Supporting Information for

Bilayer Properties of 1,3-Diamidophospholipids

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General Remarks

Starting compounds and solvents were purchased from Sigma-Aldrich/Fluka or Acros and were used without further purification. Pad-PC-Pad and its homologs were synthesized according to published protocols. [1]

Cryo-TEM (transmission electron microscopy) was carried out for $LUVET_{100}$ using a Tecnai F20 TEM (FEI, USA) at the Electron Microscopy Center, ETH Zurich.

Production of vesicles and loading with active compounds

Preparation of unloaded and CF-loaded LUVs

All compounds are purchased from Sigma-Aldrich or Avanti Polar Lipids and used without further purification unless otherwise stated. Liposome formulation is based on the techniques described by Olson.[2]

Large unilamellar vesicles by extrusion technique ($LUVET_{100}$) were prepared as follows: 3 μmol Pad-PC-Pad or Sad-PC-Sad were weighed into a 25 mL round bottomed flask and

dissolved in 1 mL chloroform. After evaporation to dryness, the film was dried for 12 h under high vacuum. 1 mL of buffer A (107 mM NaCl, 10 mM HEPES dissolved in ultra pure water, pH=7.4 (NaOH)) was added to the flask to hydrate the film for 30 min. The suspension was sequentially frozen (liquid nitrogen bath) and melted (water bath at 60 °C) five times, then extruded 11 times using a mini-extruder (Avanti Polar Lipids) and 100 nm filter-size (Whatman). Vesicles were stored at 5 °C in the dark until use. All experiments were conducted within 24 h of vesicle formulation.

For CF-loaded LUVs of Pad-PC-Pad, 1 mL of a 5 mM carboxyfluorescein solution (dissolved in buffer A) was used to hydrate the lipid film. After freeze-thawing and extrusion, CF-loaded LUVs were separated from bulk CF solution by size exclusion chromatography on a Sephadex G-50 column (7 cm x 1.5 cm).

The osmolarities of all solutions were measured with an osmometer (Fiske Micro-Osmometer Model 210) and adjusted to 220 mOsm/kg.

Measurement of CF fluorescence

A pH gradient from 7.4 (interior) to 3 (exterior) was established by adding 0.1 M HCl. The fluorescence intensity of 6CF was monitored as a function of time on a Hidex sense microplate reader at 492 nm (excitation) and 517 nm (emission) at 25 ºC.

DSC measurements

MLV suspension was prepared by hydration of 1 mg of phospholipid with 1 mL of milliQ water. Scans were performed with a TA Instruments NanoDSC by heating the sample to 60 °C, equilibrating for 30 min, followed by recording of the cooling curves

with cooling rates of 0.5, 1 and 2 °C/min.

Preparation of GUVs

1,3-Diamido phosphocholine (0.5 mg) was dissolved in 1 mL $CHCl_3$. To this, 10 μL of Liss-Rho-DPPE solution (0.25 mg of lipid in 1 mL $CHCl_3$) was added.

ITO-coated glass plates were washed with water and EtOH, and then with $CHCl_3$. 20 μL of the lipid solution was deposited on the conductive face of the plate. The $CHCl_3$ was evaporated for 1 hour at RT under reduced pressure. A second ITO slide was put on top of the first using Vitrex as sealant. The void was filled with iso-osmolar sucrose solution (224 mOs). Then the vesicles were grown for 2 h at 55 °C at 1 V and 10 Hz.

An "observation chamber" was made from 2 glass microscopy slides that were washed with water and EtOH. A square made of parafilm was laid out between the two plates and the parafilm was melted at 100 °C. The chamber was washed with casein solution (2 mg/mL) and left drying for 15 min. The solution was then replaced with HEPES buffer of the same osmolarity (200 mOs) as the sucrose solution. 10 μL of the lipid solution were added and the chamber was sealed with varnish.

GUVs were also prepared by gentle hydration methods according to published protocols using agarose[3] or PVA gels[3].

Micrographs were recorded on a Zeiss 510 META confocal microscope at RT.

SAXS measurements

A lipid dispersion (MLV, 20 wt% in MilliQ water) was transferred into glass capillaries (inner diameter 2 mm, GLAS, Germany). The small angle X-ray scattering (SAXS) was carried out at the in-house pinhole Instrument with rotating anode. The diffracted signal has been measured with a Mar CCD plate detector (Evanston, Illinois, USA). The incoming beam had a wavelength of 154 pm, and the exposure time was 2 h. The temperature was fixed at 21 °C and 40 °C during measurements. Positions of the Bragg peaks were converted into real space repeat distances of the lattice planes. In order to determine peak maxima and the full-width at half-maximum (FWHM) Lorentzian curves have been fitted to the experimental points. Correlation lengths ξ were calculated using equation 1 of the first order diffraction peak.

$$\xi = (0.88 \cdot 2 \cdot \pi)/\Delta Q \qquad (1)$$

References

1. Fedotenko, L. A.; Stefaniu, C.; Brezesinski, G.; Zumbuehl, A., Monolayer properties of 1,3-diamidophospholipids. *Langmuir* **2013,** *29* (30), 9428-35.
2. Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D., Preparation of Liposomes of Defined Size Distribution by Extrusion through Polycarbonate Membranes. *Biochim. Biophys. Acta.* **1979,** *557* (1), 9-23.
3. Horger, K. S.; Estes, D. J.; Capone, R.; Mayer, M., Films of agarose enable rapid formation of giant liposomes in solutions of physiologic ionic strength. *J. Am. Chem. Soc.* **2009,** *131* (5), 1810-9.