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Supplemental Information

Cooperative Function of LL-37 and HNP1 Protects Mammalian Cell Membranes from Lysis

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Contents

Supporting Figures

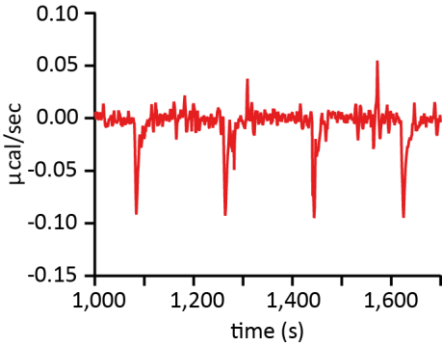

Figure S1 Reverse titration in isothermal titration calorimetry (ITC). 15 mM POPC vesicles were titrated with 100 µM LL-37 at 2 µl for each injection. During this reverse titration lipids are in excess compared to peptides, yielding an equal amount of heat at each injection. The binding enthalpy per 1 mol of peptide estimated from this reverse titration (-5.57 kcal/mol) is in agreement with that from the standard lipid-into-peptide titration shown in Fig. 3a in the main text (-5.73 kcal/mol). This validates the analysis of ITC for extracting entropy and enthalpy shown in the text. The experiment was performed in a physiological HEPES buffer solution (10 mM HEPES, 150 mM NaCl, pH = 7.4).

