Supporting Information

Impact of Glycans on Lipid Membrane Dynamics at the Nanoscale Unveiled by Planar Plasmonic Nanogap Antennas and Atomic Force Spectroscopy

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Materials and Experimental methods

Lipids, fluorescent dyes and hyaluronic acid

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and N-stearoyl-D-erythro-sphingosylphosphorylcholine 18:0 (SM) were purchased from Avanti (Avanti Polar Lipids, Inc.). Cholesterol (Chol) ≥99% was purchased from Sigma-Aldrich and the fluorescent dye DiIC18(5) solid (DiD) from Molecular Probes, Life Technologies Corporation. Pure Hyaluronic Acid (HA), MW 250k, and biotinylated Hyaluronate Biotin, MW 250k (Purity: >95%) for the dual-color fluorescence imaging in confocal mode were purchased from Creative PEGWorks. The fluorescent dye ATTO488 was purchased from ATTO-TEC GmbH and was conjugated in-house to Streptavidin.

Supported lipid bilayer (SLB) Preparation

We prepared SLBs of different lipid composition on different substrates, depending on the probing technique, i.e., glass for confocal fluorescence imaging and FCS-confocal measurements; gold-antenna substrates for FCS measurements at the nanoscale; and freshly cleaved mica for AFM and AFM-FS measurements (see sketch in Figure S2). As a control sample, we also prepared SLBs deposited on 30 nm-thick gold films. Glass coverslips were cleaned with ethanol, MilliQ water rinsing and UV/Ozone treatment. For all substrates, the SLBs were prepared following the protocol described in Ref.1. In short, small unilamellar vesicles (SUVs) of the desired lipid composition of a final concentration of 3 mM were prepared by lipid film hydration with a buffer solution of 150 mM NaCl, 20 mM MgCl₂, 20 mM Hepes at pH 7.4 prepared in ultrapure MilliQ water. In the next step, we applied interchanging heat shocks and vortexing at 60°C, followed by sonication and extrusion through 100 nm pore size filters at 60°C. Since the same SUVs were used to prepare lipid bilayers for all the experiments, a 0.1 wt% of the lipophilic fluorescent dye DiD was added to the SUV mixture. The SUVs formed into a single lipid bilayer once they were suspended onto the substrate of choice at 60 °C on a hotplate and left to settle for 30 minutes.

The lipid compositions of the single SLB examined further were DOPC alone, DOPC:SM (1:1) and DOPC:SM:Chol (2:2:1). The formed SLBs on the respective substrates were carefully rinsed with buffer solution and left to equilibrate at room temperature for another 30 minutes prior to the
experiments. To investigate the influence of HA on the lipid bilayer, the buffer solution was partly replaced by an HA solution to have a final concentration of 20 mg/mL, and left to incubate for 30 minutes, gently rinsed afterwards by replacement with the buffer solution for 2-3 times. For the incubation step the HA solution was prepared at a stock concentration of 40 mg/mL by dissolving the HA molecules in MilliQ water at ~40°C and a few minutes of vortexing. To ensure the integrity of the lipid bilayer, special attention was paid to keep it hydrated at all times and to avoid disturbing the lipid bilayer by carefully pipetting at the edge of the sample. After the rinsing step the sample was equilibrated for another 30 minutes at room temperature. For dual color confocal imaging experiments, we further labeled HA (250kMW) using 1 mol% biotin substitution of the average concentration of disaccharide units within each chain. In our case, this corresponds to approximately 6 biotins/chain (chain length of approximately 620 disaccharide units, i.e., sparse number of biotin molecules per chain). Biotinylated HA molecules were then incubated with Atto488-Streptavidin for 4 hours under constant rotation at 4°C. We adjusted the Atto488-Streptavidin concentration experimentally to obtain just enough fluorescence contrast to visualise the presence of the HA layer by confocal, while minimising the occurrence of aggregates. Note that the FCS diffusion experiments in confocal and on the nanoantennas were performed in the absence of HA fluorescent labeling to minimize the occurrence of cross-linking artifacts. Only the far-red lipid dye DiD was added as the FCS read-out signal.

The conditions of the HA incubation and rinsing steps to guarantee a well-defined HA exposure were found by testing experimentally different conditions. Fluorescence inspection by means of confocal (including z stacks) served as a first check to evaluate the quality of the HA layer. Moreover, since unbound HA molecules in solution lead to interactions with the AFM tip, we optimized the conditions to observe an effect of HA on the binary and ternary lipid bilayer mixtures while keeping tip interactions to minimum. We then applied the same conditions to the FCS diffusion measurements to guarantee the most consistent experimental approach.

