Supporting Information for

Nano-mechanical in-process monitoring of antimicrobial poration in model phospholipid bilayers.

Andrea Valsesia,[a]† *Patrizia Iavicoli,*[a] *Helen Lewis,*[b] *Cloé Desmet,*[a] *Dora Mehn,*[a] *Luigi Calzolai,*[a] *Pascal Colpo,*[a] *François Rossi* [a] *and Maxim G. Ryadnov* [b] †

Materials and Methods

Materials

Palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG) were purchased from Avanti® Polar Lipids, Inc. Salts used for the preparation of the buffer (monosodium phosphate monohydrate and disodium phosphate dihydrate), poly(diallyldimethylammonium chloride) (PDDA) and Poly(sodium 4-styrenesulfonate) (PSS) were provided by Sigma Aldrich. Filtered (0.22μm) Milli-Q® water (Millipore S.A.) was used in all of the experiments.

Liposome preparation

Unilamellar vesicles consisting of POPC:POPG (3:1 molar ratio) were prepared following a procedure described elsewhere.[S1] The lipids were dissolved in a chloroform:methanol (2:1 v:v) mixture at a concentration of 10 mg/ml. The solution was then evaporated under nitrogen flow overnight. The obtained dried film was re-suspended in 10 mL of a 10 mM phosphate buffer, pH 7.4. The preparation was alternatively vortexed for 2 minutes and sonicated at room temperature for 2 minutes. The cycle was repeated three times. The dispersion was extruded 21 times through a 100 nm polycarbonate membrane before each use.

Determination of liposome size by Dynamic Light Scattering (DLS)

The particle size distribution was measured using a Malvern Zetasizer Nano-ZS instrument. Each sample has a volume of 500 μL in a PMMA cuvette and each measurement was done at 25°C in triplicate. The refractive index of liposomes used was 1.45 and the absorption coefficient was 0.001. The number of data sets of each measurement is automatically optimized by the instrument according to the quality of the sample and the intensity of the scattered light (ideally the correlogram should have amplitude around 0.8). The data were analyzed using the manufacturer's Dispersion Technology Software (DTS version 7.01), using both the cumulant (single exponential) and multimodal analysis (multi exponential-Malvern, DTS algorithm). From the cumulant analysis, the value of hydrodynamic diameter (Z-ave in nm) and polydispersity index (PdI) were determined. If the PdI is larger than 0.5, the hydrodynamic radius is calculated from the multimodal analysis. The instrument calibration was checked by using varied sizes of Polystyrene latex (PSL) beads standards.

Peptide synthesis

All peptides were assembled on a Rink amide MBHA resin using standard Fmoc/^{t}Bu solid-phase protocols with HBTU/DIPEA as coupling reagents on an automated Liberty microwave peptide synthesizer (CEM Corp., USA). The peptides were purified using reversed phase high performance liquid chromatography (RP-HPLC). Peptide identities were confirmed by analytical RP-HPLC and MALDI-ToF MS on an Autoflex III (Bruker,

Germany). Analytical and semi-preparative RP-HPLC was performed on a JASCO HPLC system (PU-980; Tokyo, Japan) using a Vydac C18 analytical and semi-preparative (both 5 μm) columns (Grace, USA). Both analytical and semi-preparative runs used a 10-70% B gradient over 30 min at 1 mL/min and 4.5 mL/min, respectively, with detection at 280 and 220 nm (buffer A, 5% and buffer B, 95% aqueous CH_3CN, 0.1% TFA). The purified peptides were lyophilised and stored at -80°C until use. Stock solutions were prepared from lyophilised peptides dissolved in Milli-Q water.

Ultracentrifuge

Sedimentation coefficient of the vesicles was measured in phosphate buffered saline solution. The sedimentation velocity experiments were performed at 10000 rpm, 20 °C, using a Beckman Coulter analytical ultracentrifuge. Interference based data were acquired for 5 hours and analysed using the SedFit software. In order to obtain an estimate of the density of the vesicles (reciprocal partial-specific volume) the ls-g*(s) model was applied for fitting in the sedimentation coefficient range 1-500 S and considering the hydrodynamic diameter determined by DLS.

