Supporting information

Decorating nanostructured surfaces with antimicrobial peptides to efficiently fight bacteria

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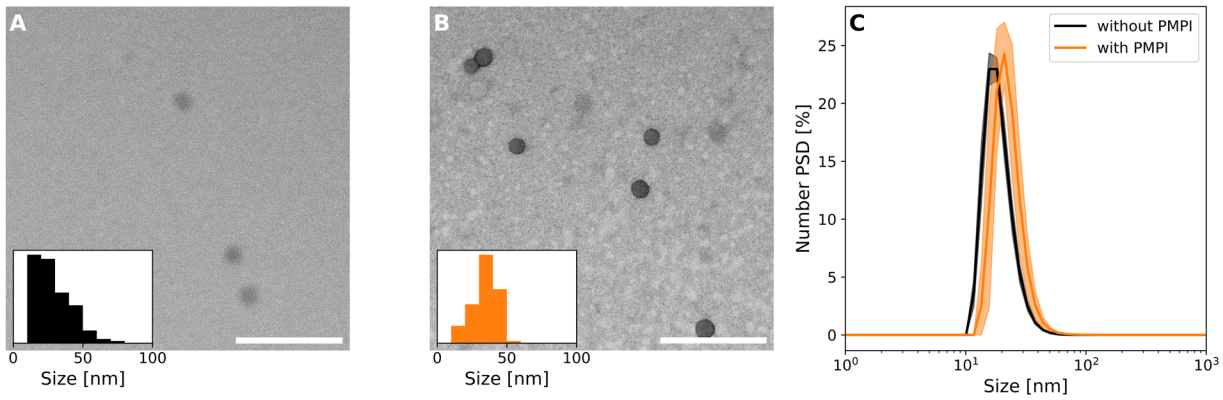

Figure S1. A) TEM micrographs with inlets of a size distribution histogram measured by image processing of A) micelles assembled from $PMOXA_{18}$-b-$PDMS_{69}$-b-$PMOXA_{18}$. and B) assembled micelles reacted to p-Maleimidophenyl-isocyanate (PMPI). Scale bars: 200 nm. C) DLS measurement of micelles without PMPI (black) and after reaction to PMPI (orange) revealed similar number particle size distributions (PSD).

Liposome leakage experiments

Material and methods

Anionic bacterial-mimicking model liposomes were self-assembled from a 75/25 mol/mol mixture of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) / 1,2-dioleoyl-sn-glycero-3-phosphoglyce-rol, monosodium salt (DOPG). DOPE and DOPG were purchased from Avanti Polar Lipids (Alabaster, U.S.A.). The lipid mixture was dissolved in chloroform and dried to a thin film under vacuum. The thin film was rehydrated with 0.1 M carboxyfluorescein (CF, Sigma, St. Louis, U.S.A.) dissolved in 10 mM Tris buffer, pH 7.4. The hydrated lipid mixture was treated with eight freeze-thaw cycles in liquid nitrogen followed by one minute heating in a 60°C water bath and 30 s vortexing. The sample solution was extruded 30 times through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) to obtain unilamellar liposomes. Non-encapsulated CF was removed by two subsequent gel filtrations (Sephadex G-50, GE Healthcare, Uppsala, Sweden) with Tris buffer as eluent. CF leakage was monitored on a SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, U.S.A.) through emitted fluorescence at 520 nm. Liposomes were diluted with Tris buffer to obtain a 10 µM lipid sample for the leakage assay. After 10 min the substrate (peptide or micelle) was

added and after another 30 min 0.8 mM Triton X-100 (Sigma-Aldrich, St. Louis, U.S.A.) was dropped into to disrupt the liposomes and obtain 100% leakage while the emitted fluorescence was monitored for another 5 min. Micelles were decorated with cysteine terminated KYE28 (Bio Peptide, San Diego) by mixing micelles and peptide together and keeping them at 4°C until gel filtration was conducted to remove non-attached KYE28.

Results

Liposomes self-assembled from DOPE/DOPG (75/25 mol/mol) are mimicking the anionic bacterial membrane and are extensively used in AMP studies. Importantly, DOPE/DOPG membranes have been demonstrated to give similar results on AMPs interactions as membranes prepared from *E. coli* lipid extracts, as well as agreeing with bacterial lysis experiments.[1] CF, in turn, is a fluorescent dye which is self-quenched at high concentrations, thus showing increased fluorescence when diluted.[2] CF was encapsulated in bacterial membrane mimicking liposomes to monitor the ability of the peptide and micelles to induce leakage in DOPE/DOPG liposomes (Figure S2A). The detergent Triton X was added in the end to determine 100 % leakage. In the self-leaking experiment, the stability of the liposomes was confirmed. The peptide free in solution caused leakage in a dose-dependent manner, reaching 100% leakage at peptide concentration of 1 μM, and 45% at 0.1 μM (Figure S2A). The micelles (10μM polymer) alone did not result in any detectable liposomes lysis. In contrast, peptide-polymer nanoparticles loaded with 1 μM peptide before purification by gel filtration resulted in 67 % leakage (Figure S2B).

