Electronic Supplementary Information

Structural Insights on Fusion Mechanisms of Small Extracellular Vesicles with Model Plasma Membranes

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Materials and methods

Manufacturing of human mesenchymal stromal cell–derived extracellular vesicles (EVs)

Human umbilical cord-derived mesenchymal stromal cells (UC-MSCs) were seeded (1560 cells / cm^2) into a CF4 cell factory system (Nunc) in fibrinogen-depleted culture medium composed of α-MEM (Sigma-Aldrich), 10 % pooled human platelet lysate (pHPL), and 5 mM (N2)-L-alanyl-L-glutamine (Fresenius Kabi). Cells were cultured at 5 % CO_2 at 37 °C. Upon reaching a cell confluence of 60 – 70 %, cells were washed with phosphate-buffered saline (PBS) solution and the growth medium was exchanged to a EV-harvest medium, consisting of α-MEM, 5 % pHPL, which was additionally EV-depleted by tangential flow filtration (TFF) using a 750 kDa hollow fibre filter (Spectrum Labs), and 5 mM (N2)-L-alanyl-L-glutamine. After 24h, conditioned medium was harvested, centrifuged at 2,500 x g for 20 mins at 18 °C, and filtered (0.22 µm). EVs were concentrated and buffer-exchanged to PBS by TFF using a 100 kDa hollow fibre filter (Spectrum Labs). Ultimately, EVs were isolated and concentrated by ultracentrifugation at 120,000 x g for 3 h at 18 °C. Resulting EV-pellets were washed with PBS and subsequently resuspended in Ringer's Lactate in an appropriate volume to achieve a dose of 20 – 40 x 10^7 / mL (cell equivalent). Resuspended EVs were centrifuged at 3,000 x g for 10 min at 4 °C, sterile filtered (0.22 µm), and stored in glass vials at – 80 °C. For the experiments we used EVs from >10 batches of isolation runs from UC-MSC whose proteomic, size and number profiles were statistically homogeneous.

Lipids for model membranes

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-d54-sn-glycero-3-phosphocholine (d54-DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (brain, porcine, SM) and cholesterol (ovine wool, > 98%) have been purchased by Avanti Polar Lipids (Alabama) and used without any further purification.

Nanoparticle Tracking Analysis:

For nanoparticle tracking analysis of EV samples in light scatter mode, EVs were diluted to a concentration of 4 – 7 x 10^7 particles / mL in PBS. Prior to EV-sample analysis, the ZetaView PMX 110 instrument (Particle Metrix GmbH, Meerbusch, Germany) was calibrated using YG-labeled 100 nm polystyrene standard beads (1:1,000,000 dilution in ddH2O). For calibration of the PMX 110 instrument, minimum brightness was appointed to 25 AU (arbitrary units), temperature to 21.5 °C, shutter to 70 AU, and sensitivity to 65 AU. To determine the size and number of particles in the prepared EV-samples, EV samples (fluorescently-labeled [1] or unlabeled) were analyzed in light scatter mode. The minimum brightness was therefore set to 20 AU, temperature to 21.5 °C, shutter to 70 AU, and sensitivity to 85 AU. Subsequently, data for two exposures at 11 measurement positions were acquired per sample. Afterwards, data for two exposures at 11 measurement positions were collected for each sample. Based on the Stokes-Einstein equation, particle sizes were determined using the ZetaView software (PMX 110: Version 8.4.2).

Macs Plex:

The MACSPlex Exosome Kit (Miltenyi) is a bead-based multiplexed FACs based assay for the analysis surface markers present on sEVs. We have used the MACSPlex kit according to the manufacturer´s instruction and following a validated standard operating procedure with 5 x 10^7 to 5 x 10^8 particles as input. Data acquisition was conducted on a FACS Canto II (BD Biosciences).

Unilamellar Lipid Vesicles Preparation

Model lipid-based membranes were prepared according to a standard procedure explained elsewhere [2] by thin film deposition, hydration and extrusion through policarbonate filters with controlled porosity of 80 nm.

The vesicles were not investigated being the scope the description of the membrane form factor. Nonetheless the extrusion procedure on 80 nm sized pore filters, is well known to bring to the formation of vesicles sized 120 nm with low polidispersity [see for example L.D. Mayer, M.J. Hope and P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, BBA 858 (1986) 161-168]. The Lipid Vesicles concentration may be obtained by considering an area per lipid of 50 $Å^2$ and a vesicle radius of 60 nm and can be estimated to be of the order of 10^{12} vesicles/mL for the 0.5 mg/mL solution used for AFM and NR experiments and of the order of 10^{13} SUV/mL for the 20 mg/mL solution used for SANS experiments.

