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Microcavity array supported lipid bilayer models of ganglioside - influenza hemagglutinin$_1$ binding

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Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), porcine brain N-(octadecanoyl)-sphing-4-enine-1-phosphocholine (SM), cholesterol (Chol), ganglioside GM1, GM3 and GD1a were purchased with maximum degree of purity (> 99%) from Avanti Polar Lipids (Alabama, USA) and used without further purification. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine labelled Atto655 (DOPE-ATTO655), sphingomyelin-ATTO647n (SM-ATTO647n) and 532-ATTO-NHS-ester (ATTO532) were purchase from ATTO-TEC GmbH (Siegen, Germany). Hemagglutinin HA_1 sub-unit (Recombinant Influenza A virus proteins, subtype H3N2, A/Aichi/2/1968, His Tag) was purchased from Invitrogen ThermoFisher. Aqueous solutions were prepared using Milli-Q water (Millipore Corp., Bedford, USA). Polydimethylsiloxane silicon elastomer (PDMS) was purchased from Dow Corning GmbH (Wiesbaden, Germany) and mixed following supplier instructions. Silicon wafers coated with a 100 nm layer of gold on a 50 Å layer of titanium were obtained from AMS Biotechnology Inc. The monodisperse polystyrene latex sphere with a diameter of 1 µm was obtained from Bangs Laboratories Inc. The commercial cyanide free gold plating solution (TG-25 RTU) was obtained from Technic Inc. All other HPLC grade reagents were obtained from Sigma-Aldrich and used as obtained.

Liposome preparation

Briefly, in this work, liposome fusion was used to form the distal lipid leaflet of microcavity supported lipid bilayers (MSLBs). To prepare the liposomes, stock solutions of all vesicle components such as DOPC, brain sphingomyelin and cholesterol 50 mg/mL each, and GM1 (1 mg/mL), GM3 (1 mg/mL) and GD11 (1 mg/mL) were prepared in chloroform and stored in sealed glass vials at -20°C. Fluorescence labelled DOPE-ATTO655 was mixed in a ratio of 50000:1 mol/mol with unlabelled lipids for fluorescence correlation spectroscopy (FLCS) studies. For electrochemical measurements, as electrochemical impedance spectroscopy (EIS) is label-free, fluorescent probe was not included during the preparation of liposomes. Aliquots of the appropriate amounts of the stock solutions were mixed in clean amber glass vials and dried under a gentle stream of nitrogen and placed under vacuum for 1 h. The lipids were rehydrated in 1 mL of PBS buffer (pH 7.4) and vortexed vigorously for at least 60 s.

Large unilamellar vesicles (LUVs) were prepared by extruding the multilamellar vesicle suspension 11x against a polycarbonate membrane (0.1 µm pore size) using a mini-extruder (Avanti Polar Lipids). The LUVs were diluted to 0.25 mg/mL. Liposomes composed of SM were extruded at 45°C, above the SM transition temperature, to guarantee that vesicles are in the fluidic state. Figure S1 shows the size distribution of the liposomes obtained by dynamic light scattering (DLS) before (Figure S1a) and after extrusion (Figure S1b).