*Planar gold nanogap antenna arrays*

Planar gold dimer antenna arrays with gaps of different sizes were fabricated in gold onto glass-coverslips according to a multistep nanofabrication procedure described in Ref. 2 in detail. The antennas consisted on gold dimers (half-sphere of 80 nm diameter each) centered in a 300×140
nm² nanoaperture boxes (see Figure S2). The central nanogap between the gold dimer provides gap sizes of 10-45 nm and creates a highly confined fluorescence hot spot. The function of the surrounding nanoaperture box is to screen for the surrounding background, further increasing the signal-to-background at the gap region.²³ For the three sizes of the gaps, and thus the effective nanoscale illumination areas, we used the same three gap areas of \((300 ± 50)\) nm², \((1080 ± 80)\) nm² and \((2025 ± 110)\) nm² as determined previously.⁴ In short, we considered the mean values of the gap sizes directly evaluated from TEM images for the x-direction. For the y-direction, we used the size of the full-width-at-half-maximum (FWHM) of the respective antenna excitation intensity profiles as obtained from FDTD simulations. The antenna-on-boxes were spaced by 4 m from each other, to allow for individual confocal excitation and detection. A single chip contains thousands of antennas of different sizes arranged in arrays, allowing to record many FCS curves from different antennas on the same SLB. Prior to the lipid bilayer deposition, the antenna substrates were carefully cleaned with ethanol, MilliQ water rinsing and UV light exposure for 1 minute followed by 3 minutes of ozone treatment.

Confocal Fluorescence Microscopy and FCS measurements

Fluorescence imaging and FCS measurements were conducted at room temperature on a commercial MicroTime 200 setup (PicoQuant) equipped with an inverted confocal microscope (Olympus 60×, 1.2 NA water-immersion objective) and a three-axis piezoelectric stage (PhysikInstrumente, Germany) allowing to select measurement regions of interest and to address individual nanoantennas.

Dual-color fluorescence imaging of the different SLBs (probed with DiD) and HA (labeled with Atto488) was performed using a linearly polarized \(λ=640\) nm picosecond laser diode (PicoQuant LDH-D-C-640) and a linearly polarized \(λ=470\) nm picosecond laser diode (PicoQuant LDH-D-C-470), both operating in continuous wave mode and coupled into the setup through the same optical fiber.

FCS measurements in confocal mode, or at the nanoscale using the antenna arrays, were performed using the same set-up and following a similar procedure by recording the intensity fluctuations of DiD embedded in the bilayer, with HA being label-free. FCS-confocal was performed at random positions of the sample. In the case of FCS-antennas, the selected nanoantenna was resonantly
excited (parallel to the dimer’s main axis) with the same linearly polarized $\lambda=640$ nm picosecond laser diode in continuous wave mode. For both, FCS-confocal and FCS-antennas the excitation power density was $\sim 2-3$ kW/cm$^2$. The emitted fluorescence signal was collected back using the same objective through a dichroic mirror and split onto two avalanche photodiodes (PicoQuant Pico Harp 300). The scattered light of the excitation laser was removed by an emission filter and a band-pass 650–690 nm filter placed before each detector. The fluorescence signal was recorded over time on a fast time-correlated single photon counting module in the time-tagged time-resolved mode (PicoQuant MPD-50CT). The typical measurement duration was 50s per run, and we calculated the correlation function $G(\tau)$ of $\sim 20$-30s time windows with the commercial software package SymPhoTime 64. The subsequent fitting step of the calculated correlation function $G(\tau)$ was performed with the SymPhoTime 64 software package as well. The fitting yields the characteristic diffusion times of the fluorescent dye, $\tau_D$, used for further quantification of the FCS measurements. For our FCS measurements in confocal and on antennas we employed a two-dimensional Brownian diffusion model, assuming a Gaussian beam profile to perform the fittings of the calculated $G(\tau)$:

$$G(\tau) = \sum_{i=0}^{n_{\text{diff}}-1} A_i / \left[ 1 + \left( \frac{\tau}{\tau_i} \right) \right]$$  \hspace{1cm} (Eq. 1)

where $n_{\text{diff}}$ is the number of independently diffusing species and $A_i$ the amplitude of the contribution of the $i$-th diffusing species with the corresponding diffusion time $\tau_i$.