Atomic Force Microscopy:

AFM imaging was carried out on a commercially available microscope (MFP-3D Stand Alone AFM from Asylum Research, Santa Barbara, CA and JPK NanoWizard III Ultra) working at room temperature in dynamic AC-mode. Commercially silicon cantilevers (BL-AC40TS-C2, Olympus Micro Cantilevers, nominal spring k 0.09 nN/nm) have been chosen for imaging in liquid. Images were acquired at 512 x 512 pixel frames at 0.6-1.0 lines/s scan speed.

For AFM analysis of individual sEVs, 10 µL of sEVs were spread onto 0.6 cm x 0.6 cm piece of freshly cleaved mica, left to incubate for 5 min, gently rinsed 3 times with PBS, and imaged by AFM in liquid.

For sEVs membrane-interaction investigation we prepared two different model membranes: one made by DOPC phospholipid and one made by DOPC+SM+Chol in 2:1:0.16 molar ratio.

100 µL of SUVs solution (0.5 mg/mL in 2 mM $CaCl_2$) of specific lipid composition were spotted on freshly cleaved mica attached to the AFM liquid chamber by ultrafast glue. The sample was left to incubate for 15 minutes at room temperature to promote vesicle adsorption, fusion and the formation of lipid

bilayer on the surface. Then, lipid membrane was gently rinsed three times with Milli-Q H_2O to remove the excess of vesicles from the liquid sub-phase before AFM analysis.

sEVs have been inserted in the AFM liquid cell and the measurements were acquired as a function of time 5 minutes after initial incubation. AFM image analyses were performed using Gwyddion, an open-source modular program for scanning probe microscopy data visualization and analysis [3]. Equivalent diameters were calculated using the approach reported by Gan et al [8].

Graphs representing AFM trace profiles and height distributions were obtained using Igor Pro software (Wavemetrics, US).

Small Angle X-Ray Scattering:

Small Angle X-Ray Scattering (SAXS) experiments have been performed at the Austrian SAXS beamline in Elettra Synchrotron in Basovizza, IT. [4] The sample to detector distance was set to 1200 mm, allowing to investigate a q-range between 0.08 and 5.5 nm^{-1}. MSC-EVs were resuspended in phosphate buffer solution (PBS), to the final concentration of $2*10^9$ sEVs in 100 µL, inserted in a quartz capillary and measured at 25°C.

Neutron Reflectometry:

d54-DMPC has been used to prepare model membranes to perform Neutron Reflectometry (NR) experiments. The use of deuterated phospholipids helps in enhancing the visibility of molecules interacting with the lipid bilayer, thanks to the induced membrane contrast change, being the scattering length density of the deuterated lipids around $7x10^{-6}Å^{-2}$, while that natural lipids around -$0.5x10^{-6}Å^{-2}$ and that of proteins around $2.5x10^{-6}Å^{-2}$.

NR measurements were carried out at the GINA neutron reflectometer at the Budapest Neutron Centre (BNC). [5,6] The solid-liquid interface was investigated at a neutron wavelength of 4.63 Å in an aluminum liquid cell (temperature controlled by water circulation to ±0.1°C) which held a 70x70x10 mm^3 Si (100) block with a smooth surface (rms roughness of 0.3 nm) for membrane fusion and

investigation. The neutron beam entered the cell from the Si block side. The single supported d54-DMPC membrane was obtained by the fusion of lipid vesicles injected in the cell at the concentration of 0.5 mg/mL. Prior to the NR experiment, the Si block of the cell was cleaned by organic solvents, then cleaned in a plasma cleaner for 15 minutes and finally washed with Milli-Q H_2O.

For sEV-based bilayer investigation, after support characterization, a solution of $3*10^9$ sEVs in 2 mL of D_2O have been injected in the measuring cell (10 mL total volume) at T = 37 °C and, after 45 min for incubation, the excess material has been gently removed by flushing D_2O in the cell. Finally, reflectivity has been measured in two contrast solvents (H_2O and D_2O).

Small Angle Neutron Scattering

d54-DMPC has been used to prepare model membranes to perform the SANS experiments. The use of deuterated phospholipids helps in enhancing the visibility of molecules interacting with the lipid bilayer, thanks to the induced membrane contrast change, being the scattering length density of the deuterated lipids around $7x10^{-6}Å^{-2}$, while that natural lipids around $-0.5x10^{-6}Å^{-2}$ and that of proteins around $2.5x10^{-6}Å^{-2}$.

Measurements were performed on the SANS-YS instrument at the Budapest Neutron Centre using two wavelengths and two sample-to-detector distances to cover a wide q-range, from 0.005 to 0.4 $Å^{-1}$. Lipid membranes have been prepared in the form of extruded monolamellar vesicles, while sEVs were resuspended in D_2O (10^{10} vesicles/ml) Samples were placed in quartz cells (produced by Hellma) and measured at 25°C.