Analytical Ultracentrifugation

Analytical Ultracentrifugation (AUC) is a powerful method used mainly in protein or macromolecule size analysis.[S2] However, the technique can be easily adapted to the characterization of nanoparticles, including liposomes with higher density than their dispersion medium. The sedimentation of the lipid vesicles in a centrifugation field was monitored by an interference optics system, allowing the generation of apparent sedimentation coefficient (s) distribution curves by using a least-squares based analysis, ls-g*(s). This can be transformed to size (D, Stokes-diameter) distributions applying the Stokes equation:

$$D = \sqrt{\frac{18\eta_m s}{\rho_p - \rho_m}}$$

Where η_m is the viscosity of the medium, ρ_p and ρ_m are the density of the particles and the density of the medium, respectively.

In case of known diameter, density of the particles can be estimated by iterative search for the density value resulting in a match between the mode of the size distribution curve and the hydrodynamic diameter (zeta average) determined by DLS. Figure S1 shows the sedimentation coefficient distribution calculated for the model vesicles.

The sedimentation coefficient distribution was calculated using the ls-g*(s) model, while the mass density was calculated by matching the particle size distribution using various partial specific volume values with the hydrodynamic diameter of the nanoparticles measured by DLS. The calculated liposome density value was 1014 Kg/m^3.

Substrate preparation

Quartz and silicon substrates were functionalized with poly(diallyldimethylammonium chloride) (PDDA) and poly(sodium 4-styrenesulfonate) (PSS). First a layer of Teflon-like (PTFE) was deposited onto the substrates using plasma-enhanced chemical vapour deposition.[S3] Then the functionalized substrates were immersed three times alternatively into a solution of PDDA 2% and PSS 2% for 2 minutes. The formation of each monolayer of polyelectrolytes (PE) was monitored by ellipsometry (J.A. Woolam, Inc) as well as the

evolution of the contact angle with water (Microdrop, France). The surface became more and more hydrophilic by increasing the number of PE layers. The surface was quenched with a PDDA layer, introducing amine groups on the surface. The final surface had a contact angle of 30±3°.

Freshly prepared liposomes were diluted (2 mg/ml in phosphate buffer, pH 7.4) and immobilized in parallel on four Au-coated QCM-D quartz crystal chips functionalized with the polyelectrolyte multilayers, terminated with PDDA.

Quartz crystal microbalance with dissipation monitoring (QCM-D) measurements

The instrument was a QCM-D E4 (Q-Sense AB, Gothenburg, Sweden), which uses four temperature-stabilized measurement cells (in parallel configuration in our case). The QCM-D instrument was able to record frequencies up to the 13th overtone. Polished AT-cut Au-coated sensor quartz discs (14 mm in diameter, 0.30 mm thick) with a fundamental frequency of 5 MHz (Q-Sense AB, Gothenburg, Sweden) were used for the QCM-D experiments. The quartz crystals were cleaned by sequential immersion in 1 M NaOH, 1 M HCl, acetone, and ethanol for 10 min at a time prior surface modification with PTFE and PE multilayers.

1) Liposomes were immobilized on the four functionalized QCM-D quartz crystals. The liposomes solution was dispensed on the quartz crystals with a peristaltic pump at a constant flow rate of 20 µl/min for 20 minutes. Then the buffer was injected to remove the unbound liposomes from the surface. The immobilization kinetics and their stability in time were monitored for 80 minutes.

2) In step 2 the antimicrobial peptides were injected at two different concentrations. The interaction of the peptides with the liposomes layer was monitored as a function of time for 240 minutes.

QCM-D curves were recorded at constant flow rate of 20 µl/min and at a concentration of 1%. The fitting of the QCM-D curves was done using the software Q-Tools (Q-sense AB, Sweden). A Voigt viscoelastic model has been used.