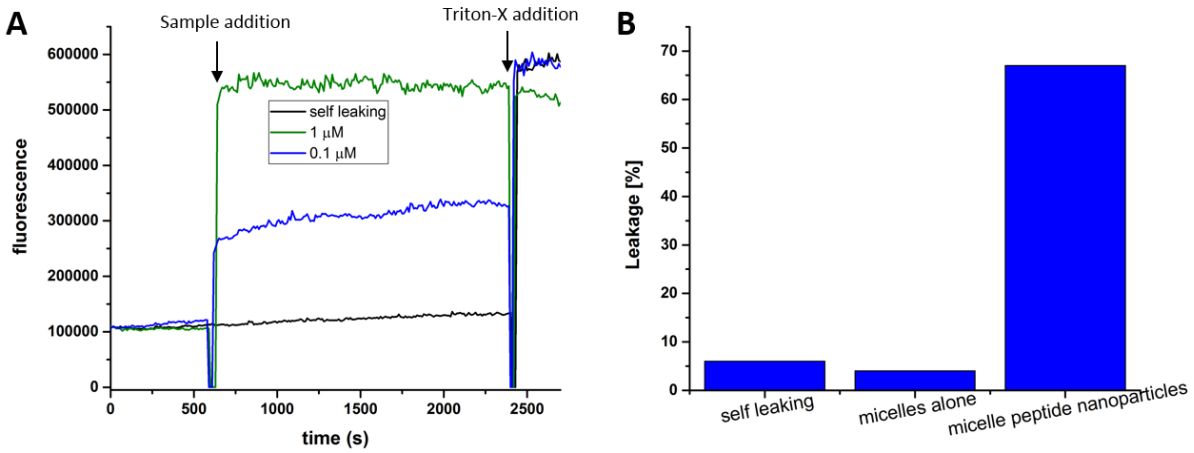

Figure S2. Leakage experiments on CF-containing DOPE/DOPG liposomes. After 10 min, the sample was added to the liposome solution and after monitoring leakage for an additional 30 min, Triton-X was added to cause 100 % leakage. Shown are results on A) Peptide alone in two different concentrations: 1 µM Peptide led to 100% leakage while 0.1 µM led to 45% leakage. B) Micelles alone did not cause any leakage; the leakage was in the range of the self-leaking of liposomes. The AMP decorated micelles caused nearly 70% leakage.

Thiol functionalization

AFM measurements provide information not only about the height, but also about phase, the latter indicating if the interaction between the AFM tip and the surface is attractive or repulsive. Thiol functionalization with a 1:1 mixture of ethyl and thiol functionalized silanes results in yellow ellipsoidal patches of 10-50 nm in diameter with increased attractive behavior towards the tip than other regions. We assumed these to be the thiol regions due to the higher hydrophilicity of the thiols compared to the ethyl groups.

