

Aqueous-filled microcavity spanning bilayers: versatile platforms offering enhanced stability and high lateral mobility to incorporated membrane protein

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Supplementary Methods:

Fabrication of microcavity PDMS substrates

Microcavity arrays were fabricated in polydimethylsiloxane using polystyrene sphere templating according to a method modified from one recently described by us.ⁱ

The polystyrene sphere template was assembled on cleaned mica by depositing 100 μ L of a 0.1% w/v dispersion of microspheres of the appropriate size in milliQ water onto a piece of mica glued to a microscope slide. This was then left to evaporate overnight to obtain a dry film of spheres on mica.

Using a two part Sylgard 184 Silicone Elastomer Kit obtained from Dow Corning, a 10:1 ratio of the elastomer to curing agent was prepared. This mixture was then degassed under vacuum for about 30 minutes and then poured over the dried film of polystyrene spheres on mica. The elastomer mixture was then cured at 150 °C for 20 minutes following which time the cured PDMS formed over the polystyrene spheres was allowed to cool. The PDMS was then peeled off the supporting mica/glass slide, and the polystyrene spheres were removed from the PDMS by immersing the substrate in THF for 10 minutes. This microcavity bearing PDMS substrate was left overnight in the fume hood to allow evaporation of any remaining THF from its pores.

This fabrication method produced porous arrays in which the diameter of the pore was approximately 35% narrower than the diameter of the polystyrene particle template, see Figure S1.

Under confocal microscopy, the aqueous filled pores appeared wider and a good match to the PS sphere template diameter in white light images, as the PDMS is transparent, reflected light is seen across the entire width of the cavity in such images. This observation confirms that the cavities, height exceeds the particle equator, (i.e. rather than being shallower than it).

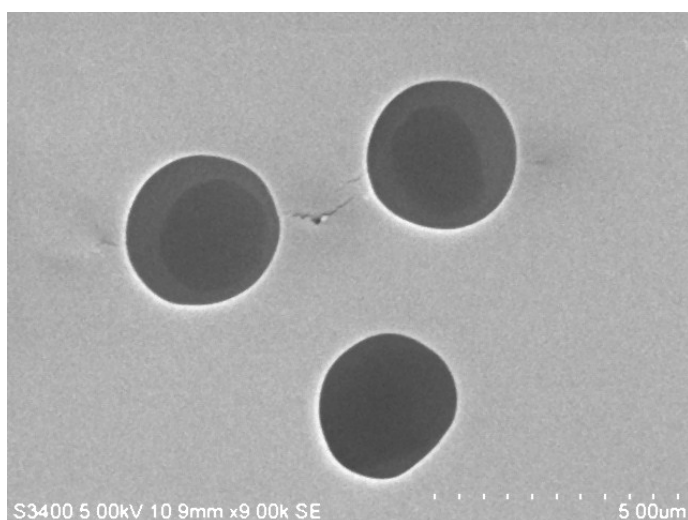


Figure S1: SEM image of spherical pore in PDMS produced by the templating method from 2.94 μm diameter polystyrene spheres. The pore size at approximately 2 μm indicates that the pore depth was greater half the height of the PS sphere, i.e. approximately 1.5 μm .

Plasma treatment and buffer filling of microcavities

The microcavity PDMS substrates were rendered hydrophilic by air-plasma treatment at an air pressure of 1000 mT for 5 minutes using Harrick PDC-002 plasma cleaner. Immediately after plasma treatment the substrates were sonicated for 30-40 minutes in 10 mM Tris buffer containing 150 mM NaCl at pH 7.4 to prevent the hydrophobic recovery of PDMS. The sonication step ensured the filling of most of the microcavities by the buffer.

Figure S2 shows the contact angle measurements for planar PDMS treated as described. Contact angle is reduced from 92° in untreated PDMS to 20° in the plasma treated substrate. The contact angle for the planar substrate is shown here because for the porous substrate the contact angle drops to zero due to aqueous filling of the pores.

