Nanoscale imaging reveals laterally expanding antimicrobial pores in lipid bilayers

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Supporting Information

Design notes

Amhelin has the typical length of AMPs characterised by comparable antimicrobial activities. The sequence is based on a repetitive *PPPHPPH* pattern (P – polar, H – hydrophobic) which promotes amphipathic helix formation and allows for the placement of *i* and *i+7* residues in close proximity in the folded helix (Figure S1). *i* and *i+7* residues are of the same type which allows hydrophobic and polar residues to segregate onto distinct regions or faces – the principle feature of AMPs. Importantly, the peptide folds only upon binding to microbial anionic membranes – another principle feature of AMPs. To achieve this, the pattern was tuned into a *CNCHNCH* repeat, where *C* is cationic and *N* is neutral polar. The pattern ensures the 1:1.5 ratio of hydrophobic and cationic residues to avoid cytotoxic and hemolytic effects of venom peptides. The neutral residues are separated in the sequence at standard *i, i+3* and *i, i+4* helical spacings and are alanines and glutamines. Having all-alanine or all-glutamine clusters may promote stronger hydrophobic interactions leading to uncontrolled aggregation which were to be avoided. Therefore, alanines and glutamines alternate in the neutral cluster which was also placed opposite to the hydrophobic face (Figure S1). Further, the sequence exhibits a canonical coiled-coil pattern in which hydrophobic side chains alternate three and four residues apart (*i, i+3* and *i, i+4* spacings). Such patterns are usually referred to as 3,4 hydrophobic patterns and are prerequisite for cooperative oligomerization. The hydrophobicity index for leucines is one of the highest (i) to strongly bind to lipids and (ii) to support cooperative AMP oligomerization in lipid bilayers.

Because the design is a helical peptide all amino acids used exhibit high helical propensity. Leucines and lysines are most common in AMPs. Leucines also provide a better control over oligomerization and aggregation and are more accessible synthetically with much lower solubility problems when compared to other hydrophobic amino acids (phenylalanines, tryptophans). Nonetheless, other amino acids showing similar hydrophobicity, hydrophilicity and helicity indexes can be used in the pattern achieving similar results. For example, another AMP sequence (AMP2) with activities comparable to those of amhelin (Table S1), comprises a combination of hydrophobic isoleucine and leucine residues that favour cooperative assembly of lower oligomers. The peptide forms expanding pores within the same timescales (Figure S6).

Materials and methods

High performance liquid chromatography. Analytical and semi-preparative gradient RP-HPLC was performed on a JASCO HPLC system using Vydac C18 analytical (5µm) and semi-preparative (5µm) columns. Both analytical and semi-preparative runs used a 10-60% B gradient over 50 min at 1 mL/min and 4.5 mL/min respectively with detection at 230 and 220 nm. Buffer A − 5% and buffer B − 95% aqueous CH$_3$CN, 0.1% TFA.

Lipid vesicle preparation. The lipids, 1,2-dilauroylphosphatidylcholine (DLPC) and 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DLPG), 75%/25% (w:w) used for liposome construction were from Avanti Polar Lipids (Alabaster, AL, USA). The lipids were weighted up, dissolved in chloroform-methanol (2:1, v:v), dried under a nitrogen stream, and placed under vacuum overnight. The resulting film was hydrated to 10 mg/ml total lipid concentration in 10 mM phosphate buffer, pH 7.4. The suspension was then extensively vortexed, sonicated (30°C), and extruded using a hand-held extruder (Avanti Polar Lipids) (fifteen times, polycarbonate filter, 0.05 µm) to give a clear solution containing small unilamellar vesicles (SUV), which were analysed (50 nm) by photon correlation spectroscopy. For solid state NMR the lipid film was rehydrated to 10 mg/ml in 20 mM HEPES buffer, pH 7. 75%/25% ratios of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) with 1,2-dimyristoyl-sn-

glycero-3-phosphoglycerol (DMPG) (all from Avanti Polar Lipids, Alabaster, AL, USA) were used to form thicker SLBs.