For fusion experiments, 500 µL of phospholipid vesicles at the concentration of 20 mg/mL have been mixed to 100 µL of EVs solution ($3x10^{12}$ phospholipid vesicles + $2x10^8$ sEVs (LC)); to 500 µL of sEVs solution ($3*10^{12}$ phospholipid vesicles+ 10^9 sEVs (MC)) and to 600 µL of EVs solution ($3x10^{12}$ phospholipid vesicles + $1.2x10^9$ sEVs (HC)).

Deuterated lipids have been chosen to highlight the presence of eventual H-containing molecules (by which sEVs are composed) within the model membranes in D_2O.

Data fits have been performed by the software SasView. [7]

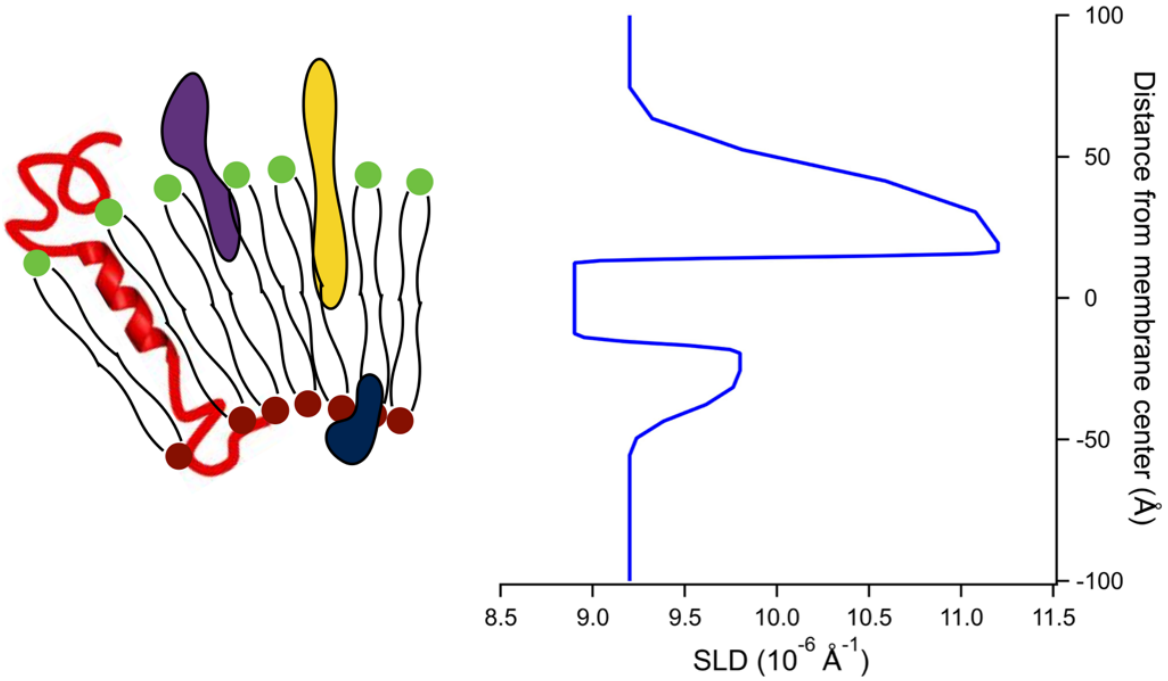


Figure S1. sEVs Scattering Length Density profile extracted from SAXS data fits obtained with a 3-layered membrane model, accounting for the proteomic component extending in the extra-vesicular solution.