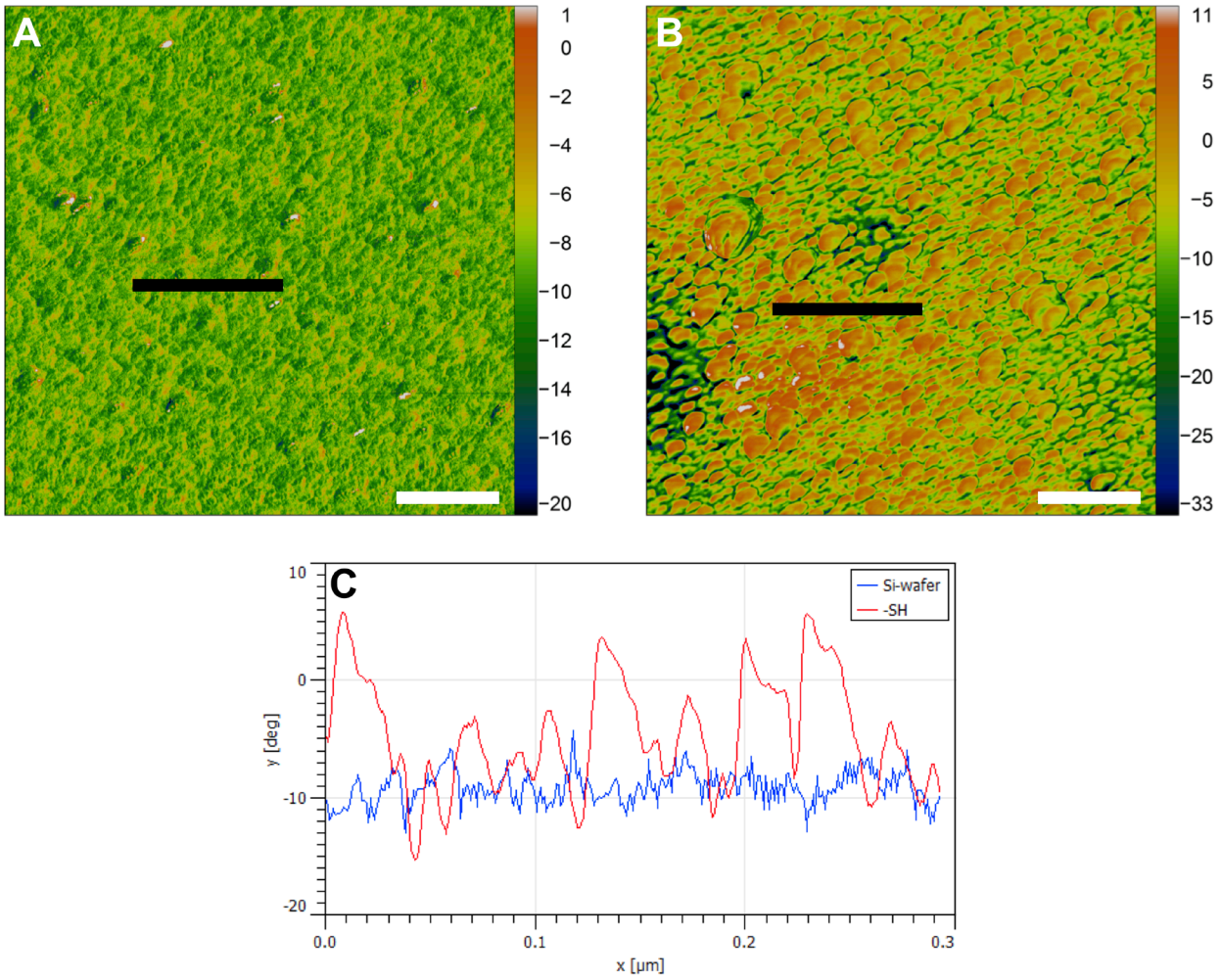

Figure S3: AFM images showing the phase (°) channel of A) untreated Si-wafer, and B) thiol-functionalized Si-wafer. Scale bars are shown in white: 200 nm. C) Measured profile lines in black (300 nm, A, B) of the phase are displayed.

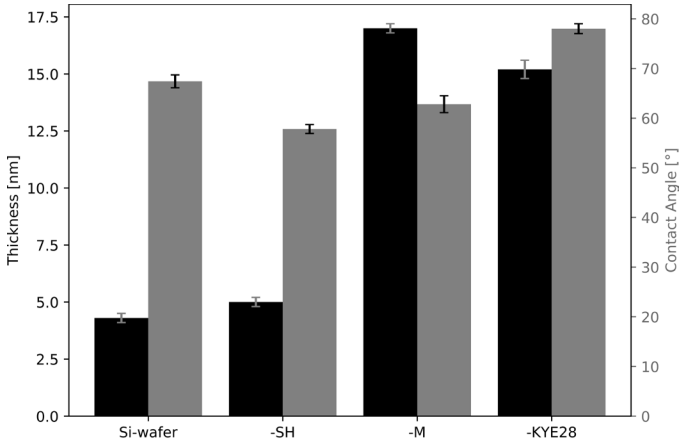

Figure S4. In situ assessment of thickness by ellipsometry (black bars) and static water contact angles (grey bars) during different stages of surface fabrication. Error bars are standard deviations of samples (n=3).

Micelle immobilized surfaces

Micelles were reacted to different amounts of PMPI to yield maleimide-exposing micelles. Similar fluorescence intensities after immobilizing Bodipy-stained, maleimide-exposing micelles were obtained for micelles reacted to different concentrations of PMPI. Significantly lower fluorescence and therefore fewer micelles on the surface, was noted for micelles without maleimide (Figure S5). The surface roughness was calculated from the atomic force microscopy (AFM) micrographs. AFM revealed that the less PMPI used for maleimide grafting, the rougher the final surface, although complete absence of maleimide resulted in the lowest roughness. The surface corrugation is indicative of individual micelles being immobilized as individual micelles are larger and stick out from the surface. Together, these results show that for the successful immobilization on thiol-functionalized surfaces, maleimide-exposing micelles are crucial.

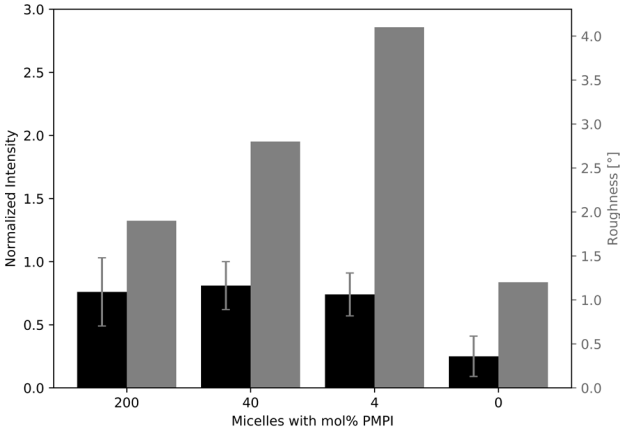

Figure S5: Confocal laser scanning microscopy intensities (black) comparison and AFM calculated roughness results (grey), comparing immobilized micelles treated with different mol% PMPI (0-200) prior to immobilization.