Photon Correlation Spectroscopy. Vesicles were re-suspended to a final concentration of 1 mg/mL and were analysed on a Zetasizer Nano (ZEN3600, Malvern Instruments, Worcestershire, UK). Dynamic light scattering batch measurements were carried out in a low volume disposable cuvette at 25°C. Hydrodynamic radii were obtained through the fitting of autocorrelation data using the manufacture's software, Dispersion Technology Software (DTS version 5.10).

Sample preparation for surface imaging. Oxidised silicon substrates (University Wafer), were cut in to ~1 cm^2 pieces and ozone-cleaned. The SUV solution was poured over the substrate in the culture dish with shaking for 2 hours to enable lipid bilayer deposition on the surface. The substrates were plunged into a dish of water, and kept with shaking for 3 min (3 times) to remove vesicle excess (27). The surface lipid bilayer was then placed into a dish and treated with a 10-µM peptide solution (10 ml) for 30 min, followed by washing to remove peptide excess (the step was omitted for control samples that did not require peptide treatment). The substrate without drying was transferred into a 2-ml container filled with water which was plunged into a chamber with liquid nitrogen to quickly freeze the entire sample. The lid of the container was replaced with a perforated lid and the sample was freeze-dried. The same procedures were performed for both supported lipid-bilayer and bare substrates. Bacterial cells were imaged directly following peptide treatment and washing.

Preparation of supported lipid bilayers for AFM in water. Supported lipid bilayers were formed on mica as described elsewhere (26) from a solution of 1.5 mg/ml DLPC:DLPG (3:1) in 150 mM NaCl, 20 mM HEPES pH 7.4 buffer with 20 mM MgCl$_2$ and 20 mM CaCl$_2$. After absorption, the solution was then washed three times with buffer, to remove the unfused vesicles from solution. Amhelin was introduced into the 100-µl fluid cell (JPK Instruments, Berlin, Germany) at a final concentration of 0.5 µM.

Minimum inhibitory concentrations assay. Minimum inhibitory concentrations (MIC) were determined by broth microdilution on *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* K12, *Staphylococcus aureus* ATCC 25723, *Micrococcus luteus* NCIMB 13267 and *Bacillus subtilis* ATCC 6633 according to the

Clinical and Laboratory Standards Institute. Typically, 100 µl of 0.5 - 1 x 10^6 CFU per ml of each bacterium in Mueller Hinton media broth (Oxoid) were incubated in 96 well micro-titre plates with 100µL of serial two-fold 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). Minimum inhibitory concentrations (MIC's) were defined as the lowest peptide concentration after 24 hours at 37°C. All tests were done in triplicate.

Stain-dead antimicrobial assay. *E. coli* culture (1 ml) was centrifuged to give a cell pellet, which was washed twice with 10 mM phosphate buffer (pH 7.4) before being reconstituted in phosphate buffer to give OD600nm=0.008. 1 ml of the solution was dispensed in a 2-well glass chamber (LabTek) with diluted (1/500) propidium iodide (PI) (1mg/ml, from Invitrogen). The chambers with surface-settled bacteria (60 min) were mounted on a confocal microscope (Olympus) equipped with an incubation chamber at 37°C. PI fluorescence emission was monitored at 625 nm for 60 min (3 frames/min) using an appropriate filter after the addition of peptide (1 ml). Recorded videos (XYZ) were analysed using Fidji software to plot the number of fluorescent (stain-dead) cells as a function of time (Fig. S1).

Hemolysis assay. Hemolysis was determined by incubating 10% (v/v) suspension of human erythrocytes with peptides. Erythrocytes were rinsed 4 times in 10 mM PBS, pH 7.2, by repeated centrifugation and re-suspension (3 min at 3000 x g). Erythrocytes were incubated at room temperature for 1 h in either deionised water (fully hemolysed control), PBS or with peptide in PBS. After centrifugation at 10.000 g for 5 min, the supernatant was separated from the pellet and the absorbance measured at 550 nm. Absorbance of the suspension treated with deionised water defined complete hemolysis. The values given in Table S1 correspond to concentrations needed to kill a half of the sample population (50% lysis of human erythrocytes) and are expressed as median lethal concentrations – LC_{50}. All tests were done in triplicate.