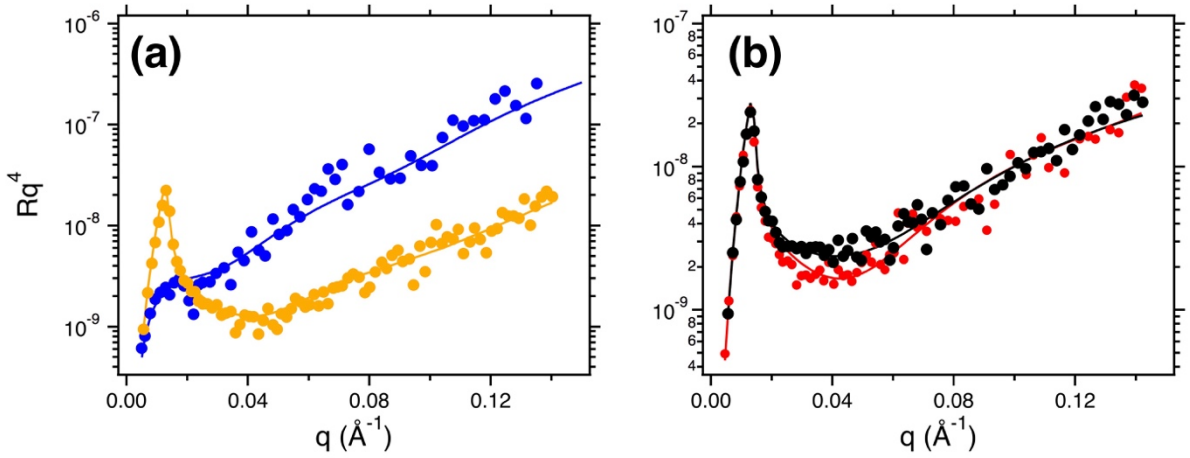


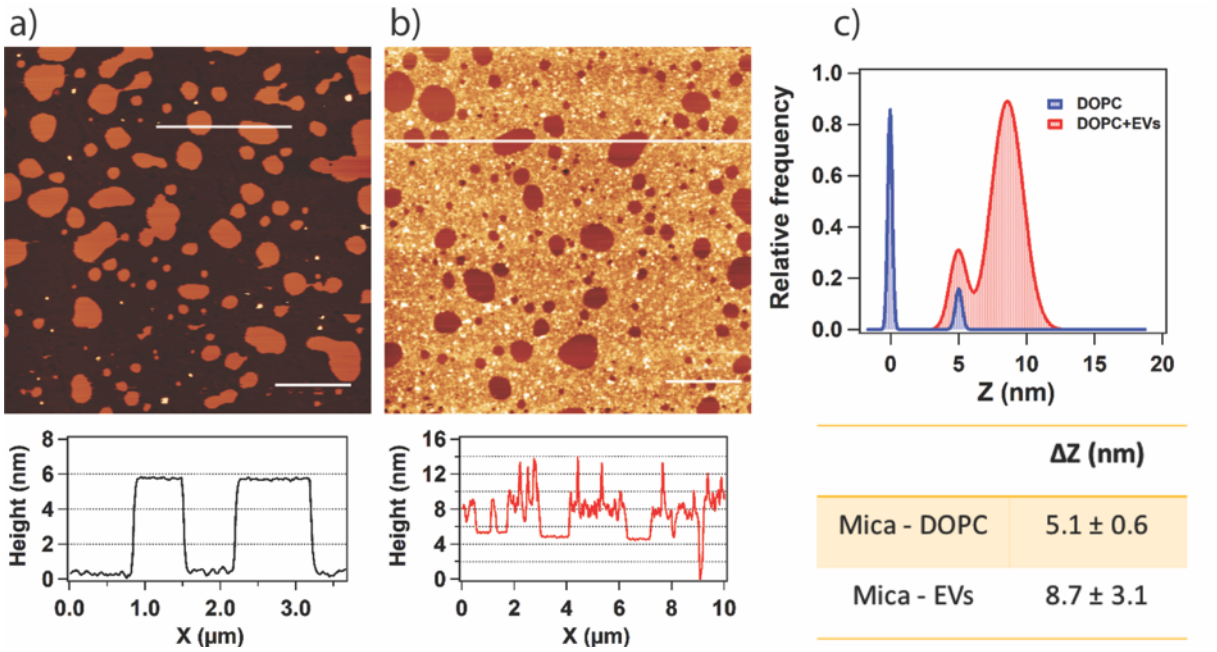
Figure S2. Neutron reflectometry from EVs based supported bilayers. a) Experimental data and relative fits of sEVs based supported membranes investigated in two contrast solvents: H_2O (blue) and D_2O (orange). The system has been investigated at 37°C. Best fit parameters are reported in Table S1. b) Experimental data and relative fits of d54-DMPC SLB before (black) and upon addition of sEVs (red). The system has been investigated at 37°C. Best fit parameters are reported in Table S2.

	Thickness (± 2 Å)	SLD (± 0.2 E^{-6} $Å^{-2}$)	Roughness with previous layer (± 2 Å)
SiO_2	1	3,4	2
solvent	5		6
membrane	69	2	2

Table S1. Best fit parameters for NR curves of sEVs based supported bilayers

DMPC				
	Thickness (± 2 Å)	**SLD (± 0.2 E^{-6} $Å^{-2}$)**	**Solvent penetration (± 3% vol)**	**Roughness with previous layer (± 2 Å)**
SiO_2	1	3.4		2
solvent	5			3
Heads in	7	1.75	15	2
Chains	28	7.1	4	2
Heads out	7	1.75	15	3
DMPC + EVs				
	Thickness (± 2 Å)	**SLD (± 0.2 E^{-6} $Å^{-2}$)**	**Solvent penetration (± 3% vol)**	**Roughness with previous layer (± 2 Å)**
SiO_2	1	3.4		2
solvent	5			5
Heads in	7	1.8	15	4
Chains	40	6.1	4	2
Heads out	7	1.8	15	7

Table S2. Best fit parameters for NR curves of DMPC and DMPC + sEVs supported bilayers.