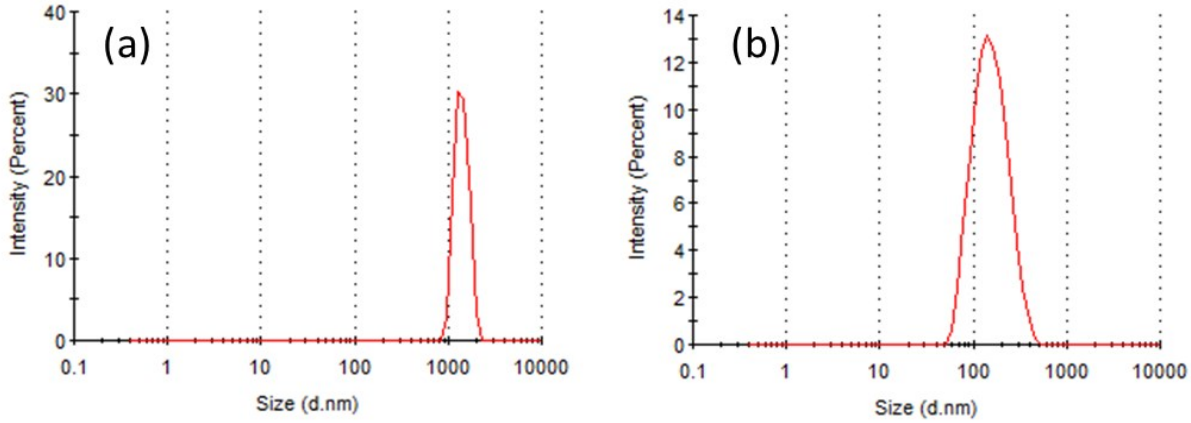

Figure S1 – Size distribution of liposomes obtained by DLS (a) before and (b) after extrusion against polycarbonate membranes of 0.1 µm diameter pore size. The average size of liposomes after extrusion was 120 nm with polydispersity index (PDI) of approximately 0.1.

Fluorescent labelling of HA_1

For FLCS measurements, HA_1 was labelled with ATTO532 NHS-ester (ATTO-Tec GmbH) following the method suggested by the seller. The conjugation was performed by reacting to the succinimidyl ester moiety of ATTO dye with terminal amines in the protein at basic pH. To avoid ester hydrolysis and therefore inactivation of the dye, ATTO532 NHS-ester was diluted in anhydrous DMSO (HPLC grade). Briefly, HA_1 was diluted in PBS buffer (pH 8.3) to the final concentration of 0.2 mg/mL. ATTO532 ATTO532 NHS-ester was added to 400 µL of HA_1 in a molar ratio of 3:1 of dye to protein to ensure adequate reaction with protein. The reaction was conducted under gentle agitation for 1 h at room temperature in the dark. The labelled protein was separated from unreacted dye by dialysis in a centrifugal filter membrane (AMICON 10 kDa) at 10000 RPM for 15 min. The process was repeated 6 times by the addition of 1 mL of PBS buffer pH 7.4 each time. The labelling of HA_1 was determined by UV-VIS spectroscopy (Figure S2) and by the lateral diffusion of the protein in solution measured by FLCS by fitting the autocorrelation functions (ACFs).

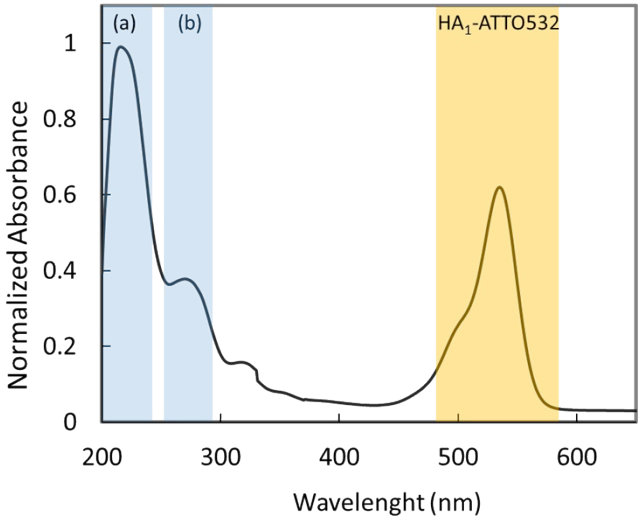

Figure S2 – Normalised Absorbance spectrum of labelled HA_1 after reaction with ATTO532-NHS-ester. The insertions (a) and (b) indicates the maximum absorbance of trypsin and tyrosine with the probe maxima marked in yellow.[1,2]