A

PPPHPPH**PPPHPPH**PPPHPPH

amhelin   KQKLAKLKAKLQKLKQKLAKL
           i   i+7

AMP2   KIAKLKAKIQKLKQKIAKLK

CNCHNCH**CNCHNCH**CNCHNCH
           i   i+3
               i   i+4

B

C
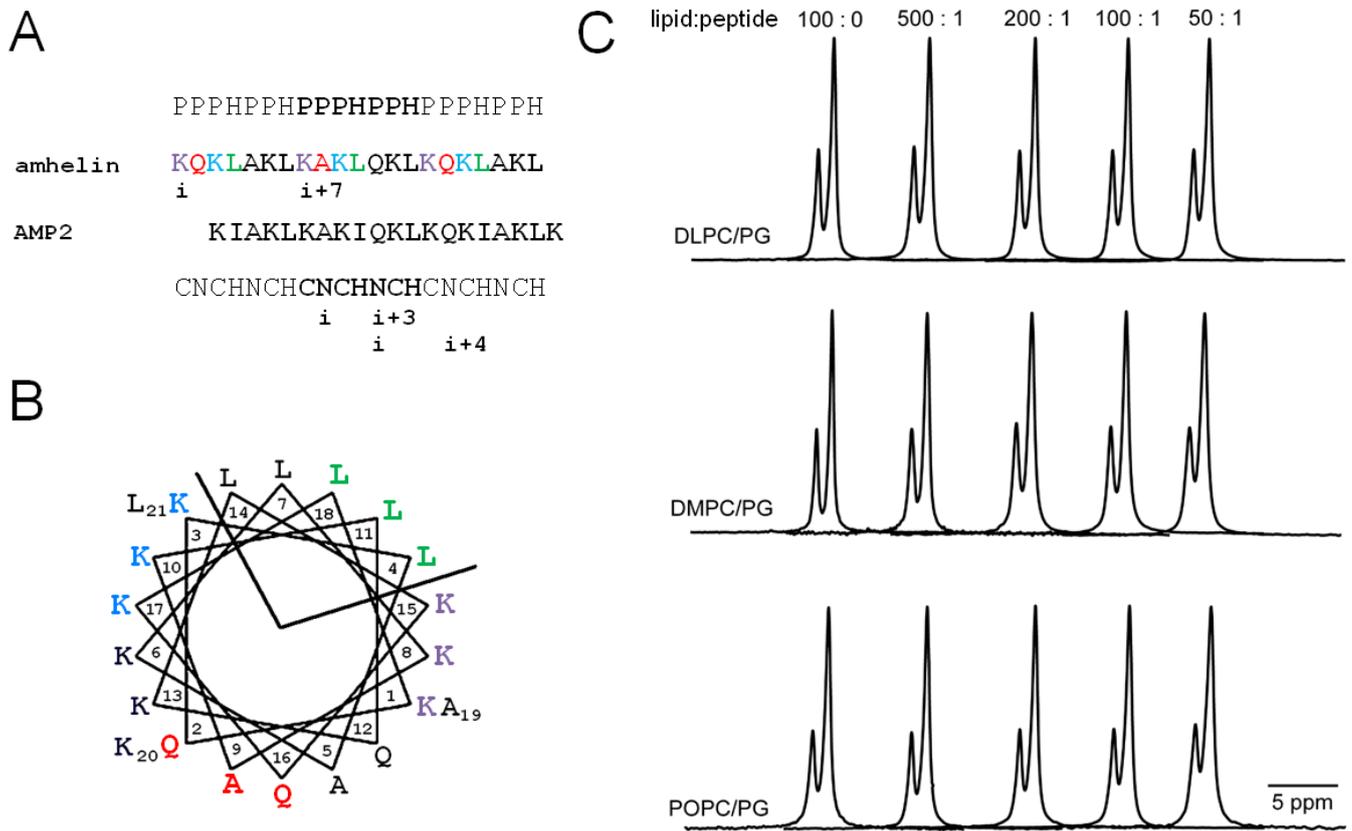
Figure S1. Peptide design. (A) Linear amhelin and AMP2 sequences aligned with the repetitive heptad patterns, PPPHPPH (top) and CNCHNCH (bottom). *i, i+7* amino-acid pairs of the same type are colored. Different colors denote different pairs. *i, i+3* and *i, i+4* helical spacings are shown for N residues only for clarity. (B) Amhelin sequence configured onto an α-helical wheel with 3.6 residues per turn