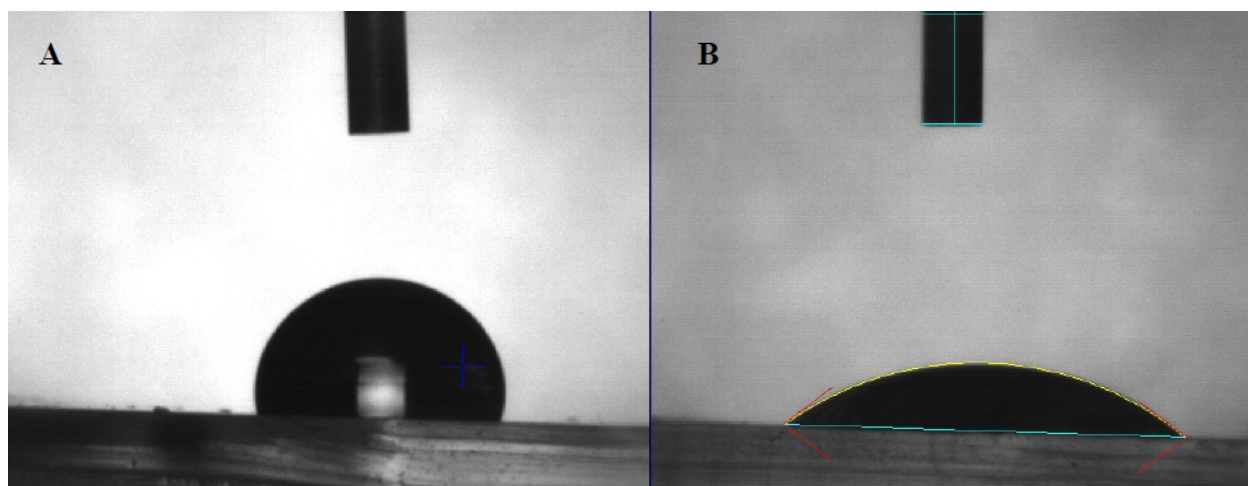


Figure S2: Water contact angle measurements for planar plasma treated PDMS left and following plasma treatment.

Spanning of the microcavities by the Lipid bilayer and fabrication of the flow chamber

Bilayers were spanned onto the microcavity substrates using a combination of Langmuir-Blodgett (LB) and vesicle fusion techniques. A monolayer of DOPC was formed onto the air-water interface of a NIMA 120m Langmuir Trough by spreading 100 μL of a chloroform solution of the appropriate lipids at 50 mg/mL. For confocal measurements DOPE-Carboxyfluorescein at a concentration of 1 mol% was included as a lipid probe whereas for FLIM and FLCS measurements DOPE-ATTO 532 at a concentration of 0.001mol % was included as the probe. DOPE was used here as the probe lipid as it is available commercially. At the low loadings it was used at here it would not be expected to influence the phase of the bilayer. The chloroform was allowed to evaporate for 20 minutes after which the lipids were compressed at a barrier speed of 30 cm/min to a surface pressure of 30-32 mN/m. Surface

pressure was measured using a paper Wilhelmy plate. Holding the surface pressure constant, the substrate was rapidly lowered into the dipping well at a dipper speed of 67 mm/min and slowly withdrawn at a dipper speed of 5 mm/min to allow the transfer of a monolayer onto the surface. Once the monolayer was formed, two holes were punched at the extremities of the PDMS and at this stage the substrate was glued to a cover glass slide using epoxy glue to obtain the flow chamber.

Teflon tubing was attached to the two holes to allow the circulation of solution into the flow chamber. To obtain Small Unilamellar Vesicles (SUVs), 20 μ l of DOPC or DOPC/DOPS in chloroform containing the appropriate dye was evaporated under N₂ to yield a lipid film on the inner wall of a glass vial. The vial was then placed in vacuum for at least 2 hours to fully remove the last traces of solvent after which the lipids were hydrated in buffer and vortexed for 5 minutes. This solution was then extruded over a 100 nm polycarbonate membrane obtained from Avanti Polar Lipids to obtain vesicles of mean hydrodynamic radius of 100 nm as measured by DLS. SUVs were then injected into the flow chamber and allowed to remain in contact with the monolayer for about 15-20 minutes to allow their fusion to obtain a bilayer, after which the chamber was rinsed with the buffer.

Labeling of Glycophorin A with TAMRA

Glycophorin A from blood type B negative obtained from Sigma-Aldrich was solubilized in HEPES buffer at pH 8.0 at a concentration of 0.5 mg/ml, TAMRA-NHS (5-Carboxy-tetramethylrhodamine N-succinimidyl ester) obtained from Sigma-Aldrich was solubilized in DMF at a concentration of 2 mg/ml. This dye solution was then added to the Glycophorin solution at 5-fold molar excess and allowed to incubate at room temperature for 2 hours. After this time, the unbound dye was separated from the TAMRA-conjugated glycophorin by

size-exclusion chromatography using a Sephadex G-25 column. The eluted labeled protein was concentrated using an Amicon-3000 filter.