Electrochemical Impedance Spectroscopy

Electrochemical impedance measurements were performed on a CHI 760e bipotentiostat (CH Instruments Inc., Austin, TX) in a three-electrode cell consisting of a Ag/AgCl (1 M KCl) reference electrode, platinum coiled wire as a counter electrode and the gold substrate with microcavity array served as the working electrode. 1 mM $[Fe(CN)_6]^{3-/4-}$ was used as an internal redox probe in 0.1 M KCl electrolyte solution in PBS buffer. The EIS recording was performed in the frequency region between 10^4 and 0.01 Hz with a bias potential of 0.26 V vs Ag/AgCl. The impedance spectra were fit to an equivalent circuit model (ECM) as described previously [3] and the same has been shown in Figure 1a (inset) main text. In the circuit, R_S is the solution resistance, R_M and CPE_M represents the resistance and constant phase element of the membrane and R_C and CPE_C represents the cavity resistance and constant phase element of the gold substrate. The constant phase elements (CPE) were used in the equivalent circuit to provide to account for the heterogeneity of the SLBs in microcavity array. The quality of fit was assessed from χ^2 values and visual inspection. The impedance of the CPE can be calculated using Eq. (S1);

$$Z_{CPE} = \frac{1}{Q(j\omega)^{\beta}} \tag{S1}$$

where Q and the exponent β represent respectively the CPE and an empirical constant related to the frequency dispersion. When β close to 1, it behaves like an ideal capacitor, and when $\beta < 1$, the

capacitance depends on the frequency. From our fitting using the ECM model, we obtained a β value in the range of 0.93-0.96. Note the membrane capacitance values are reported are not a true capacitance value rather it is CPE (Q) values which do not depend on any particular frequency unlike a true capacitance value (which is frequency dependent). Note, the relative change in resistance and capacitance values are not normalized with respect to electrode surface area.

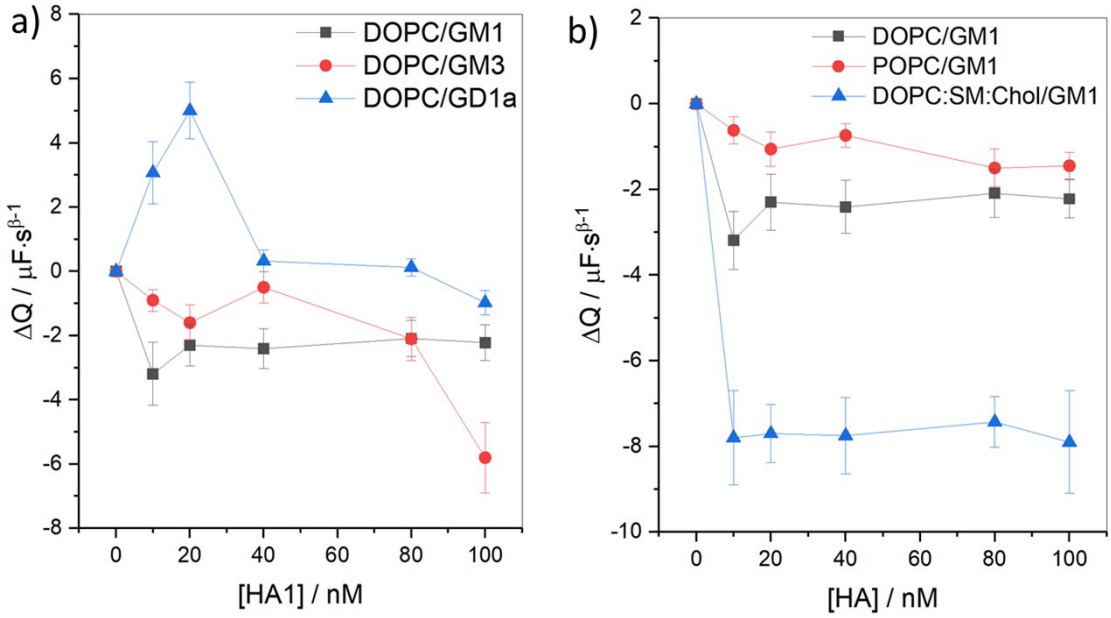

Figure S3 – a) Representative relative change in capacitance of DOPC membranes with varied embedded receptors upon HA_1 interaction. b) relative change in capacitance of different membrane compositions with GM1 receptor upon HA_1 interaction.