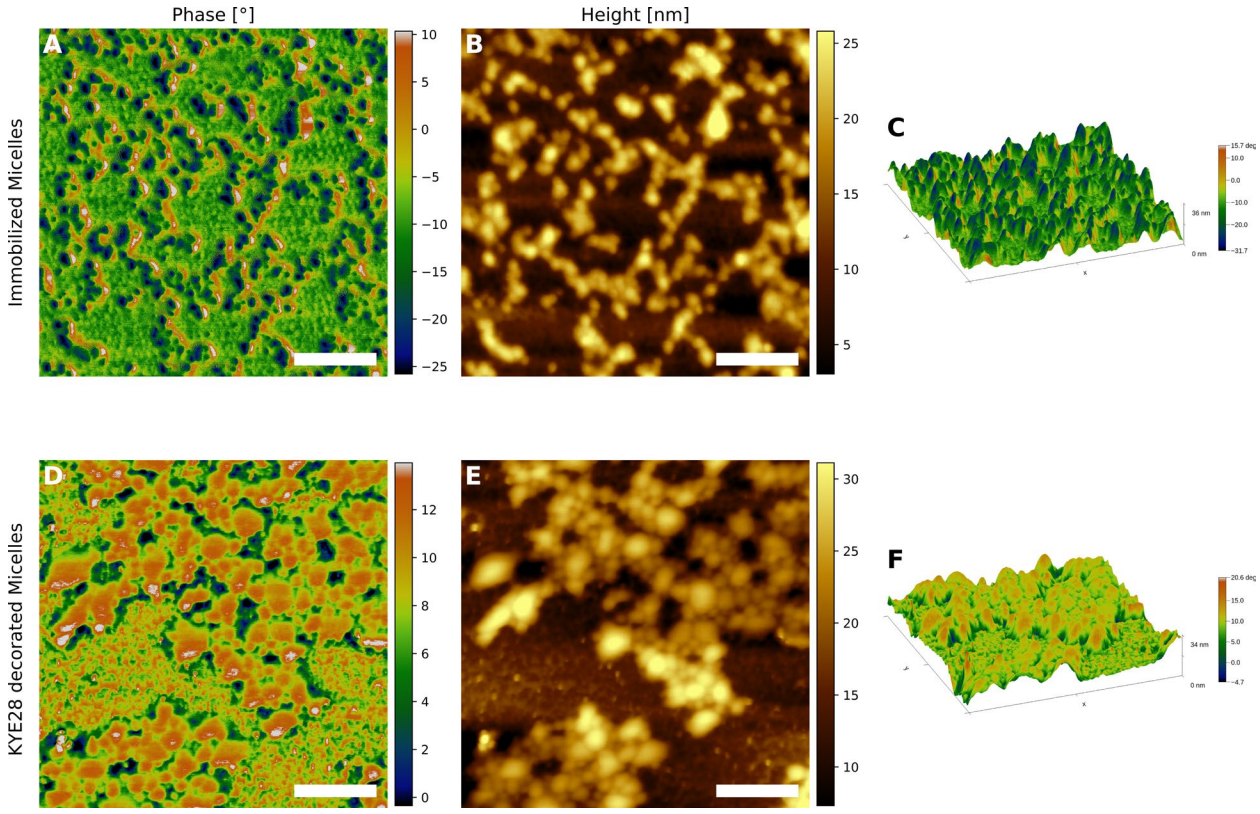

Figure S6: AFM micrographs recorded in air of immobilized micelles. A) and D): Phase contrast images, B) and E) height images, and C) and F) 3D-images of height with phase as overlay of immobilized micelles (top), and of a surface after KYE28 adsorption (bottom). Scalebars are A-B) 400 nm, and D-E) 200 nm. C) Axis length is 2 μm, and F) axis length is 1 μm.