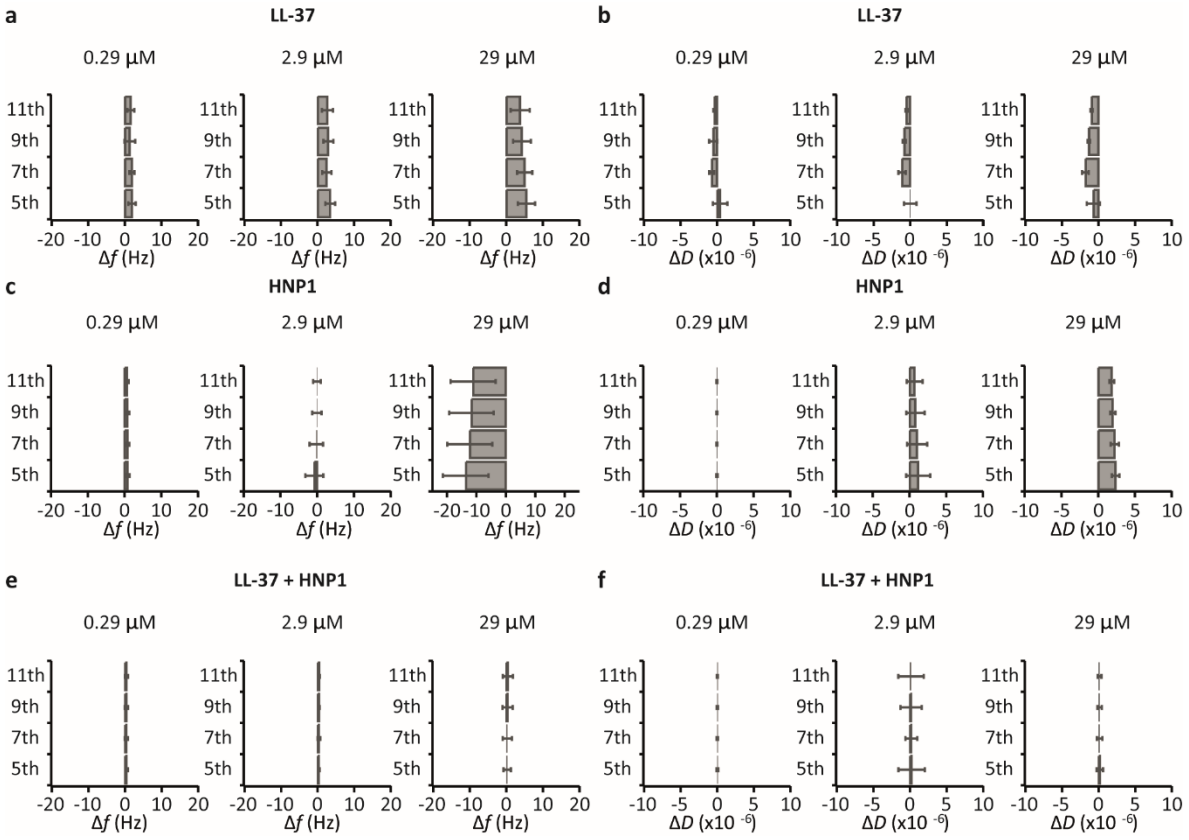

Figure S2 Overtone analysis of the quartz crystal microbalance (QCM) experiment shown in Fig. 3e in the main text.

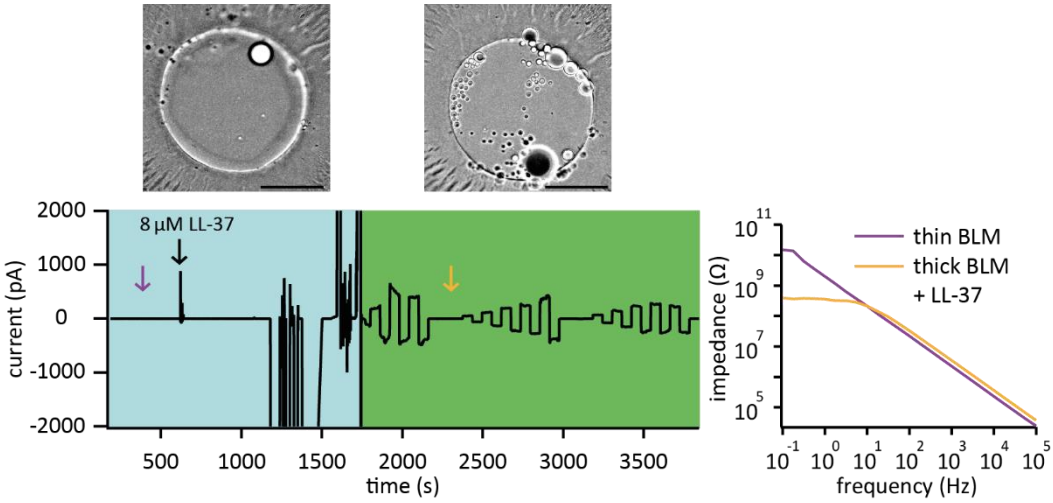

Figure S3 Comparison of conductance measurements at a fixed voltage and impedance spectra taken in between. During this experiment, the bilayer thickened from 3.0 nm to 10.4 nm as the impedance spectra taken at the beginning (purple) and in the middle (yellow) indicate a decrease in the capacitance. Nevertheless, the transmembrane conductance remained even after the bilayer thickened. Scale bar 50 µm.