Hill-Waud binding model:

The relative change in membrane resistance from EIS data for different lipid compositions versus HA_1 binding concentrations (see Fig. 2a, main text) were fit iteratively to the Hill-Waud expression, as defined by equation (S2) and the extracted fit data are shown in Table S1.

$$\Delta R = \frac{\Delta R_{sat}(C)^n}{(K_D)^n + (C)^n} \qquad \text{(S2)}$$

where ΔR is the change in membrane resistance, defined by $R_M^0 - R_M^{HA1}$, ΔR_{sat} is absorption capacity or change in resistance at maximum surface loading that relates to the number of available binding sites, K_D is the empirical apparent equilibrium dissociation constant, C is the concentration of the HA_1 and n (dimensionless) is the Hill coefficient of cooperativity.[4] The "n" constant in the Hill-Waud model

(Eq. S2) relates to the strength of the adsorption or often relates to cooperative effect.[5] n<1 indicates chemically mediated absorption i.e., multiple binding sites with a different affinity (negative cooperativity), n =1 reverts the expression to Langmuir isotherm, where all binding sites are equal, i.e. non cooperative equilibrium binding, and n > 1 indicates positive cooperativity.

Table S1. Data obtained for HA_1 by fitting relative change in resistance (ΔR) of different lipid compositions to Hill-Waud model.

membrane composition	K_D **(nM)**	ΔR_{sat} **(MΩ)**	***n***	R^2
DOPC/GM1	41.3±9	2.11±0.27	1.81	0.97
DOPC/GM3	23.96±4	0.25±0.02	1.45	0.98
DOPC/GD1	17.47±2	1.66±0.08	2.56	0.97

Binding of HA_1 to MSLBs in absence of GSLs

Control experiments were carried out to bare lipid membranes comprised of DOPC and DOPC/SM/Chol (4:4:2) without GSLs. The results obtained have not shown non-specific binding of HA_1 to MSLBs in absence of GSLs as the membrane resistivity remains constant after exposing the lipid bilayers to HA_1 for 30 min. Figure S4 shows the results obtained for bare DOPC bilayer (Figure S4a) and to the complex lipid bilayer (Figure S4b).

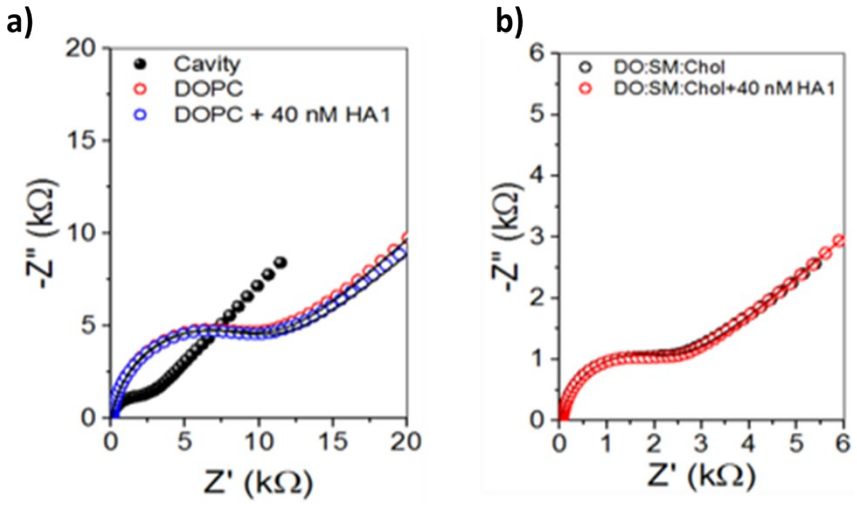

Figure S4 – Electrochemical Impedance Spectroscopy of control experiments without GSLs. (a) Symmetric DOPC lipid bilayers and (b) symmetric lipid bilayers comprised of DOPC/SM/Chol (4:4:2). Non-specific binding was not observed after reacting to the lipid bilayers with HA_1 (40 nM) for 30 min.