KYE28 synthesis and purification

Material and methods

The peptide KYE28 (KYEITTIHNLFRKLTHRLFRRNFGYTLR) was synthesized by microwave-assisted automated solid phase peptide synthesis (Liberty Blue, CEM Corporation). Cl-MPA ProTide resin (0.2 mmol g-1, 200 mesh, CEM) was used for the synthesis with *N,N*-dimethylformamide (DMF) (J.T. Baker) as the solvent. 9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids Ile, Lys(Boc), Tyr(tBu), Asn(Trt), Leu, and Phe were purchased from Novabiochem and Gly, Thr(tBu), Arg(Pbf), His(Boc) from Iris Bio. Fmoc-protected amino acids were de-protected with piperidine (20% v/v, 3 mL) (Sigma), followed by coupling the next amino acid by amide bond formation in the presence of N,N'-Diisopropylcarbodiimide (DIC)

(0.25 M, 1 mL. Sigma), and a mixture of OxymaPure (CEM) and DIEA (0.5 M and 0.05 M, 0.5 mL). The microwave power was set to reach 90°C (50°C for Histidine) during de-protection and coupling. The first amino acid was coupled to the resin in a more basic environment, a mixture of diisopropylethylamine (DIEA) (Sigma) and KI (1 M and 125 mM, 1 mL) (Sigma). After microwave assisted synthesis of the complete sequence, the peptide-bound resin was transferred to a syringe vessel, rinsed with dichloromethane (DCM, VWR), and allowed to dry in air. Resin cleavage and amino acid side chain de-protection was accomplished by adding cleavage solution (4 mL) consisting of trifluoroacetic acid (TFA, VWR), H_2O and Triisopropylsilane (TIS, Fluka) (95 %/2.5 %/2.5 %) and stirring for 4 h (RT, 80 rpm). After collection of the TFA mixture containing the cleaved peptide, the resin was washed several times with cleavage solution and the supernatants combined with the peptide solution. The peptide was precipitated in ice cold diethyl ether (40 mL) purchased from Sigma and pelleted by centrifugation (5 min, 2300g). The supernatant was removed and the precipitated peptide resuspended in ice cold diethyl ether (repeated 3x). The precipitated peptide was finally dissolved in 0.1 % TFA in water and lyophilized. The peptide was purified by semi-preparative reverse phase HPLC (Shimadzu) on a Proto 300 (Higgins Analytical) C18 column (250 mm x 10 mm) with 5 µm particle size at a flow rate of 4 ml min^{-1}. The mobile phase used was a mixture of acetonitrile (MeCN) and water (H_2O), each acidified with 0.1 % v/v TFA. The run was initiated with 5 % MeCN increasing linearly to 25 % over the first 4 min. Then, up to minute 26, a linear gradient from 25 % to 38 % MeCN was applied to collect KYE28 around minute 24 (Figure S8A). After this, the column was washed with 95 % MeCN up to minute 30 and decreased again back to 5 % over half a minute where it stayed until the end of the run at minute 37. UV-Vis absorbance was simultaneously measured focusing on $\lambda = 214$ nm. The solvents were evaporated in a rotary evaporator, followed by lyophilization.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-Tof-MS) analysis of the peptide was performed on a Bruker Microflex system. A saturated α-cyano-4-hydroxycinnamic acid (purchased from Sigma) solution (1:1, v/v, H2O:MeCN, 0.1 % TFA) was mixed with 100 pmol μL^{-1} of purified peptide solution in H_2O at a sample to matrix ratio of 1:20 and 1 µL of the mixture was dropped onto the target plate and allowed to air dry.

Results

The AMP KYE28 was synthesized by Fmoc solid-phase peptide synthesis with microwave heating having the advantage of reduced synthesis time and chain aggregation.[3] This peptide was chosen as a model peptide due to its antimicrobial and anti-endotoxic properties.[4] A yield of up to 22 % after HPLC purification was achieved. The mass was confirmed by MALDI ToF-MS, where the single and double charged species are observed at 3591 m/z and 1792 m/z, respectively (Figure S7B).The peptide could be purified successfully as visible by the HPLC elugram of the purified KYE28 solution (Figure S9).