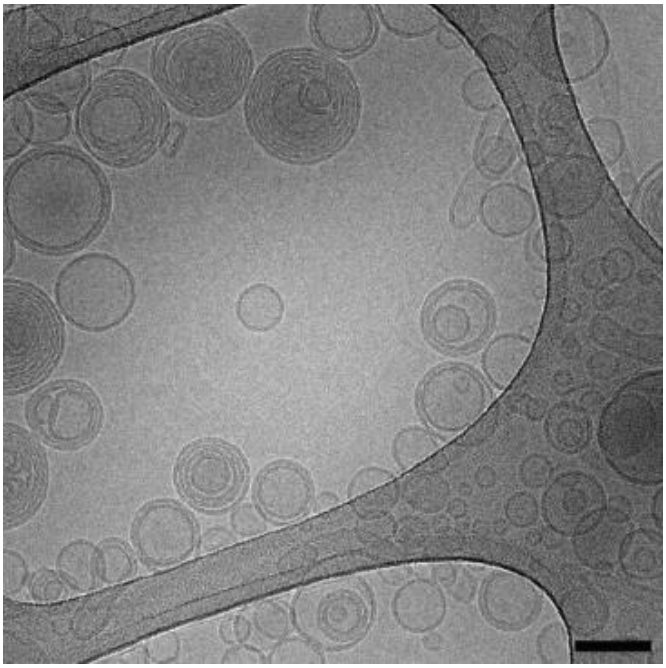

Figure S4 A cryo-transmission electron microscopy (cryo-TEM) image of POPC vesicles. Scale bar 100 nm.

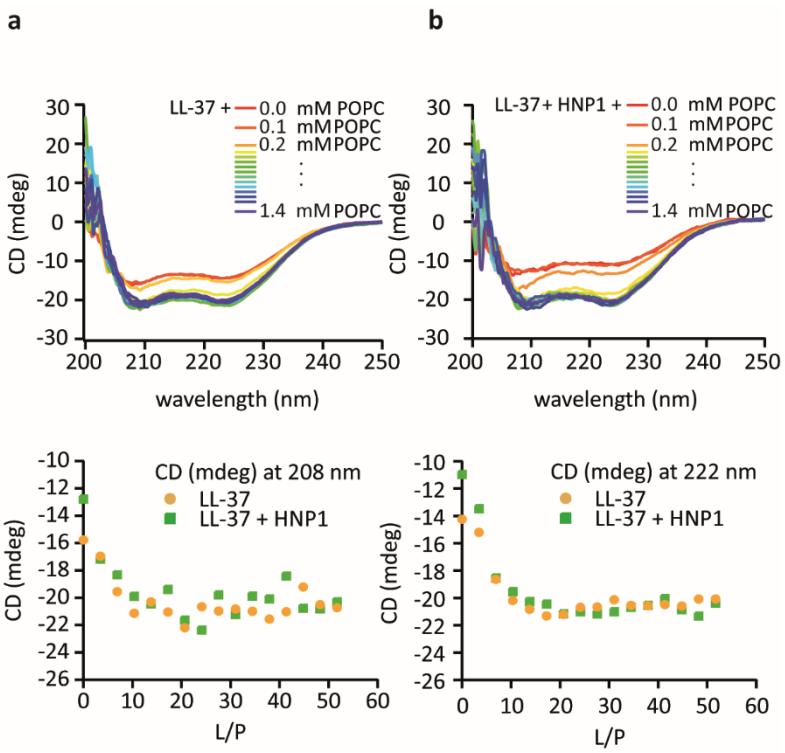

Figure S5 CD spectra of (a) LL-37 alone and (b) in pair with HNP1 titrated with POPC vesicles and the corresponding analysis of the intensity at 208 nm and 222 nm as a function of lipid-to-peptide ratio. 29 μM of LL-37 or LL-37 + HNP1 (58 μM at a final concentration) was titrated with 15 mM of POPC vesicles at 2 μl for each injection. The last injection corresponds to the final POPC concentration at 1.4 mM. For the spectra of LL-37 + HNP1 mixture in solution, the spectra of HNP1 alone in solution is subtracted. For the spectra of LL-37 + HNP1 mixture in vesicles, the spectra of HNP1 in vesicles at the corresponding lipid-to-peptide ratio is subtracted. No significant difference was observed between LL-37 and LL-37 + HNP1 mixture. The experiment was performed in a physiological HEPES buffer solution (10 mM HEPES, 150 mM NaCl, pH=7.4).