	ΔZ (nm)
Mica - DOPC	5.1 ± 0.6
Mica - EVs	8.7 ± 3.1

Figure S3. Atomic Force Microscopy topographic images and corresponding line profiles of a partial DOPC SLB before (a) and after addition of sEVs (b). We report in (c) the height histograms relative to the two AFM images, to highlight the height of the DOPC SLB (a) and the protrusion of sEVs SLB inside the DOPC SLB.

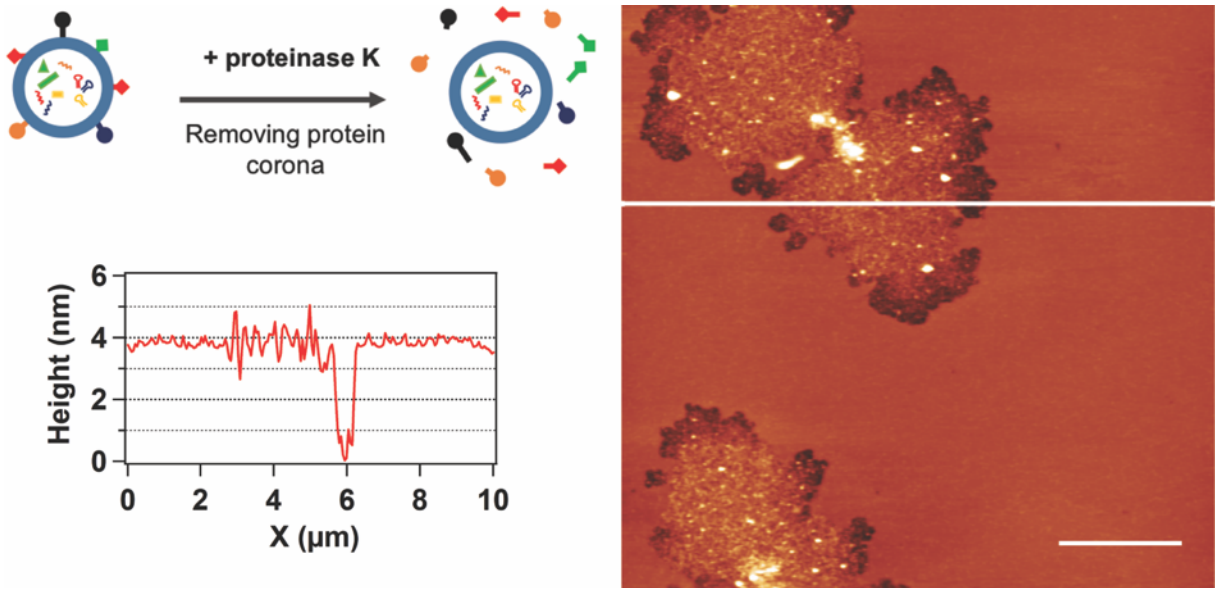


Figure S4. Atomic Force Microscopy topographic image and corresponding line profiles of DOPC SLB after addition of sEVs previously treated with proteinase k, following the protocol reported by Skliar et al. (Ref. 59 Main manuscript). We can observe the presence of small depressions at the interface between the sEVs' patches and the DOPC bilayer. A similar behaviour has been observed by Tero et al. (Ref. 58 Main manuscript) that attribute the depression to differences in lipid phases/composition in the bilayer. It might be the case that at the interface between the Ld phase and the sEV's patches a mixed phase could cause such discontinuity.

	SLD heads in (E^{-6} Å^{-2})	Thick heads in (Å)	SLD chains (E^{-6} Å^{-2})	Thick chains (Å)	SDL heads out (E^{-6} Å^{-2})	Thick heads out (Å)
DMPC	1.7	6	6.4	30	1.7	8
DMPC+ EVs LC	1.9	6	6.3	28	1.9	12
DMPC+ EVs MC	1.8	5	6.3	25	2	13
DMPC+ EVs HC	1.9	5	6.5	23	2.1	14

Table S3. Best fit parameters for SANS scattering patterns for bulk d54-DMPC SUVs before and after the interaction with sEVs at low, medium and high concentration