Incorporation of Glycophorin A into vesicles:

Glycophorin A (GpA) was incorporated in lipid vesicles by the method of MacDonald and MacDonald.ⁱⁱ To 20 μ l of DOPC at 50 mg/ml in chloroform/methanol (2/1), containing DOPE-Atto655 at a 1:100,000 dye: lipid ratio, 2 μ l of labeled Glycophorin A at a concentration of 0.5 mg/ml in 10 mM HEPES buffer was added. This mixture was evaporated to form a dry lipid-protein film. The dried glycoprotein containing lipid film was then kept in vacuum for an hour to ensure removal of traces of the solvent, following which it was hydrated in the working Tris buffer at pH 7.4 and gently sonicated for 5 minutes. This mixture was then extruded over 100 nm polycarbonate membranes to obtain GpA containing vesicles.

Integrin labeling

The manufacturer provided the integrin in a buffer solution (pH 7.4) containing 20 mM Tris, 100 mM NaCl, 1 mM CaCl_2 , 0.05% NaN_3 , 0.1% Triton X-100, and 50% glycerol. To preclude a chemical reaction between Tris and the dye NHS ester, the foregoing buffer was exchanged for an HEPES buffer solution (pH 8). Such exchange was performed via a 48 hour dialysis against 1×10^3 times the original buffer volume. After dialysis, ATTO 655 NHS ester was dissolved in DMF to obtain a concentration of 2 mg/ml. This solution was incubated with the integrin solution for 3 h under stirring at room temperature; a five-fold excess of dye was utilized. Afterwards, unbound dye molecules were eliminated by size exclusion chromatography using a Sephadex G-25 column.

Detergent mediated insertion of integrin

The lipids were completely solubilized in a HEPES reconstitution buffer (10 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, 0.1% Triton X-100, pH 7.4) with a final detergent: lipid ratio of 2:1 (w/w). The final lipid concentration was normally 1 mM. α IIb β 3 in HEPES reconstitution buffer was then added to the detergent-lipid solution at the required concentration, normally 1 integrin molecule for every 8000 lipid molecules. The protein containing solution was shaken at room temperature for 30 minutes to fully ensure that all lipid has been solubilized and that the solution was homologous. The solution was then incubated at 37 °C for 1 hour 30 minutes. Detergent was removed by the addition of 100 mg of biobeads per ml of solution for 3 hours 30 minutes in a slow shaken vial. The biobeads were then removed and replaced by a fresh batch of 100 mg biobeads per ml of solution for 30 minutes (slowly shaken). Once complete, biobeads were removed and the reconstituted proteo-liposomes were stored at 4°C.

Ultra-centrifugation

Proteo-liposomes in a HEPES buffer were loaded into an ultracentrifuge tube and centrifuged at 100,000 g for 1 hour at 4 °C in order to pellet the vesicles. The pellet was then gently washed with deionized H₂O before being re-suspended in deionized H₂O at a lipid concentration of 10 mM.

Liposome Electro-formation

The lipid was then pipetted in 2 μ l droplets on the conductive side of an indium tin oxide (ITO) glass slide. To partially dehydrate the lipid droplets, the slide was placed in a vacuum desiccator in the presence of a saturated sodium chloride solution for at least 2 hours. After partial dehydration, the electroformation cell was assembled and filled with electroformation buffer (10 mM HEPES, 150 mM NaCl, 1 mM CaCl₂, 100 mM sucrose, pH 7.4). For electroformation, an AC electric field provided by a pulse generator was applied overnight

across the chamber and was linearly increased to 1 V over the first hour before being held constant. The frequency was kept at 500 Hz throughout.

GUV disruption

The GUV containing solution was diluted 1:1 in disruption buffer (10 mM HEPES, 150 mM NaCl, 30 mM CaCl₂, 100 mM glucose, pH 7.4) and immediately injected into the preassembled cavity array (lipid monolayer already transferred via LB method). The GUVs were incubated at room temperature for 1 hour before the cell was flushed with HEPES buffer. This process yields a lipid monolayer with integrin properly inserted within.