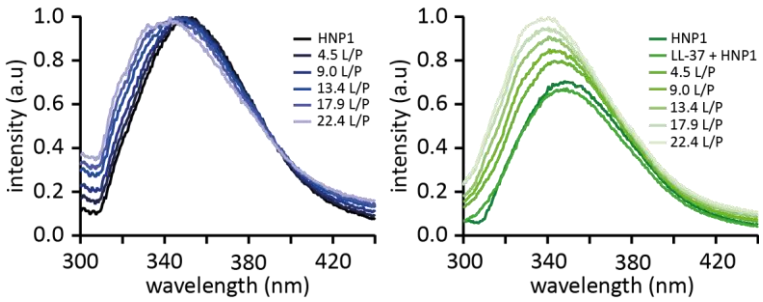

Figure S6 Fluorescence emission of Trp residue in HNP1 measured during the titration with POPC vesicles with or without LL-37. HNP1 or its mixture with LL-37 at 2.9 μM (in case of mixture 2.9 μM HNP1 + 2.9 μM LL-37) were titrated with POPC vesicles at 1.3 mM with 4 μl for each injection. The experiments were performed in 10 mM HEPES buffer solution with 150 mM NaCl, pH = 7.4.

METHODS

Materials

HEPES buffer solution was prepared with 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Fluka, Switzerland) with addition of either 150 mM sodium chloride (Sigma Aldrich, Switzerland) or 2 M potassium chloride (Sigma Aldrich, Switzerland) in ultra-pure water filtered through MilliQ Gradient A10 filters (Millipore AG, Switzerland). The pH was adjusted to 7.4 using 6M NaOH (Sigma Aldrich, Switzerland). Prior to use, buffer was sterile filtered through 0.22 µm pore size PVDF membrane (Merck, Switzerland). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, #850457) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBDPE, #810145) were purchased from Avanti Polar Lipids. n-decane (reagent plus ≥ 99%) and hexane (reagent plus ≥ 99%) were purchased from Sigma Aldrich (Germany). Fluo-3 AM FluoroPure^{TM} grade (Invitrogen, #F23917) were purchased from Thermo Fischer Scientific. Human Neutrophil Peptide-1 (HNP1, #4025473) was purchased from Bachem (Switzerland), while Human LL-37 Cathelicidin (LL-37, #AM-001) was purchased from IscaBiochemicals (United Kingdom). Both peptides were dissolved and stored according to instructions provided by the manufacturers.

Cell culture

Primary Human Umbilical Vein Endothelial Cells (HUVEC) from pooled donors, proliferating, were purchased from PromoCell (PromoCell, #12253) and cultured in complete Phenol-red free (PRF) medium (PromoCell, #C-22215 supplemented with Growth Medium supplementPack, #C-39210) according to manufacturer's instructions. Madin-Darby Canine Kidney (MDCK) cells were a gift from Prof. Aurélien Roux (University of Geneva) and cultured in high glucose

DMEM Glutamax^{TM} (Gibco, #61965), supplemented with 10% fetal calf serum (Bioconcept, Switzerland, #2-01F10-I) and 1% penicillin streptomycin (Gibco, #15140122).

Intracellular calcium response monitored with Fluo-3 indicator

The cells were loaded with membrane permeable ester of calcium indicator Fluo-3 AM, which upon entry into the cytosol undergoes hydrolysis by intracellular enzymes into membrane impermeable form. After loading into the cells in the dark and leaving the culture dish for 40 min to reach the cells quiescence, the time lapse imaging was performed at 37 °C by a confocal laser scanning microscope (CLSM, confocal A1, Nikon, Japan) with an oil immersion objective (CFI PLAN APO LBDA 60X, Nikon, Japan). The microscope is integrated with perfect focus system, active vibration isolation platform (i4 series, Accurion, Germany) and an acoustic enclosure equipped with a temperature controller (JPK Instruments, Germany). After taking some frames without peptide, the AMPs were added either individually or in a pair at a final concentration of 29 µM, and the time-lapse images were recorded. To monitor changes in fluorescence of Fluo-3 indicator, 488 nm laser excitation wavelength and a FITC A1 emission filter (525/50) were selected. Images were taken with 1024 pixels resolution and were further analyzed with ImageJ software.