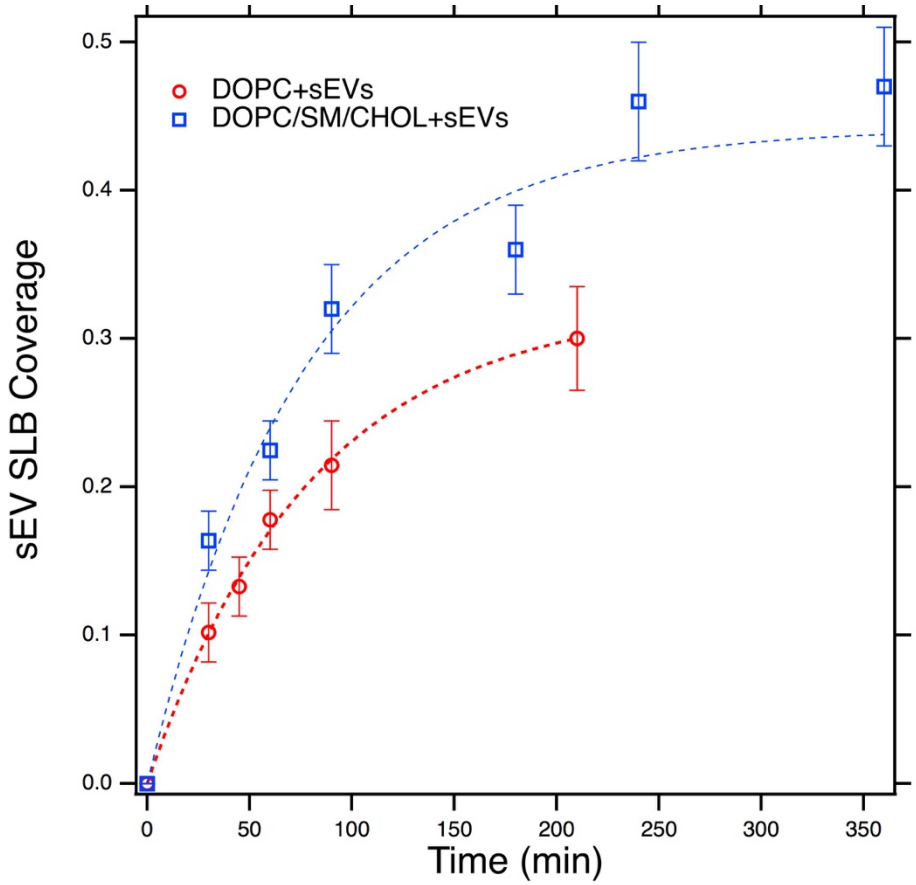


Figure S5. Evolution in time of the sEV SLB coverage with respect to the total area of the artificial SLB for DOPC (red dots) and DOPC/SM/Chol (blue squares) systems. The dashed lines are fitting curves with a 1st order Langmuir model, $\Theta=A*(1-e^{-t/\tau})$, where Θ is the coverage, A is the maximum coverage and τ is the Langmuir adsorption model time constant. Fitting results for DOPC: A=0.32+/-0.05, τ=80+/-20 minutes. Fitting results for DOPC/SM/Chol: A=0.44+/-0.03, τ=77+/-11 minutes.