Fluorescence Lifetime Correlation Spectroscopy

FLCS measurements were performed at PDMS-MSLBs (Figure S5) platform where the labelled phospholipid DOPE-ATTO655 was introduced to the distal leaflet during the liposome fusion. The conjugated HA1-ATTO532 was inserted in the microfluidic device by the tubing port connection and was left to equilibrate for 1 hour. After, the microfluidic chamber was rinsed with 1 mL of PBS buffer in order to remove any unreacted protein. The autocorrelated data obtained for labelled DOPE-ATTO655 and bound HA1-ATTO532 were fitted to 2D diffusion model (Eq. S3).

$$G(\tau) = \frac{1}{N}\left[1 + \left[\frac{\tau}{\tau_D}\right]^{\alpha}\right]^{-1} \quad \text{(S3)}$$

The lateral diffusion of labelled HA1-ATTO532 and the free ATTO532, e.g. unbound dye in PBS buffer, were calculated by fitting the ACFs obtained in for 10 nM solution of each molecule to a 3D model (Eq. S4). The Equation S4 includes a κ term, which defines the shape of the confocal volume.

$$G(\tau) = \frac{1}{N}\left[1 + \left[\frac{\tau}{\tau_D}\right]^{\alpha}\right]^{-1}\left[1 + \left[\frac{\tau}{\tau_D}\right]^{\alpha}\frac{1}{\kappa^2}\right]^{-1/2} \quad \text{(S4)}$$

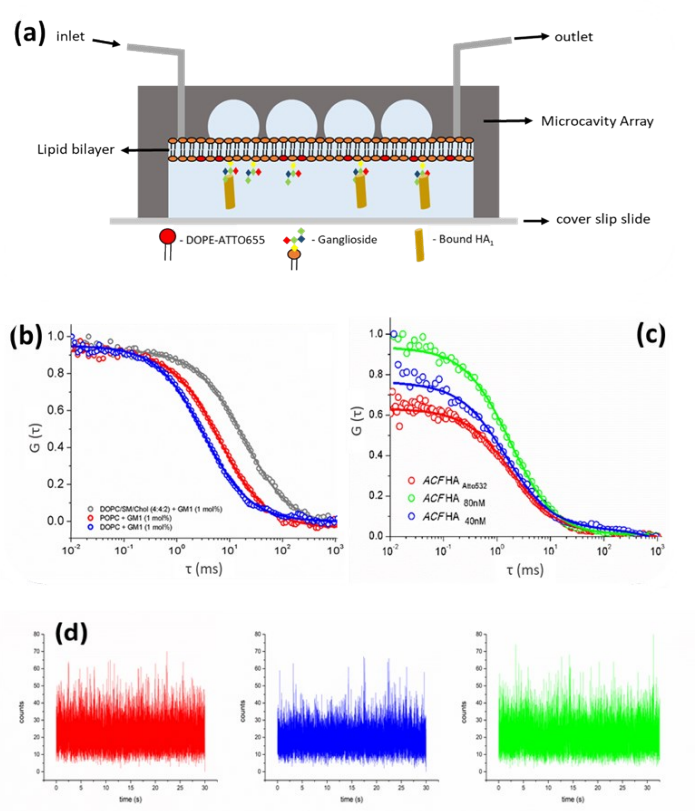

Figure S5 – (a) Schematic representation of bound HA_1 to gangliosides at MSLBs. (b) Representative autocorrelation functions (ACFs) obtained for labelled DOPE-ATTO655 incorporated into DOPC (blue circles), POPC (red circles) and DOPC/SM/Chol (4:4:2) (grey circles) MSLBs doped with GM1 (1 mol%). (c) Representative fitted ACFs of bound HA_1 to DOPC lipid bilayer doped with GD1a (1 mol%) at different HA_1 concentrations, indicating the absence of protein aggregation. (d) Intensity time-trace obtained at different concentrations of HA_1-ATTO532 (10 nM (red), 40 nM (blue), 80 nM (green)) to DOPC bilayer containing GM3 (1 mol%).