Isothermal Titration Calorimetry (ITC)

POPC dissolved in chloroform was dried under a stream of nitrogen and left in a vacuum overnight for completely removing the organic solvent residuals. Obtained lipid films were rehydrated in buffer solution (10 mM HEPES, 150 mM NaCl, pH=7.4) at a final concentration of 15 mM, left for 2h and went through high power titanium tip sonicator treatment (Omni Sonic Ruptor 400 Ultrasonic Homogenizer, Omni International) until solution became transparent, followed by ultracentrifugation to separate titanium particles from the vesicle solution. The measurements were carried out on VP-ITC Microcalorimeter (MicroCal. Inc.,

Northampton, MA) with a chamber volume of 200 µl. Prior to each experiment, the sample cell was cleaned following the manufacturer's protocol. To investigate peptide-peptide interaction in aqueous solution, 40 µM HNP1 was titrated with 400 µM LL-37 peptide and heats of reaction were recorded. To account for heats of dilution, control experiment was performed, where 400 µM LL-37 was titrated into a buffer solution, and the result was subtracted from the data presented in Fig. 2. For the lipid to peptide titration in Fig. 3, relevant peptide solution at a final concentration of 40 µM in 10 mM HEPES, 150 mM NaCl was placed in the sample cell and left to equilibrate for 30 min at 25°C. POPC vesicle solution was loaded into an injection syringe with a purge and re-fill procedure in order to dislodge any air bubbles remaining in the syringe. During the experiment 2 µl vesicle suspension was injected into stirred peptide solution with 180 s intervals, at the reference power set to 6 µcal/sec, and the resulting heat change was measured. As a control experiment, to account for heats of dilution, vesicle solution was injected into a buffer solution without peptide, and this result was subtracted from the data shown in Fig. 3. In a separate experiment in Fig. S1, the reverse titration was performed, where peptides (100 µM LL-37) were injected into lipid vesicle suspension (15 mM POPC) with the same conditions as mentioned above. The binding enthalpy per 1 mol of peptide was calculated after subtraction of heat from a control experiment, where LL-37 was injected into a buffer solution. Data processing (baseline subtraction, peak integration, correction for sample dilution) was performed with MicroCal Origin software. Vesicle size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using Zetasizer Nano series (Nano-ZS, Malvern Panalytical) right after the ITC measurements. All the experiments were performed in HEPES buffer solution (10 mM HEPES, 150mM NaCl, pH = 7.4).

Circular Dichroism (CD) spectroscopy

CD spectra were recorded with a Jasco CD-J-815 spectropolarimeter using a quartz cuvette with path length of 1 mm. Each CD spectrum was recorded from 240 nm to 190 nm with 50 nm/min scanning speed, 1 nm bandwidth, 4 sec digital integration time at 25 °C at least for 10 times to improve the signal to noise ratio by averaging. Peptides at 29 µM were incubated in HEPES buffer solution (10 mM HEPES, 150 mM NaCl, pH = 7.4) for 1 h before measurement at 25 °C. For studying the secondary structure of peptides in solution, first the spectra for LL-37 and HNP1 were separately measured. Next, they were mixed at the final concentration of 29 µM for each, left for 1 h to stabilize and the spectra was taken. In Fig. 2c the spectra from LL-37 and LL-37 + HNP1 are shown, where for the mixture spectra from HNP1 is subtracted. For studying the secondary structure of peptides in vesicles, first the spectra from LL-37 in HEPES buffer solution was taken, followed by a sequential injection of 2 µl 15 mM POPC vesicle suspension prepared by sonication, where CD spectra was measured after each injection. The same procedure was repeated with HNP1 and the peptide mixture. LL-37 spectra and LL-37 + HNP1 spectra after the subtraction of HNP1 spectra at each concentration were presented in Fig. S5.