Confocal and FCS measurements:

Confocal imaging were done on a LSM 50, Zeiss confocal fluorescence microscope using a 64× oil immersion objective (NA = 1.4), and 488 nm excitation from an argon ion laser. The collected signal was separated using a 505 nm long-pass filter, yielding reflectance ($\lambda < 505$ nm) and fluorescence images ($\lambda > 505$ nm).

Fluorescence Lifetime Correlation Spectroscopy (FLCS) and Fluorescence Lifetime Imaging (FLIM) measurements were performed using a MicroTime 200 fluorescence lifetime microscope system (PicoQuant GmbH, Germany). The time-tagged time-resolved methodology was employed. The excitation sources used were 532-nm PicoTA (PicoQuant GmbH, Berlin, Germany) and 640-nm LDH-P-C-640B diode lasers (PicoQuant GmbH, Berlin, Germany) operating at a 10 MHz repetition rate. Their laser beams were directed towards a z532/635rpc dichroic mirror and focused on a water immersion objective (UPlanSApo 60x, NA 1.2 water/CC1.48, Olympus). The sample fluorescence was collected through the same objective and filtered by the aforementioned dichroic mirror, as well as by an HQ550LP AHF/Chroma filter (Olching, Germany) for the 532-nm laser, or a BLP01-

635R-25 Semrock filter (New York, USA) for the 640-nm laser. Finally, the sample fluorescence passed through a 50- μm pinhole and reached a MPD SPAD detector (PicoQuant GmbH, Berlin, Germany). To precisely locate the cavities, reflectance images were recorded by replacing the long-pass filter (either HQ550 LP AHF/Chroma or BLP01-635R-25 Semrock filters) by an OD3 neutral density filter (Chroma, Olching, Germany). The autocorrelation functions were obtained using Fluorescence Lifetime Correlation Spectroscopy (FLCS), which eliminates both detector after-pulsing and contributions arising from spurious signals.

Intensity-time curves:

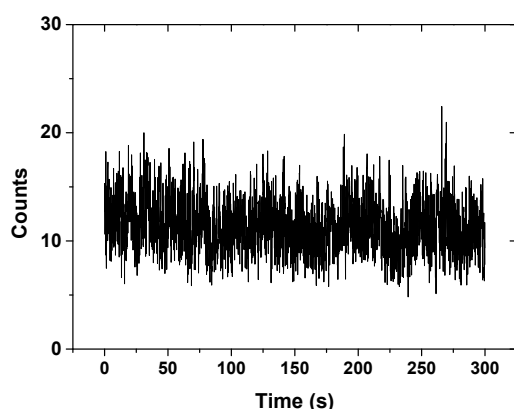


Figure S3: Fluorescence intensity-time curve measured over a cavity for TAMRA labelled Glycophorin A in a DOPC bilayer. Cavities were formed on PDMS by templating 2.94 μm spheres as described above. The excitation wavelength is 532 nm and data was recorded over 300s. The stable intensity over the measurement time indicates that GpA is mobile in the bilayer over a cavity.

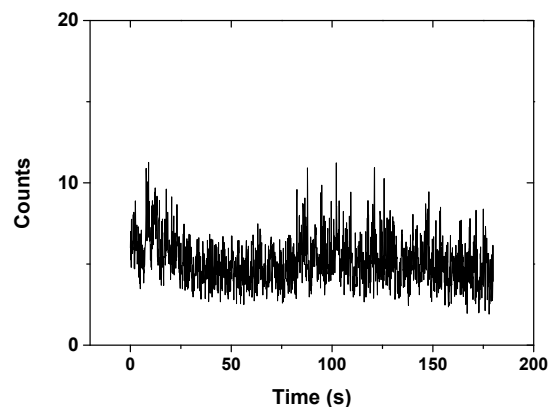


Figure S4: Fluorescence intensity-time curve for a point measurement performed on ATTO655 labelled α IIb β 3 integrin suspended in a DOPC bilayer above a cavity. The excitation wavelength is 532 nm and the data was recorded over 180s. The stable fluorescence signal over the measurement time confirms that the integrin is mobile in the bilayer over a cavity.

References:

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- ⁱ C. Mallon, R.J. Forster, T.E. Keyes. Fabrication of Gold Sphere to Cuboid Nanoarrays using PDMS Templates *Chem. Commun.* 2011, **47**, 7605 - 7607
 - ⁱⁱ McDonald, R. I., and R. C. McDonald. Assembly of phospholipid vesicles bearing sialoglycoprotein from erythrocyte membrane *J. Biol. Chem.*, 1975, **250**, 9206-9214