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A membrane fusion assay based on pore-spanning lipid bilayers

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Materials. All lipids POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt)), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), DPhPC (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine) and DPPTE (1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (sodium salt)) were purchased from Avanti Polar Lipids (Alabastar, AL, USA). Texas Red DHPE (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) was obtained from Sigma-Aldrich (Taufkirchen, Germany) and Oregon Green DHPE (Oregon Green 488 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) was purchased from Molecular Probes (Eugene, OR, USA). Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt) was obtained from Acros Organics (Geel, Belgium). The water used was ion exchanged and millipore-filtered (Milli-Q-System, Millipore, Molsheim, France; specific resistance $R > 18\text{M}\Omega\ \text{cm}^{-1}$, pH 5.5). Porous silicon substrates were received from fluXXion B.V. (Eindhoven, NL).

Preparation of micro-BLMs. Porous silicon substrates with pore diameters of 1.2 µm were coated with a thin titanium layer (2.5 nm) and a gold layer (60 nm) using a sputter coater with a thickness control unit (Cressington sputter coater 108auto, Cressington MTM-20, Elektronen-Optik-Service, Dortmund, Germany). The surface was rendered hydrophobic by chemisorption of DPPTE. To achieve this, the substrate was incubated with a 0.5 mM ethanolic solution of DPPTE at 8° C over night. Afterwards, the substrates were rinsed with ethanol and mounted in a Teflon chamber filled with Ca^{2+}-containing buffer (10 mM HEPES, 100 mM KCl, 20 mM $CaCl_2$, pH 7.4). Micro-BLMs were formed by the application of a small drop of lipids dissolved in organic solvent. In detail, a mixture of DPhPC and POPE (2:1) was dissolved in *n*-decane at a concentration of 2 % (*w/v*) and doped with either 1 or 2 mol% Oregon Green DHPE. The lipid-solvent mixture was applied to the porous silicon substrate surface with a paint brush. During the thinning process micro-BLMs are formed.

Vesicle preparation. Large unilamellar vesicles were prepared by the extrusion method. Lipid films composed of POPC/POPS/Texas Red DHPE (79/20/1 or 78/20/2) were prepared from chloroform solution in a glass test tube. The organic solvent was evaporated under a stream of nitrogen and the lipid films were dried in vacuum for at least 3 hours. For vesicle preparation, the lipids were dissolved in sucrose-buffer (10 mM MES, 200 mM sucrose, 100 mM KCl, pH 6.5). After 20 min incubation, the solution was vortexed three times for 30 s every 5 min. The suspension of multilamellar vesicles was extruded 31 times through a polycarbonate membrane with pores of 1000 nm in diameter. In case of content release experiments 2.5 mM pyranine was added to the sucrose-buffer. Unencapsulated dye was removed by gel filtration

of the extruded liposomes using a Sephadex G-25 column (GE Healthcare, Buckinghamshire, UK).

Confocal laser scanning microscopy. An upright confocal laser scanning microscope (Zeiss LSM 710, Zeiss, Jena, Germany) equipped with a water immersion objective with 63× magnification (Zeiss, W Plan-Apochromat, N.A. = 1.0) was used for fluorescence imaging. In case of experiments without pyranine, a 488 nm Ar-laser was used to excite the fluorescent dyes. Emission light was spectrally separated and collected by photo multiplier tubes for Oregon Green in the range of 500-560 nm and for Texas Red at 600-680 nm. In the presence of pyranine, the sample was simultaneously excited with three different lasers with emission at 405 nm (diode), 488 nm (Ar^+) and 561 nm (DPSS). Emission was collected at the following wavelengths: pyranine: 430-510 nm, Oregon Green 515-554 nm, Texas Red 660-750 nm. Data acquisition started immediately after the addition of 0.5-2 µL vesicle suspension to the micro-BLMs. Images of 256 × 128 pixels related to an area of 86 × 43 µm² were recorded with a time resolution of 115-156 ms.

Scanning ion conductance microscopy. Topographic information of the micro-BLMs before and after fusion was obtained by the SICM technique. The principle of imaging pore-spanning lipid bilayers by SICM was described previously (Böcker, M. et al. *Langmuir*, 2009, **25**, 3022). SICM measurements were performed using a commercially available SICM instrument (ICnano, Ionscope, London, UK). Nanopipettes with typical inner diameters of 100 nm were made from glass capillaries (borosilicate glass, inner diameter 0.58 mm, outer diameter 1.0 mm, with filament, Science Products, Hofheim, Germany) using a Brown-flaming electrode puller (P-97, Sutter Instruments, Novato, CA, USA). The pipette mounted on a three-axis piezo translation stage was filled with Ca^{2+}-containing buffer and placed into the buffer filled Teflon chamber in which the micro-BLMs were prepared. A constant voltage of 100-150 mV was applied between two Ag/AgCl-electrodes, of which one is located inside the nanopipette, while the other one is placed into the electrolyte bath. The resulting ion current through the pipette was measured and was used as input to a feedback loop, controlling the *z*-scanner. The measurements were performed in hopping mode (Novak, P. et al. *Nature Methods,* 2009, **6**, 279). The total resistance was typically in the range of 150−500 MΩ. After approaching the surface, the nanopipette was scanned over the surface of the sample in *x*- and *y*-direction. The lateral and vertical positions of the nanopipette were measured producing topographic images. Several SICM-images were recorded on different positions of the substrate. Then, 2 µL of LUV suspension were added to the sample and after 20 min of incubation, the substrate was rinsed with Ca^{2+}-containing buffer to remove the excess of vesicles. Afterwards, the sample was scanned again on different positions of the substrate. To make sure that the incubation with sucrose buffer, in which the LUVs were prepared or rinsing with Ca^{2+}-containing buffer does not affect the pore depths, control experiments were performed. Therefore, micro-BLMs were incubated with sucrose-containing buffer and rinsed with Ca^{2+}-containing buffer. Several SICM-images were recorded before and after this procedure. For determination of the height differences, data were processed using the Gwyddion Software.