showing amino-acid clustering and *i, i+7* pairs (colored as in (A)). (C) ^{31}P MAS ssNMR spectra of three different AUVs mixed with amhelin at different lipid-peptide ratios. Full width half height ratios of PC and PG peaks from 100:0 to 50:1 lipid-peptide ratios show increases by 2.8% (DL), 21% (DM) and 42% (PO).
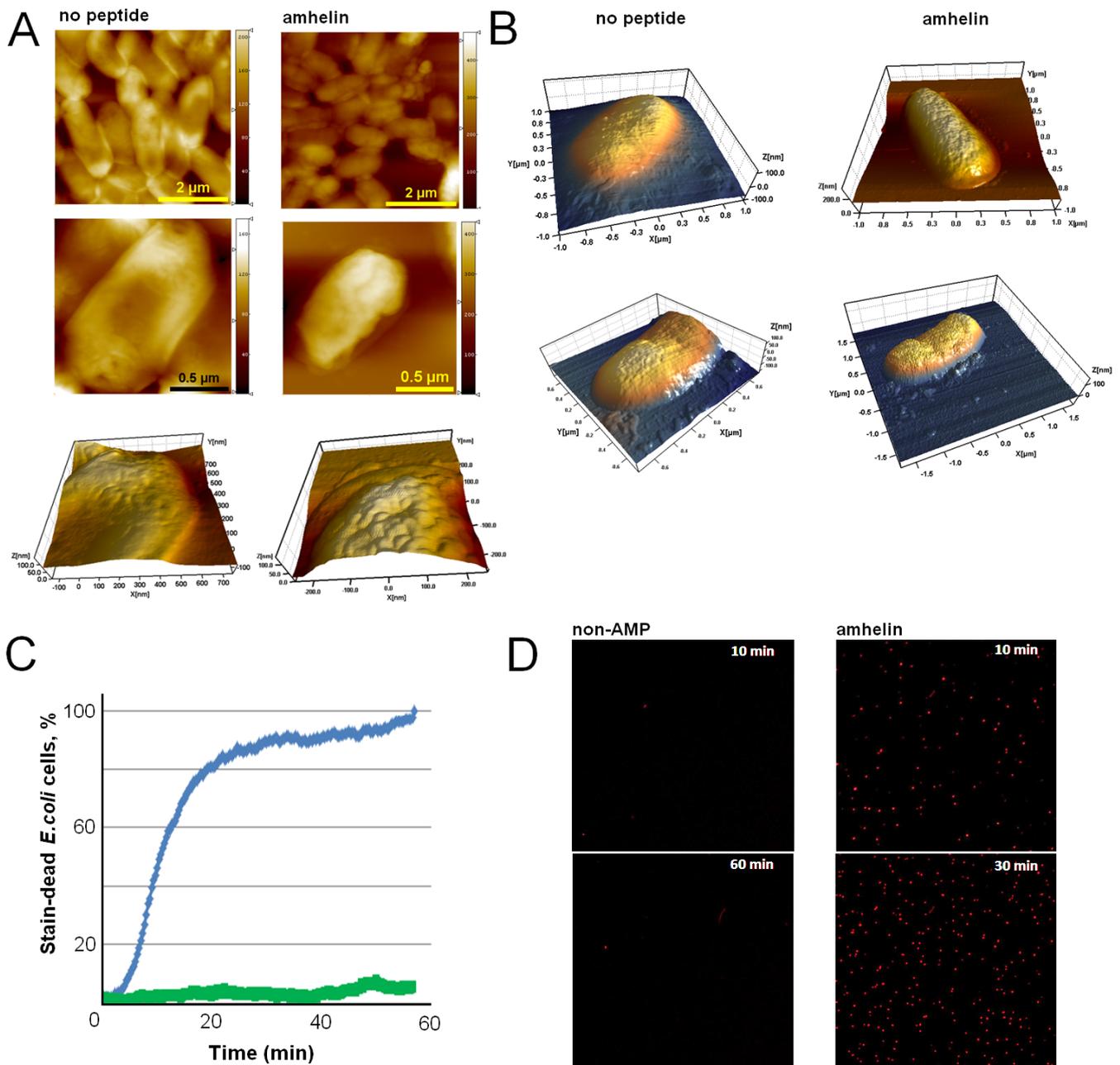
Figure S2. Antimicrobial activity of amhelin. (A) Topographic in-air AFM images of *E. coli* cells with and without amhelin including high-mag 3D images of individual cells. (B) Low-mag 3D images of bacterial cells incubated with amhelin. (C) Average number of stain-dead