This measured value for the density of the liposomes, 1014 Kg/m^3 was used as a fixed value in the mechanical model. The value for the shear elastic modulus was fixed at E_{layer} = 1 KPa.[S4] The fitting routines were carried out using D_{Layer} and E_{layer} as fixed parameters and the viscosity V_{Layer} and thickness Th_{Layer} as tuned parameters. The F-D curves from two overtones (F7 and F9) were chosen for the fitting. The fitting routine was stopped upon reaching the minimum value for the χ^2 of 8 x 10^7. This is reasonable given that the viscoelastic modelling includes a number of ideal mechanical elements which are far away from the reality of a complex system. Furthermore, the obtained value for the $\chi 2$ is the result of the statistical deviation from 4 functions per routine (F7, F9, D7 and D9) for thousands of time points.

Atomic force microscopy imaging

AFM images were acquired with a NT-MDT Solver instrument by scanning the sample using a standard soft silicon cantilever (k = 0.01 Nm) with a tip with nominal radius < 10 nm. The object identification was performed using the Nova control software of the instrument. Note: once adsorbed on the surface, liposomes remained intact upon air drying.

1) The lateral size (radius) was calculated taking into account the tip shape and dimensions with method described in [S5]. The average measured diameter was D_{ave} = 150±42 nm.

2) The average height was measured by counting the number of object per each height interval with a resolution of 5 nm. The number of objects was then weighted by the height and averaged. The average height was H_{ave} = 122±7 nm.

Dark Field Microscopy imaging in real time

DFM images were acquired using a Leica microscope with DF configuration in reflection mode using a 10X objective with NA = 0.45 and a field of view of 0.3 X 0.35 mm^2. The liposomes were immobilized on a silicon chip coated with the same functional layer used for the QCM-D experiments. The silicon chip was coupled to a transparent liquid cell (Ibidi, Germany), which was connected to a precision peristaltic pump. Note: since the DF imaging efficiency is proportional to the 6 power of the diameter of the objects, only the largest liposomes (> 100 nm) could be clearly visualized.

Minimum inhibitory concentrations assay

Minimum inhibitory concentrations (MICs) were determined by broth microdilution on *E. coli*, *S. aureus*, *B. subtilis* and *S. enterica* according to the Clinical and Laboratory Standards Institute. Typically, 100 μL of 0.5–1 × 106 CFU per ml of each bacterium in Mueller Hinton media broth (Oxoid) were incubated in 96-well microtiter plates with 100 μL of serial twofold dilutions of the peptides (from 100 to 0 μM) at 37 °C on a 3D orbital shaker. The absorbance was measured after peptide addition at 600 nm using a Victor 2 plate reader (Perkin-Elmer). MICs were defined as the lowest peptide concentration after 24 h at 37 °C.

Table and Figures

Table S1. Fitted mechanical parameters at 60 min post-injection.

Quartz crystal cell number	Th_{layer}, nm	V_{layer} x 10^{-3}, Kg/ms
1	53	2.08
2	54	2.02
3	58	2.20
4	54	2.22

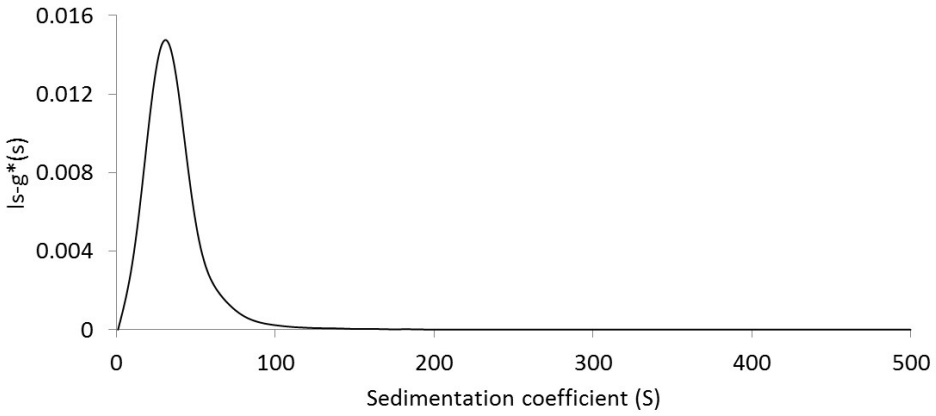

Figure S1. ls-g*(s) sedimentation coefficient distribution of the vesicles.