Fluorescence spectroscopy

The fluorescence emission spectra from Trp-26 residue in a hydrophobic region of HNP1 was measured before and after adding LL-37. Experiment was performed on FluoroMax-4 Spectrofluorometer (Horiba Scientific) with 280 nm excitation wavelength, emission between 300 to 500 nm, 1 nm step size and 5 nm excitation and emission slit widths in cuvette with 10 mm path length. Prior to measurement, HNP1 was dissolved in HEPES buffer solution (10 mM HEPES, 150 mM NaCl, pH = 7.4) at 2.9 µM and left 1h at 25 °C. After taking a spectrum, LL-37 was added at a final concentration of 2.9 µM and the spectra was collected every 5 minutes to monitor the possible time evolution of the signals. In addition, the effect of POPC lipid

addition was monitored by injecting POPC vesicles every 5 minutes to HNP1 alone or in combination with LL-37 (Fig. S6).

Vesicle preparation for FRAP, QCM-D and EIS experiments

POPC and 0.2 % mol of NBD-PE dissolved in chloroform were mixed and dried under a stream of nitrogen and left in a vacuum overnight for completely removing the organic solvent residuals. Obtained lipid films were rehydrated in a buffer solution (10 mM HEPES, 150 mM NaCl, pH=7.4) at a final concentration of 1 mg/ml POPC, left for 2h and extruded for 31 cycles through two 50 nm polycarbonate membranes (Whatman Nuclepore Track-Etched Membranes, #800308) at room temperature to make small unilamellar vesicles (SUV).

Fluorescent Recovery After Photobleaching (FRAP)

Glass coverslips were cleaned in a bath sonicator with acetone followed by ethanol and MilliQ water, dried under nitrogen flow and activated in an oxygen plasma cleaner (TePla IoN 3MHz Plasma System, Germany) for 30 min. To form supported lipid bilayers, activated glass surface was incubated with the vesicle suspension for at least 20 min followed by extensive rinsing with buffer solution to eliminate any remained fluorescence vesicles. To study the influence of peptides on membrane lateral fluidity, individual types of peptides or their mixture were added at 2.9 μM and the supported lipid bilayers were incubated for 40 min followed by rinse. Time-lapse images were taken using Nikon Eclipse Ti-E inverted fluorescence microscope (Nikon, Japan) with 60x/1.40 oil-immersion objective (Plan Apo, Nikon). The microscope was equipped with a CCD camera (DS-Qi2, Nikon) and 482/35 excitation (nm) and 536/40 emission filters (nm) were used. The contrast and the brightness were adjusted and the images were

presented with false colors in the figures. Diffusion coefficients were extracted based on double normalization presented elsewhere(1).

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

QCM-D measurements were performed with Q-SENSE (Biolin Scientific, Sweden) instrument by using Quartz Crystal, 5 Hz, AT cut, Gold electrode with 300 nm silicon-dioxide coating (QS-QSX318, Biolin Scientific, Sweden). Prior to use, these sensors were cleaned with 2% SDS, rinsed with ultrapure water (MilliQ), dried under a nitrogen stream and activated with an UV/ozone cleaner for 20 minutes. In order to investigate the mass and the viscoelastic properties, changes in resonance frequency and energy dissipation were recorded in real-time at four overtones of the fundamental resonance frequency at 25 °C. After the bilayer formation, peptides were injected sequentially at concentrations from 0.29 µM to 29 µM, where at each concentration bilayers were incubated with the peptides for 40 min and were rinsed with a buffer solution. The frequency and the dissipation shown in the overtone analysis in Fig. S2 are the changes between the signal after forming a lipid bilayer and that after rinse at each peptide injection normalized to overtones.