cells incubated with amhelin (blue) and the non-AMP, QIAALEQEIAALEQEIAALQ, (green) as a function of time. (D) Fluorescence microscopy images of PI-stained *E. coli* cells. Incubation conditions: 10 μM peptide, 30 min, at OD600nm=0.008.
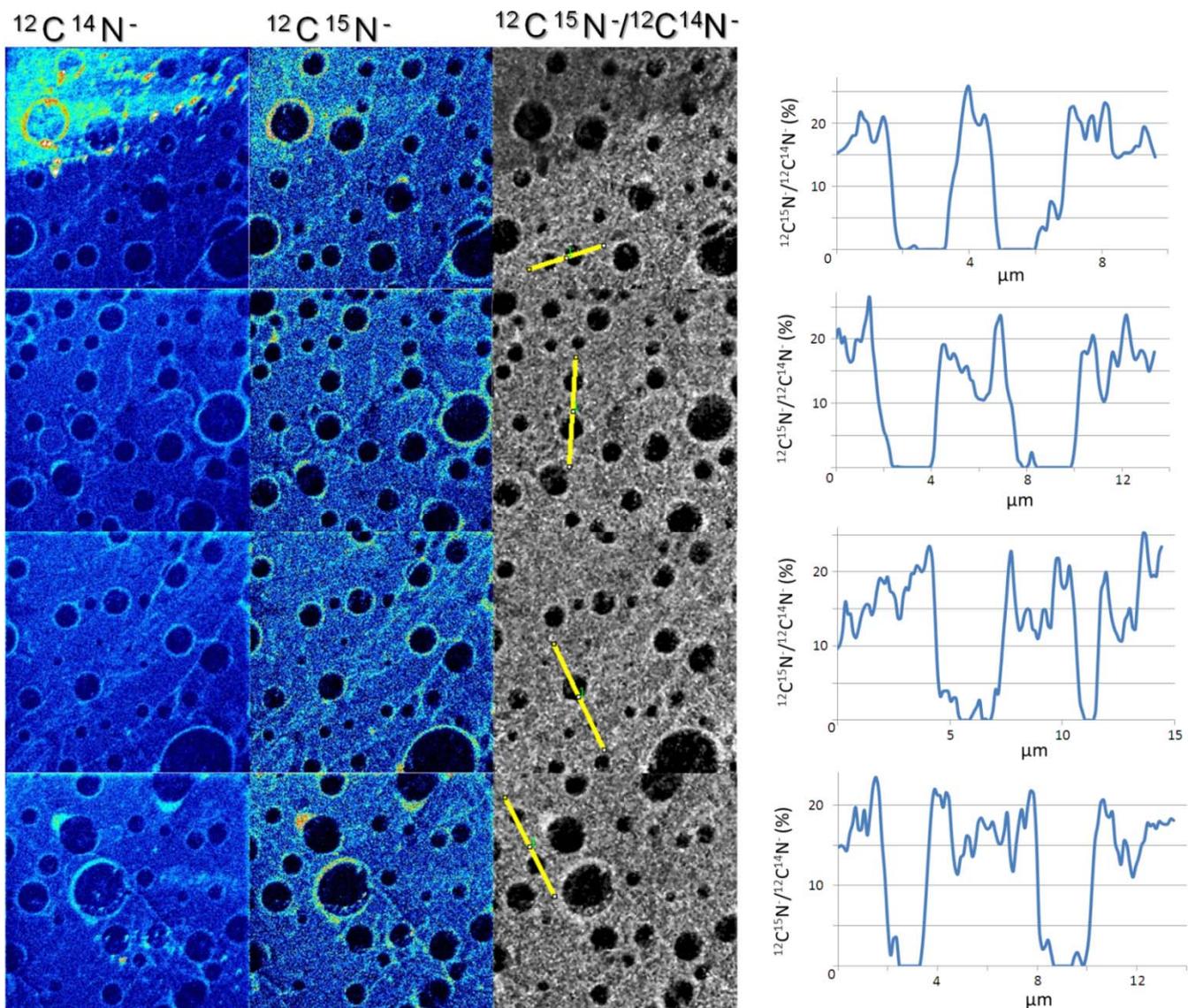
Figure S3. Nano-SIMS images and line scans of supported lipid bilayers treated with ^{15}N-amhelin. The line scans plot $^{12}C^{15}N^-/^{12}C^{14}N^-$ ratios along the yellow lines in corresponding images. The natural abundance of ^{15}N is 0.37%. Image sides are 30 μm (256 x 256 pixels). Incubation conditions: 10 μM peptide, 30 min, pH 7.4, 20 °C.

