to judge the phase state. Analyses of phase states under asymmetric conditions were performed at least 3 times on independent batches in case an alteration was observed. The imaging of GUVs captured on the microfluidic device was done using confocal laser scanning microscopy with a 63x/1.2 NA objective (TCS SP5, Leica, Wetzlar, Germany).

GUV miscibility transition temperatures

To determine the miscibility temperatures, the vesicles were introduced into a custom-made chamber with temperature controlled by circulating water connected to a thermostat. During analysis, the temperature was increased by 2 °C every 5 – 10 min (depending on observation time required) until the vesicles in the investigated batch appeared homogenous. To check for hysteresis, a representative sample of GUVs with sucrose inside and outside was cooled down again after analysis. The fraction of phase separation was checked at T = 19 °C and T = 13 °C and was the same as during the period of temperature increase. Due to different density gradients in case of asymmetric solution conditions vesicles would float up to the cover glass or settle onto the microscope slide. Therefore, objective lenses with different working distances needed to be used, namely 40x/0.75 NA for short-distance and 63x/0.9 NA for long-distance. A control experiment was performed using both objectives to assess the phase state statistics and revealed no significant differences depending on the objective resolution and the location of the vesicles in the chamber. GUVs were incubated for at least 2 minutes at each temperature before observation. In order to assess the fraction of homogenous

membranes y, between 20 and 40 vesicles were analyzed at each temperature and in total 3 independent batches were analyzed. The resulting data were fitted to the sigmoidal Boltzmann function:

$$y = \frac{A_1 - A_2}{1 + e^{T - T_{mix}/dT}} + A_2 \quad (1)$$

where y represents the fraction of uniform GUVs, A_1 the fraction at the lowest temperature, A_2 the fraction at the highest temperature, T the temperature, and T_{mix} the temperature at half-maximal y. As y is the fraction of homogenous GUVs, A_1 and A_2 were fixed to 0 and 1, respectively, corresponding to the experimental situation. The miscibility transition was assumed to be sigmoidal and therefore once the fraction value was measured to be 0 at a certain temperature, all fraction values below that temperature were assumed to be 0. Vice versa, once the fraction value reached 1 at a particular temperature, all fraction values above this temperature were assumed to be 1.

Fabrication of the microfluidic device

The multi-layered PDMS microfluidic device was fabricated as previously described (2). The upper layer serves as a control layer to hydraulically actuate circular valves, while the lower layer contains the GUV solutions. Briefly, PDMS oligomer and curing agent were mixed at a ratio of 10:1 and poured onto the silicon wafer master for the upper layer (feature height: 20 µm) to a final thickness of 5 mm. The same PDMS mixture was spin coated onto the silicon wafer for the lower layer (feature height: 20 µm) at 2000 rpm to a height of 40 µm. Both were then cured at 80 °C for 3 h. The upper layer was then cut to size and 1 mm holes were punched using a 1 mm Biopsy puncher (Biopsy puncher, Miltex, Plainsboro, New Jersey) for the hydraulic pressure. The upper layer section and the lower layer wafer (with PDMS) were then exposed to an air plasma (1 min, 0.5 mbar, PDC-002, Harrick Plasma, Brindley, Ithaca) to allow bonding and aligned under a microscope by hand. After 80 °C for 2 h, the two bonded PDMS layers were removed from the wafer and 1.5 mm fluidic access holes were punched. To complete the device and seal the fluidic channels, 170 ± 10 µm glass coverslips were bonded to the lower side using the same air plasma and left for 30 min at 60 °C.

Operation of the microfluidic device for complete fluid exchange

Using centrifugation (900 x g, 10 min) the upper and lower layers were filled with the same solution with which the GUVs were prepared. This ensured a bubble-free environment within the channel network. Delivery of GUV suspension and solutions from a reservoir attached to the microfluidic device was achieved using a syringe pump in withdrawal mode (neMESYS, cetoni, Korbußen, Germany). After loading the device with GUVs (5-10 µl/min, 15-30 min), the valves were closed (3 bar from a custom pressure controller) for at least 1 h prior to imaging to prevent fluidic shear and allow the GUVs to reach equilibrium. With the valve still closed, the GUV suspension in the reservoir was exchanged for the desired external vesicle solution and sucked into the device (20 µl/min, 5 min). The flow rate was subsequently reduced to 2 µl/min and the valves

were opened for 2 s. After the exchange, the valves were closed once more to allow the GUVs to reach equilibrium and imaging was performed after at least 1 h.

Phase-separated to homogenous membrane via complete external solution exchange

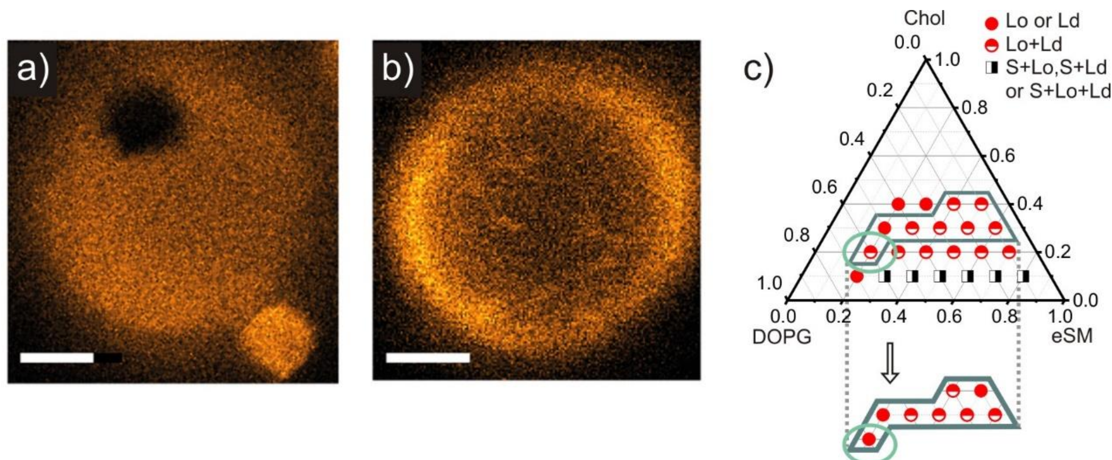

Figure S1. Confocal images of a single GUV (DOPG/eSM/Chol 60/20/20) captured within a microfluidic device. (a) Image of the vesicle with symmetric salt/salt (in/out) showing Lo/Ld phase coexistence. (b) Image of the vesicle after a full exchange of the external solution to salt/sucrose (in/out) when the vesicle exhibited a single-liquid state. Scale bars: 2 µm. (c) Phase diagram (as in Fig. 1C in the main text) for symmetric salt/salt conditions; the lower polygonal section is obtained for asymmetric salt/sucrose (in/out) conditions after twentyfold dilution with sucrose solution. The composition of the vesicle shown in (a, b) is encircled.

References of Supplementary Information

1. Dimova, R., S. Aranda, N. Bezlyepkina, V. Nikolov, K. A. Riske, and R. Lipowsky. 2006. A practical guide to giant vesicles. Probing the membrane nanoregime via optical microscopy. J. Phys.: Condens. Matter 18:S1151-S1176.
2. Robinson, T., P. Kuhn, K. Eyer, and P. S. Dittrich. 2013. Microfluidic trapping of giant unilamellar vesicles to study transport through a membrane pore. Biomicrofluidics 7.