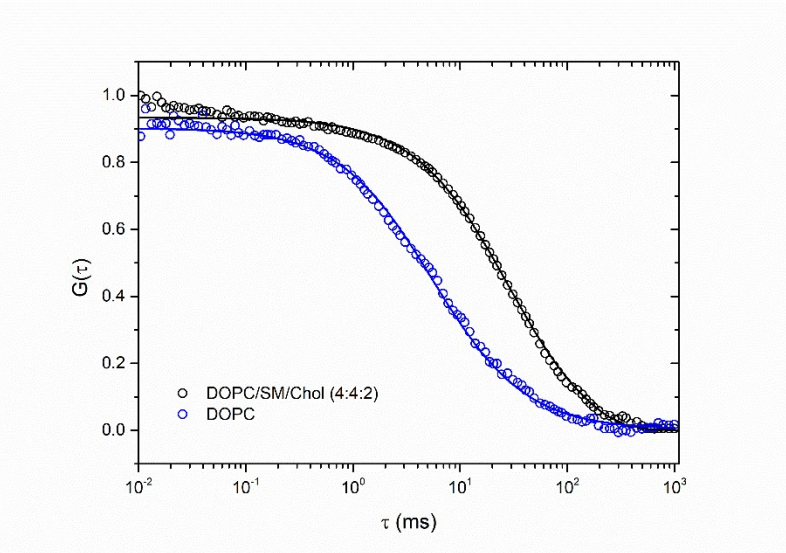

Figure S6 – FLCS of labelled SM-ATTO647n obtained from DOPC (blue circles) and DOPC/SM/Chol (4:4:2) (black circles) MSLBs.

Saffman-Delbrück (SD) model:

Due to the suspended character of planar MSLBs, where both leaflets are in contact with bulk aqueous phase, we assume the Saffman-Delbrück model (Equation S5) applies and this was used to estimate the diameter of the membrane associated complex.

$$D = \frac{KT}{4\pi\mu h}\left(\ln\left(\frac{\mu h}{\mu' r}\right) - \gamma\right) \qquad \text{(S5)}$$

Where D is the lateral diffusion obtained from FLCS, K is Boltzmann constant, T is the absolute temperature, r is the radius of the cylindrical membrane inclusion, h is the membrane thickness, γ is Euler-Mascheroni constant (approx. 0.577), μ and μ' are the membrane viscosity and the bulk solution viscosity, respectively. The parameters used to estimate the membrane associated complexes were membrane height h = 3.8 nm, viscosity of the surrounding media μ' = 0.001 Pa.s and membrane viscosity μ = 0.05 Pa.s.

Table S2. Lateral diffusion of labelled HA_1-ATTO532 obtained after binding to lipid membranes containing GM1 (1 mol %) in different lipid systems, from FLCS. The lateral diffusion of DOPE-ATTO655 decreases with membranes fluidity, here expressed by the DOPE diffusivity.

Lipid bilayer + GM1 (1 mol %)	Lateral Diffusion ($\mu m^2 s^{-1}$)		N_{HA1}
	HA_1-ATTO532	DOPE-ATTO655	
DOPC	5.0 ± 0.4	10.0 ± 0.5	6.3 ± 0.6
POPC	3.3 ± 0.4	6.3 ± 0.6	3.5 ± 0.4
DOPC/SM/Chol (4:4:2)	1.8 ± 0.5	3.0 ± 0.4	2.0 ± 0.6

References

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