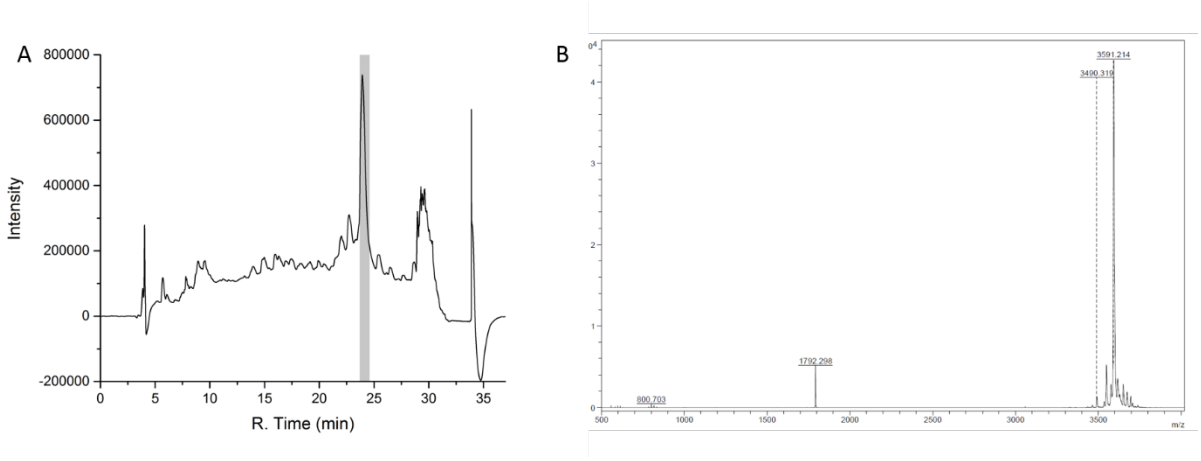

Figure S7: A) HPLC elugram. The grey area was collected. B) Linear positive MALDI-ToF spectra of the collected KYE28.

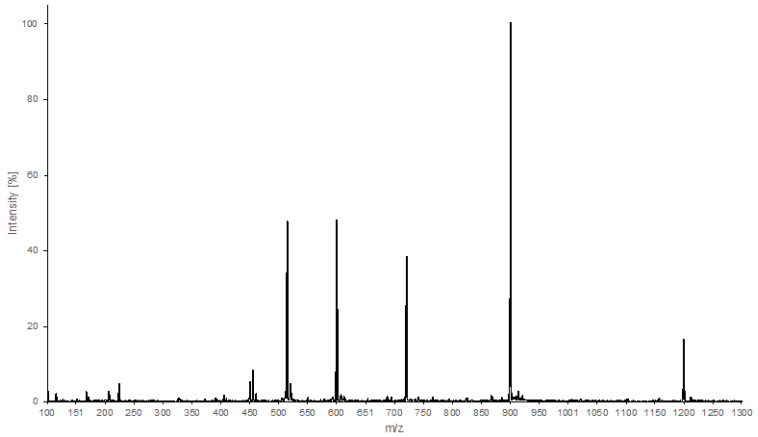

Figure S8: Positive electron spray ionization mass spectroscopy image of KYE28 after 1.5 years stored at rt. With the 5 largest peaks M+7 to M+3 (left to right).

Modification of surface-attached micelles with antimicrobial peptide KYE28

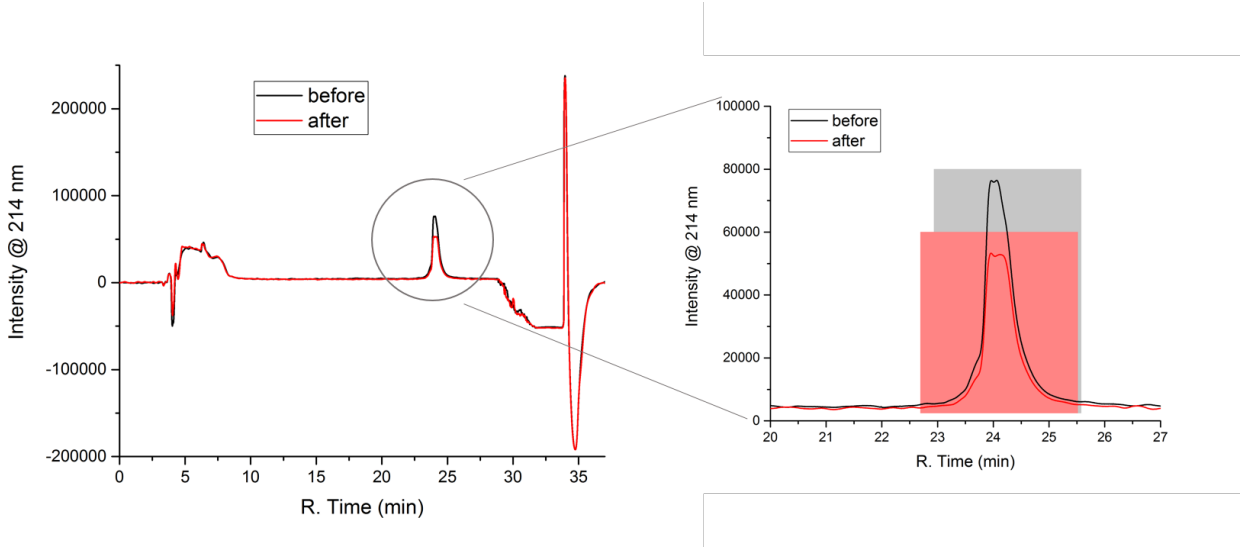

Figure S9: HPLC elugram of the KYE28 solution before immersing the micelle immobilized surface in it (black) and after immersing the surface in it (red) to let the peptide adsorb on the immobilized micelles.