$^{12}C^{14}N^-$ $^{12}C^{15}N^-$ $^{12}C^{15}N^-/^{12}C^{14}N^-$
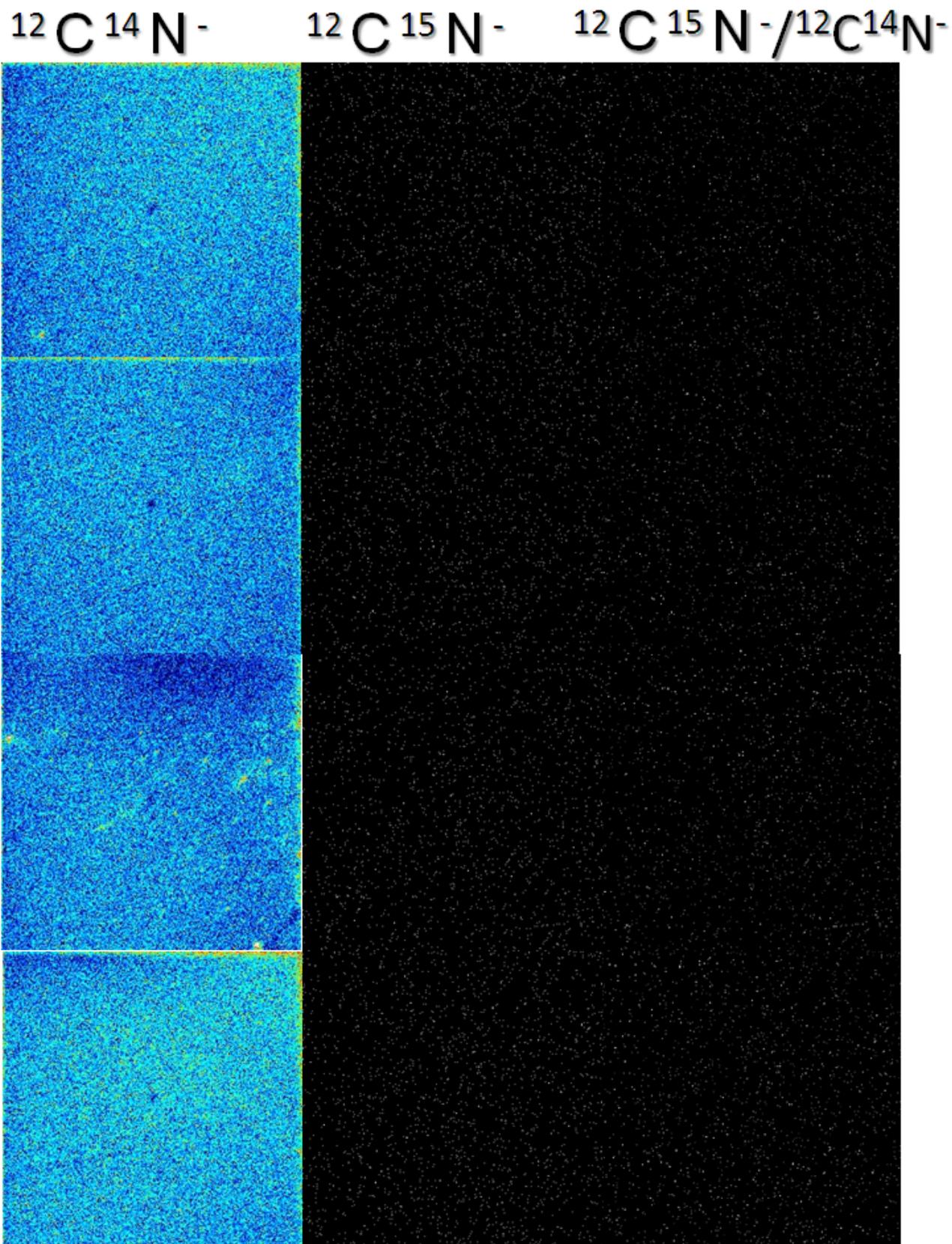
Figure S4. Nano-SIMS images of supported lipid bilayers without peptide treatment. Image sides are 30 μm (256 x 256 pixels). $^{12}C^{14}N^-$ images were set to royal color scale (0-30), and $^{12}C^{15}N^-$ images were set to grey color scale (0-1). Ratios ($^{12}C^{15}N^-/(^{12}C^{14}N^-+^{12}C^{15}N^-)$) of the above images are 0.367%, 0.364%, 0.372 