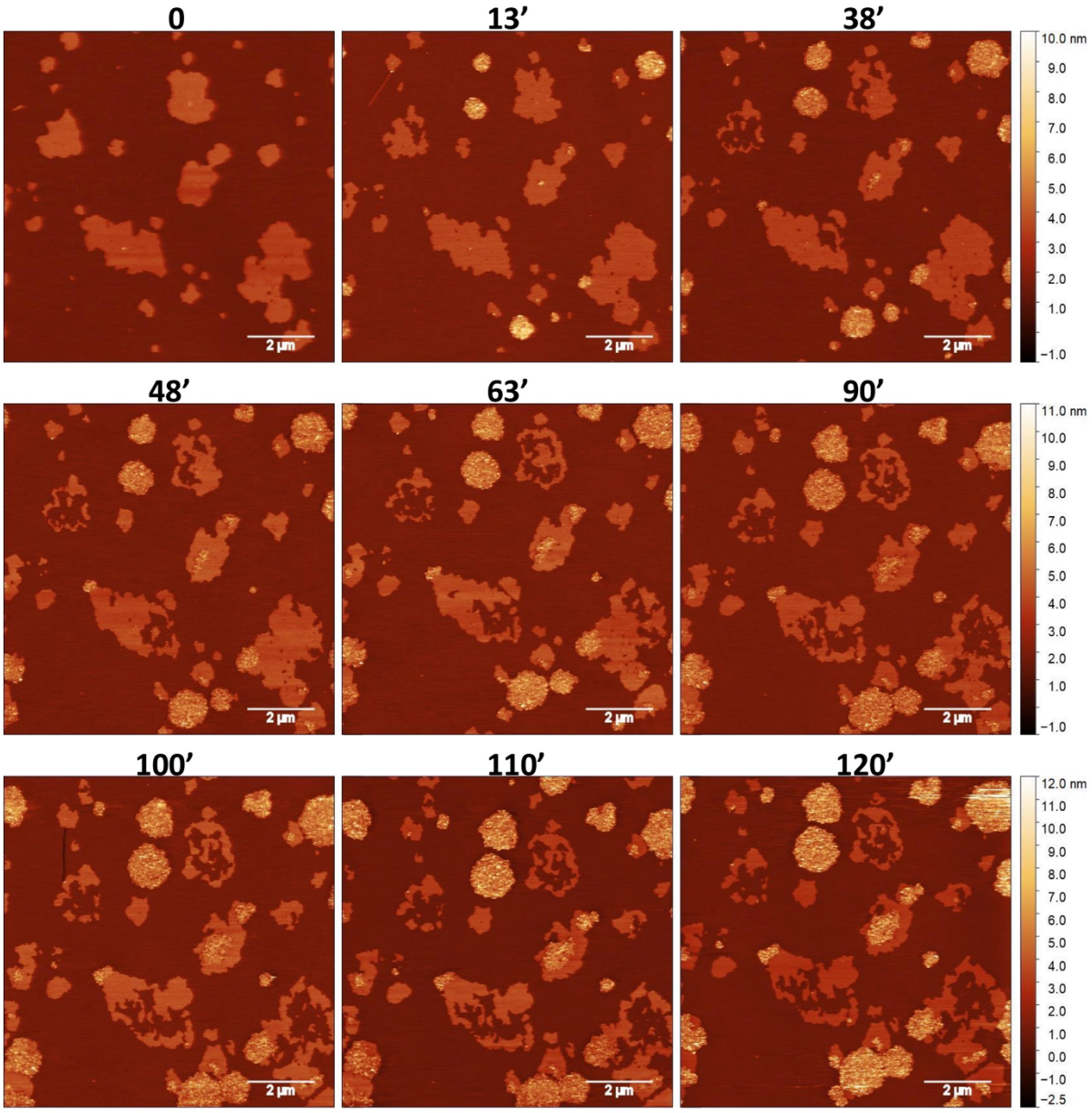


Figure S6 AFM topographic images of a DOPC:SM:Chol (2:1:0.15) SLB before (a) and after addition of sEVs when Lo phase is not directly connected to the sEVs' islands. We report the temporal evolution of the formation of EVs SLB showing the images after 13, 38, 48, 63, 90, 100, 110, 120 minutes. We can clearly follow the growth of sEVs' patches over time with a re-shaping of the Lo phase. The sEVs' islands grow in lateral dimension and sometimes coalescence in larger islands.