Surface analysis by XPS showed changes in the C1s core level spectra for each surface, demonstrating chemical differences. Contributions around 286.4 eV and 288.2 eV for C-N and O=C-N,[5] respectively, were found in the PMOXA block of the polymer in the micelles, as well as in the peptide backbone. For the peptide-decorated surface, more components were expected due to the different amino acids present in KYE28. Histidine, Arginine, and Glutamic acid (287, 289 eV) were shifting the peak towards higher binding energies.[5] A long peptide then increases the complexity of the peak with many different contributions and therefore more components at different binding energies are needed to model it (Figure S10).

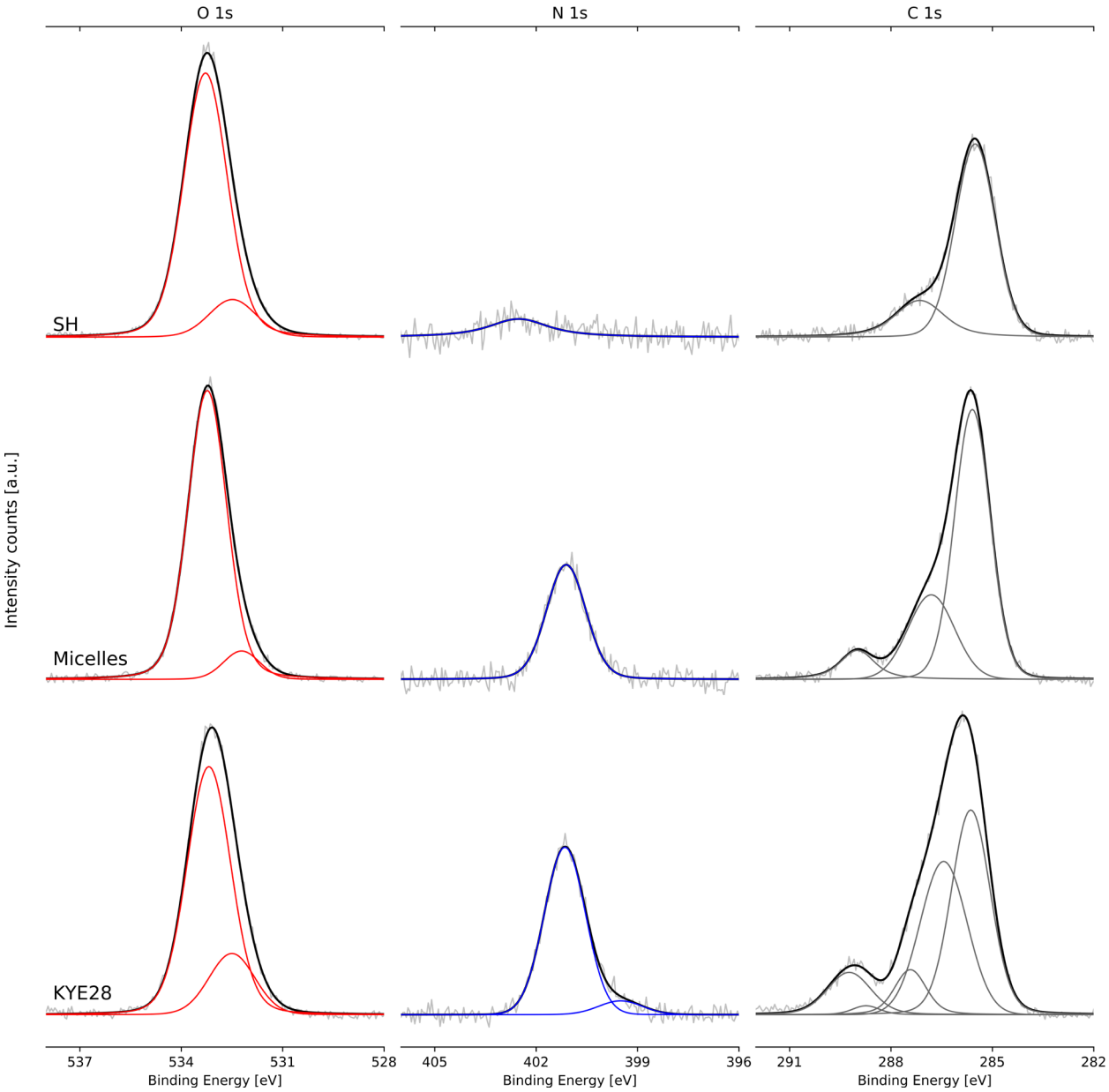

Figure S10: XPS O-1s, N-1s, and C-1s core level spectra of thiolated, micelle immobilized and peptide decorated surfaces. Measurements are displayed in a semi-transparent line while the fitted sum of the components is in black and in color the components for O 1s, N 1s, and C 1s, respectively.