and 0.369% respectively. Incubation conditions: no peptide, 30 min, pH 7.4, 20 °C.
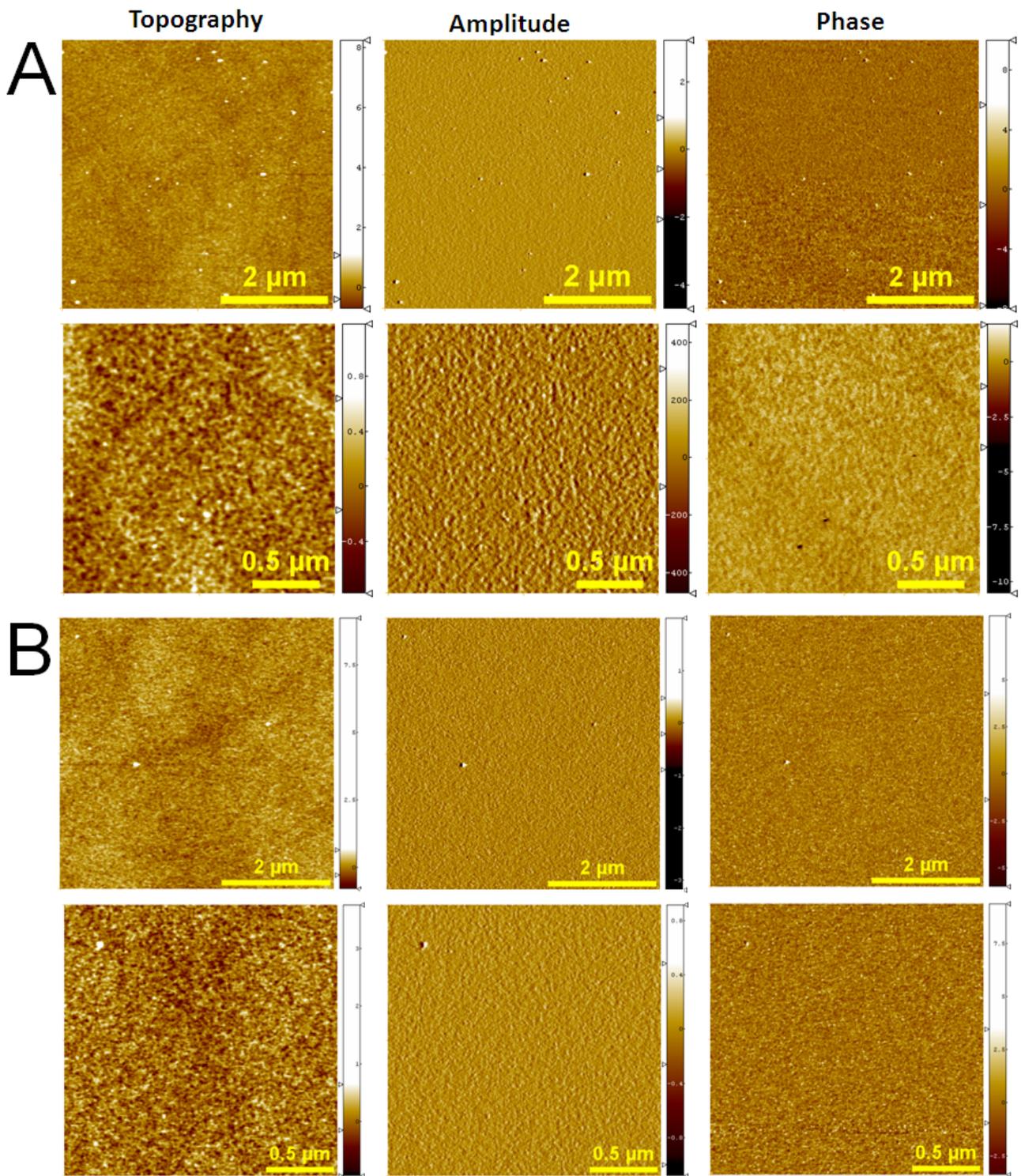
Figure S5. In-air AFM images of control surfaces. (A) Supported lipid bilayers treated with the non-AMP. (B) Bare silicon wafer substrates treated with amhelin. Incubation conditions: 10 μM peptide, 30 min, pH 7.4, 20 °C.
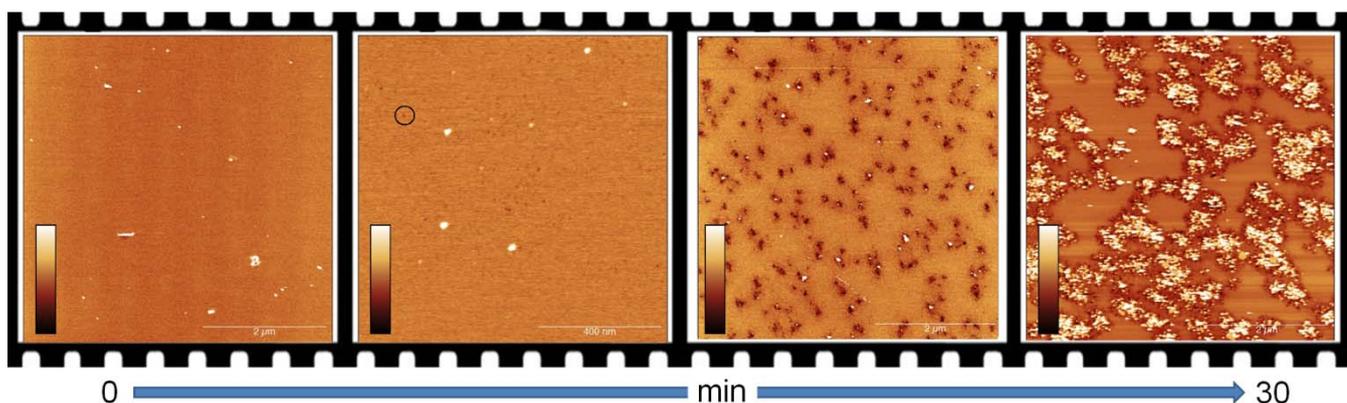
Table S1. Biological activity of amhelin.

Minimum inhibitory concentration (MIC), μM[a]					HE (LC$_{50}$), μM[b]
E. coli (K12)	*P. aeruginosa* (ATCC27853)	*B. subtilis* (ATCC 6633)	*S. aureus* (ATCC6538)	*M. luteus* (NCIMB 13267)	
3	12	6	>50	<12	>>600

[a]non-AMP was inactive (>>250 μM); AMP2 has activities comparable with those of amhelin; [b]human erythrocytes

Video legends

Video S1. 100-ns molecular dynamics simulation of an amhelin insert.

Video S2. 100-ns molecular dynamics simulation of a model octameric amhelin pore.