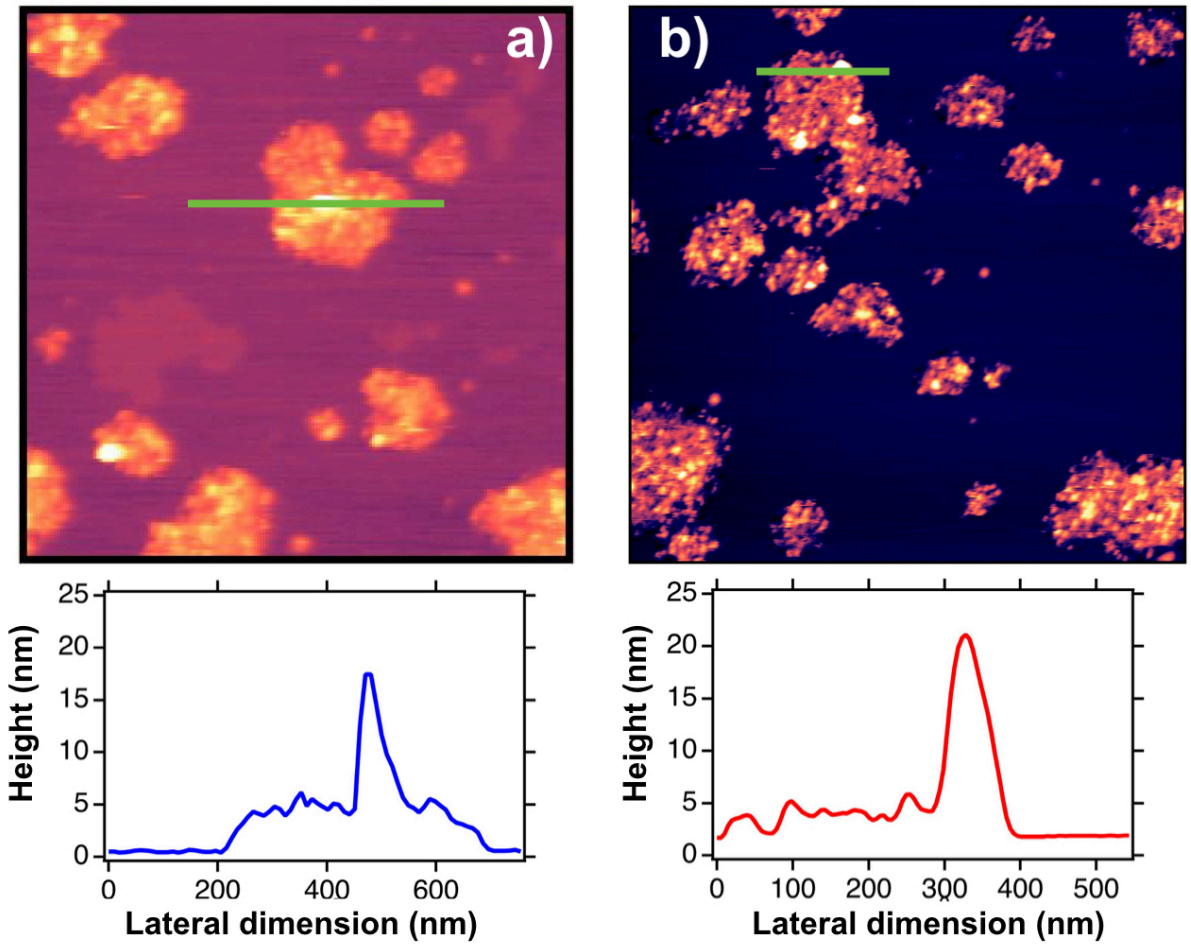


Figure S7 AFM topographic images and corresponding line profiles of a DOPC:SM:Chol (2:1:0.15) (a, blue profile) and DOPC (b, red profile) SLBs 15 minutes after addition of sEVs. We can spot few intact EVs on top of the sEVs' islands protruding 15-20 nm from the bilayer (as also observed in Figure 1d).

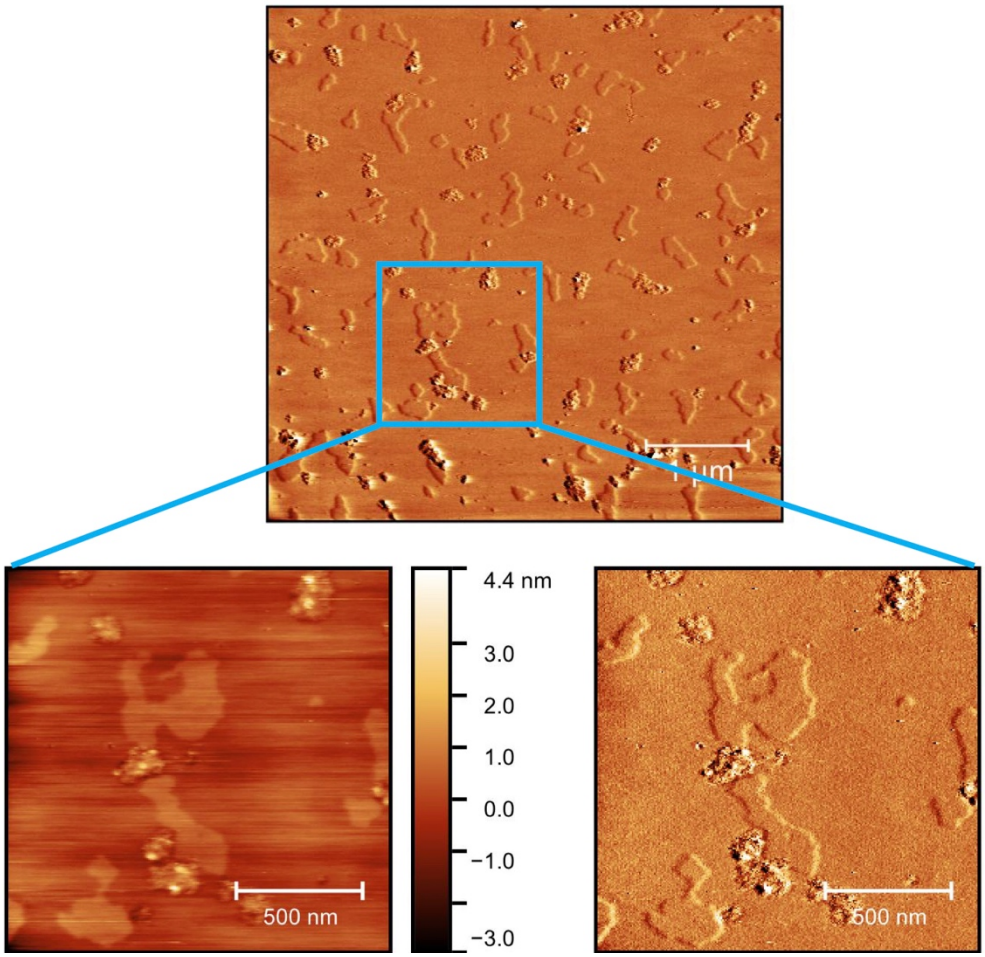


Figure S8 AFM amplitude and topography images of a DOPC:SM:Chol (2:1:0.15) SLB 5 minutes after addition of sEVs. The amplitude images, highlighting both the borders of liquid order phase rafts and of sEVs islands, clearly show that sEVs islands tend to locate at the interface between liquid ordered and liquid disordered phases. We analyzed 262 sEVs' islands from independent experiments and we calculated the percentage of the ones in contact with the borders of liquid ordered phase towards the total number of sEVs' islands. The analysis shows values ranging from 58 to 78% with an average value +/- standard deviation of 67+/-8%, confirming the preferential docking in close proximity of the Lo domains.

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