Electrochemical impedance spectroscopy (EIS)

Prior to use, highly conductive (0.001-0.005 Ω·cm) boron-doped (p-type) silicon wafers (University Wafer Inc.) with orientation <100> were cut into approximately 1 cm x 3 cm, cleaned with isopropanol, MilliQ water, and blow-dried with nitrogen. The electrode was mounted inside a half-home-made electrochemical chamber with a 3-electrode setup (Ag/AgCl as a reference and platinum as a counter electrode). The chamber volume is roughly 150 µl and

an active working electrode area is around 0.95 cm^2. To form supported lipid bilayers over this silicon working electrodes, vesicles were injected into the flow cell immediately after extrusion and incubated for 2 h at room temperature, followed by rinse with a buffer solution. To trigger the vesicle rupture efficiently for forming defect-free bilayers with a resistance value in kΩ range, the highly conductive silicon wafers were activated with an oxygen plasma cleaner (TePla IoN 3MHz Plasma System) for 30 minutes immediately before the experiment. After the bilayer formation, peptides at the concentrations from 0.29 µM to 29 µM were injected into the flow cell sequentially, where at each concentration the bilayer was incubated for 40 minutes, rinsed with a buffer solution, and impedance spectra were measured with a 10 mV sinusoidal voltage at 0 V offset potential with Autolab PGSTAT302N (Metrohm, Switzerland). Obtained spectra were analyzed by Nova 1.11 Software (Metrohm, Switzerland) with an equivalent electrical circuit presented in Fig. 4a.

Impedance Spectroscopy data analysis

The measured data was analyzed after fitting with the following equivalent circuit R_{buffer} ($R_{ch}C_{dl}$)($R_{LB}C_{LB}$), where R_{buffer} represents a serial resistance of buffer solution, R_{ch} corresponds to charge transfer resistance and C_{dl} is the double layer capacitance. The bilayer formation is represented by an additional bilayer resistance R_{LB} and capacitance C_{LB}. The extracted bilayer resistance and capacitance were further converted into defect areas $A_{defects}$ and bilayer thickness d_{LB}, respectively. First, the bilayer thickness was calculated. As a bilayer behaves like a capacitor, we could convert the capacitance values into bilayer thickness by using the following formula $C = \frac{\varepsilon_0 \varepsilon A'}{d_{LB}}$, where ε_0 is the space permittivity ($8.85 \cdot 10^{-12}$ F/m), ε is relative permittivity of the dielectric hydrocarbon region in lipid bilayers (2-4)(2, 3). A' is the area of the working electrode covered by lipid membrane (0.95 cm^2) and d_{LB} is the bilayer thickness.

For the calculations of the defect area A_{defect} the following equation was used:

$$A_{\text{defect}} = \rho \frac{d_{\text{LB}}}{R_{\text{LB}}},$$

where ρ is the buffer resistivity (73.5 Ohm·cm)(4), d_{LB} is the membrane thickness (3.7 nm) and R_{LB} corresponds to the bilayer resistance.

Painting solution for conductance experiments

For the single channel conductance measurement lipid painting solution was prepared as follows: small volume of POPC lipids in chloroform was dried under nitrogen flow and left for two hours under vacuum to remove remaining solvent. Then, the lipids were re-suspended in 1:1 mixture of hexanes isomers and n-decane at a final concentration of 25 mg/ml, vortexed and used immediately in the conductance experiment.

Single Channel Conductance Experiment with Black Lipid Membranes (BLM)

The detail of the used homemade electrochemical chamber is described elsewhere (5). In brief, it contains two chambers (*cis* and *trans*) separated by a 25 µm thick Teflon sheet with a single pore (ø = 50-100 µm) that spans horizontally. This horizontal chamber design enables the optical microscopy to be coupled during the electrophysiological recordings. An Ag/AgCl electrode is connected to each chamber through an agar salt bridge (2-electrode setup). The whole chamber was filled with HEPES buffer solution (2M KCl, 10 mM HEPES, pH = 7.4). Painting solution (~ 2 µl) was brushed over the pore to form a black lipid membrane (BLM) and its spontaneous thinning was monitored by combining bright field microscopy (Nikon Eclipse Ts2R, Nikon, Japan) equipped with DS-Ri2 camera (Nikon, Japan) and impedance