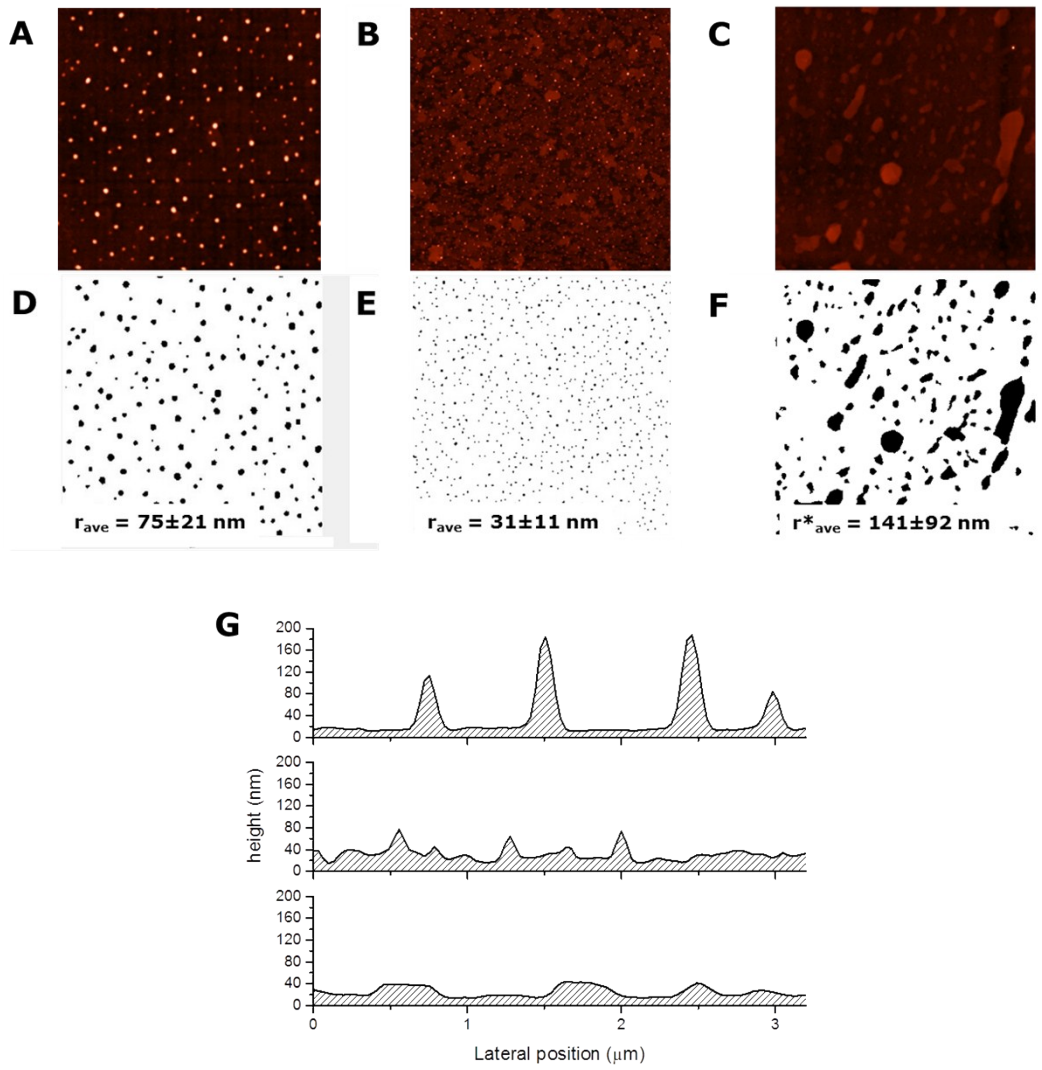

Figure S2. 10 x 10 μm AFM scans for functionalized silicon wafer after (a) liposomes adsorption (b) 40 minutes in contact with AMP-S at 1.4 μg/ml and (c) 120 minutes in contact with AMP-S at 1.4 μg/ml. Vertical scale for a, b, and c is [0 nm, 120 nm]. (d), (e) and (f) represent the objects identification for the images in (a), (b) and (c) respectively using a threshold value of 80 nm of height for (a) and (b) and 40 nm for (c). (g) Profile along a line containing some liposomes for (a), (b) and (c) respectively

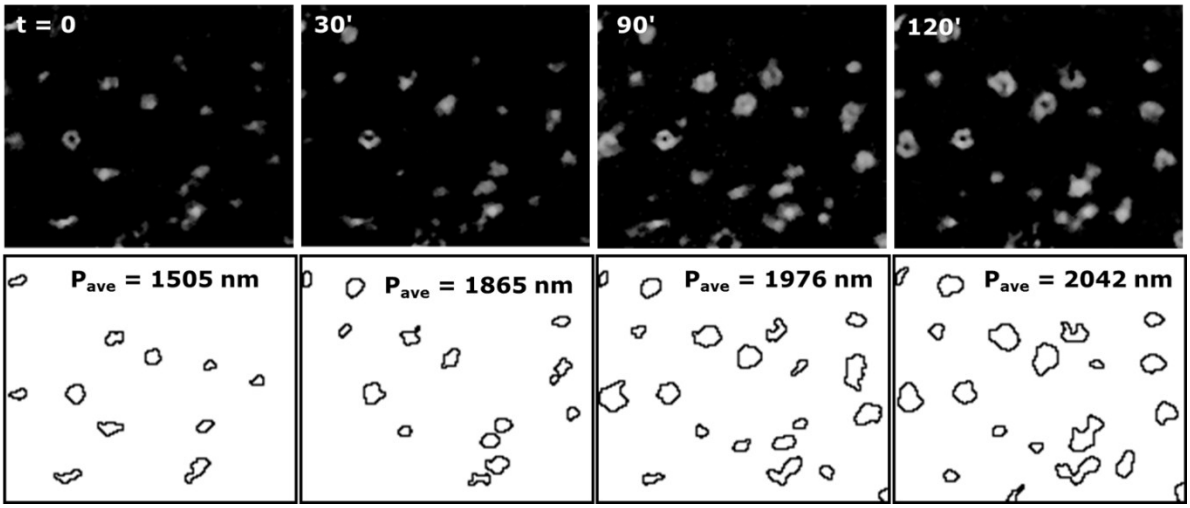

Figure S3. Real-time monitoring of tilamin-treated liposomes. Dark-field micrographs of the liposomes at 1.4 μg/ml peptide concentration. An image sequence was taken with a frame resolution of 2 minutes.

S1. P. Iavicoli, P. Urbán, A. Bella, M. G. Ryadnov, F. Rossi and L. Calzolai, *Journal of Chromatography A*, 2015, **1422**, 260-269.

S2. J. L. Cole, J. W. Lary, T. P. Moody and T. M. Laue, *Methods in cell biology*, 2008, **84**, 143-179.

S3. L.-M. Lacroix, M. Lejeune, L. Ceriotti, M. Kormunda, T. Meziani, P. Colpo and F. Rossi, *Surface Science*, 2005, **592**, 182-188.

S4. G. Ohlsson, A. Tigerström, F. Höök and B. Kasemo, *Soft Matter*, 2011, **7**, 10749-10755.

S5. J. Canet-Ferrer, E. Coronado, A. Forment-Aliaga and E. Pinilla-Cienfuegos, *Nanotechnology*, 2014, **25**, 395703.