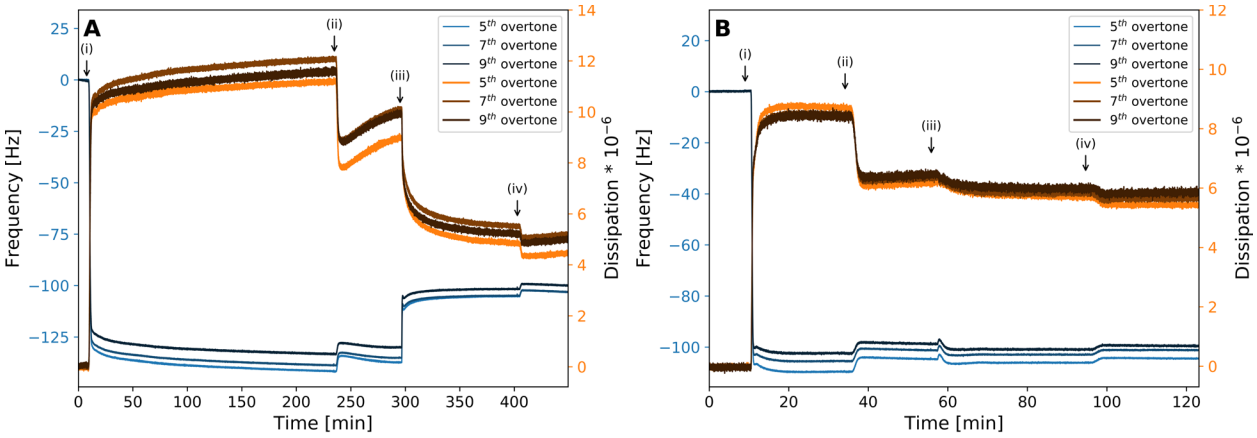

Figure S11: A) QCM-D diagram. Blue colors show the frequencies of the 5th, 7th, 9th overtone whereas the brown colors show the dissipations of the same overtones. (i) Injection of micelles dispersed in H_2O, (ii) 1st washing with H_2O, (iii) injection of KYE28 dissolved in H_2O, and (iv) 2nd washing with H_2O. B) QCM diagram. Repetition of the experiment displayed in A.

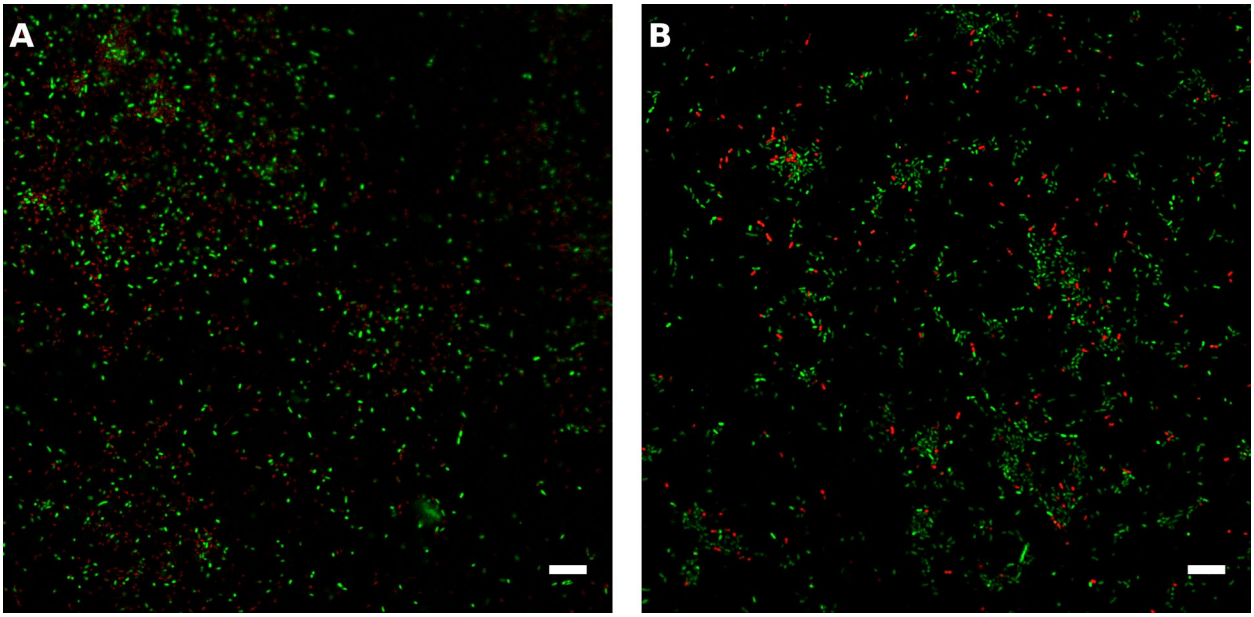

Figure S12: Static biofilm assay. Surfaces were visualized by CLSM after staining the adhering bacteria with LIVE/DEAD stain. Bacteria with a damaged membrane were stained with propidium iodine (red, dead), whereas bacteria with intact membranes were stained with cell permeable Syto9 (green, alive). A) Si-wafer, and B) KYE28 on a thiolated surface. Scale bars, 10 µm.

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