spectroscopy. When the estimated bilayer capacitance density (capacitance per area determined by the capacitance value extracted from the impedance spectroscopy divided by the bilayer area estimated from the bright field microscopy) was above 0.6 μF/cm^2 and the bilayer resistance was in GΩ range, further experiments were performed. Electrical measurement was recorded with an Autolab PGSTAT302N potentiostat equipped with a FRA32M and ECD Modules for low current detection. First, to monitor the incorporation of the peptides to the bilayers, peptides were added to the *cis* compartment, while keeping the voltage at 50 mV. Next, a voltage step sequence, 0 mV, 25 mV, -25 mV, 50 mV, -50 mV, 75 mV, -75 mV, 100 mV, -100 mV, 125 mV, -125 mV, 0 mV, where the potential was halt for 60 s at each potential was applied. The appearance of the bilayer was being monitored simultaneously by bright field microscopy. Before and after each voltage step sequence, impedance spectroscopy was performed to confirm membrane capacitance and resistance. Obtained impedance spectra were analyzed by Nova 1.11 Software (Metrohm, Switzerland).

For single channel current monitoring, the voltage was fixed at 50 mV or -125 mV (Fig. 4e) without voltage sweep. The obtained step currents *I* were expressed as a conductance *g* based on the Ohm's law and were further converted into diameters by the following Hille equation(6):

$$g^{-1} = \frac{4 \cdot l \cdot \rho}{\pi \cdot d^2} + \frac{\rho}{d},$$

where g (= 50.4 pS) is the conductance from the single pore calculated from *I*, l (=3.7 nm) is the bilayer thickness, ρ (= 0.0481 Ωm) is the solution resistivity, from which the diameter of the single pore d was calculated.

Cryo-Electron Microscopy

Sample preparation: POPC lipids dissolved in chloroform were dried under nitrogen and left in desiccator for overnight, re-suspended in a buffer solution (10mM HEPES, 150mM NaCl, pH=7.4) at a final concentration of 15 mM, and filtered through 220 nm pore size membrane prior to use. Vesicles were prepared by 41 cycles of extrusion through two polycarbonate membranes with 100 nm pore size just before the experiment. Four samples were imaged: POPC vesicles, POPC vesicles incubated with LL-37, HNP1 and their 1:1 molar mixture. For the vesicle sample with peptides, POPC vesicles were incubated with each peptide or their mixture at the final vesicle concentration of 1.5 mM and the final LL-37 or HNP1 concentration of 150 μM, where in case of the mixture 150 μM of LL-37 and 150 μM of HNP1 were mixed, for 30 minutes at room temperature prior to vitrification. This makes the lipid to peptide ratio L/P=10 for the samples with individual peptides and 20 for that with peptide mixture.

Sample preparation for image acquisition with Krios G3i Cryo-EM

CryoEM grid (TED Pella 200 mesh 2x2) was treated with air plasma for 30 s and 10 mA current (Plasma Leica EM ACE 200). Then 4 μl of each sample was applied to the grid at 100% humidity, 1.5 s blot time, at 4 °C and vitrified in liquid ethane/nitrogen (Vitrobot, FEI).

Image acquisition: Frozen samples were imaged using Thermo Scientific Krios G3i Cryo-Transmission Electron Microscope (Cryo-TEM) operated at 300 kV. Samples were imaged in tomography mode from -60° to 60° every 2°.

Sample preparation for image acquisition with Talos Cryo-EM

CryoEM grid (Lacy Carbon Only TED PELLA) was treated with air plasma for 40 s. Then 4 μl of each sample was applied to the grid and vitrified at 90% humidity, 3 s blot time. Samples

were imaged with FEI Talos Cryo-Transmission Electron Microscope operated at 200 kV with Ceta CMOS camera.

Supporting references

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