**FCS analysis**

In the case of point-measurements in confocal mode, we measured the fluorescent DiD signal on multiple random locations of the same SLB and for each SLB and calculated the respective correlation functions $G(\tau)$. The $G(\tau)$ were fitted according to Eq. 1 for a single component ($n_{\text{diff}} = 1$). From the fittings we obtained the characteristic diffusion times of the DiD dye, $\tau_D$, in a diffraction-limited spot with a size of $\omega = 285$ nm. The diffraction-limited spot size $\omega$ was independently determined in calibration measurements with Alexa 647N dye (40 nM) in solution. From there, we calculated the characteristic diffusion coefficients $D$ by applying $D = \omega^2 / (4 \times \tau_D)$. The results are shown in Table 1 for the different SLB compositions with and without HA.
In the case of the antenna measurements we performed two-component fittings of $G(\tau)$ according to Eq. 1, assuming $n_{\text{diff}} = 2$ to account for the fast diffusion through the nanometric gap region of the dimer antenna, and for the slower contribution of molecules diffusing through the nano-aperture as we have previously applied.\textsuperscript{4}

**AFM and AFM-based force spectroscopy (AFM-FS)**

AFM imaging and force spectroscopy were carried out in contact or AC mode using an MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA) in liquid environment. Silicon nitride SNL probes (Bruker AFM Probes, Camarillo, CA, USA) were used for the measurements (nominal spring constant $k=0.35$ N/m, maximal forces of 65 nN) calibrated extemporaneously applying the equipartition theorem (thermal noise routine)\textsuperscript{5}. The scan rate was set to 1 Hz and 256×256 pixels were imaged regardless of the image size. The images were minimally processed to enhance the contrast by performing a plane fit at order 0, flattening at order 1, and then another plane fit again. For the AFM-FS experiments, force-distance curves were recorded on a 2×2 µm$^2$ area of interest that had previously been imaged, by approaching and retracting the cantilever tip to the sample at constant velocity of 1 µm/s. Force-distance curves were acquired in the force map mode, using an array of 32×32 pixels (pixel size of 63 nm) over the area of interest. All AFM measurements were carried out at room temperature.
Supplementary figures

**Figure S1.** Confocal FCS on a ternary lipid bilayer on a glass coverslide in presence of HA. (a) Representative fluorescence image of the DOPC:SM:Chol (20mol%) lipid membrane (magenta) and (b) in presence of HA (green). x-z scans are included below the images at the positions indicated by the white lines. (c) Merged x-y image together with the same merged z-projection as shown in (a,b). (d) Three representative fluorescence profiles taken at three different locations on the sample, as highlighted by the while lines in (c).
**Figure S2.** Experimental approach to investigate the influence of HA on nanoscale lipid organization. (a) Illustration of how the biological membranes have been prepared on different substrates for the different experiments. On glass coverslip for confocal FCS (top), on nanogap antenna platform for nanoscale FCS (bottom left) and mica (bottom right) for AFM imaging. For the fluorescence experiments on the glass coverslip in confocal and on the nanogap antenna hotspots, the biological membranes were prepared by incorporating the lipophilic fluorescent dye DiD, shown in red. (b) TEM images of nanogap antennas of 10 and 35 nm gap size (left) and of a 5 × 5 antenna array, of 10 nm gap sizes (right). (c) AFM imaging and force spectroscopy approach. (d) FCS setup, experiment and analysis principle of DiD diffusion on the different bilayers. Confocal fluorescence imaging of HA and the bilayer was performed by sparsely labeling HA with the Atto-488 dye and the bilayer with DiD. All the FCS and AFM data were recorded in the absence of HA labelling.
Figure S3. Confocal fluorescence imaging of DOPC:SM:Chol (2:2:1) deposited on a gold substrate (30 nm gold evaporated on cleaned coverslide), in presence of HA. (a) Representative fluorescence image of DOPC:SM:Chol SLB; (b) corresponding HA image. (c) Merge image. (d-f) Corresponding zoom-in regions of the square box 1 highlighted in (a-c). (g-i) Corresponding zoom-in regions of the square box 2 highlighted in (a-c).
Figure S4. AFM topography images of a DOPC bilayer before (a) and after (b) the addition of HA.
Figure S5. Force-Distance (F-D) curves of DOPC:SM (1:1) SLB in presence of HA, from the force-map shown in Figure 3f. (a) 10 representative F-D curves (approach and retraction) exhibiting a characteristic discontinuity at the breakthrough force of the corresponding histogram shown in Figure 3h. (b) 10 representative F-D curves (approach only) from the force-map shown in Figure 3f which do not exhibit a characteristic discontinuity, thus compose the 40% of data curves not included in the histogram in Figure 3h. In the zoom-in for one F-D curve the approach (black) and retraction (grey) curve are displayed.

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