Supporting Information

# Engineering persistent antimicrobial pseudo-capsids

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## Materials and Methods

**Peptide synthesis and purification:** L- and D-triskelions – (RRWTWE)-βA-K(RRWTWE)-K(RRWTWE)-am – were assembled on a Liberty microwave peptide synthesizer (CEM) using standard Fmoc/$^{t}$Bu solid-phase protocols with HBTU/DIPEA as coupling reagents on a Rink amide MBHA resin. Fmoc-K(Mtt)-OH was used to enable orthogonal conjugation via a tri-functional dendritic hub – βAKK-am. Carboxyfluorescein-labelled triskelions were made on resin by coupling carboxyfluorescein to the N-terminus of βA-RRWTWE-βAKK, with fully protected side chains, on the resin. The remaining two arms were then assembled on resin following Mtt removal. Magainin 2 and cecropin B were synthesized as peptide amides on a Rink amide MBHA resin and LL37 was assembled as a peptide acid on a Fmoc-S($^{t}$Bu)-Wang resin. After post-synthesis cleavage and deprotection (95% TFA, 2.5% TIS, 2.5% water) all peptides were purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Peptide identities were confirmed by analytical RP-HPLC and MALDI-ToF.

MS $[M + H]^{+}$: cecropin B – *m/z* 3834.6 (calc.), 3836.0 (found); LL37 – *m/z* 4493.2 (calc.), 4495.8 (found); magainin 2 – *m/z* 2465.9 (calc.), 2467.0 (found); L-triskelion – m/z 3090.5 (calc.), 3092.3 (found); D-triskelion – m/z 3090.5 (calc.), 3092.8 (found); carboxyfluorescein-L-triskelion – m/z 3518.9 (calc.), 3519.7 (found).

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. 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 214 nm (buffer A, 5% and buffer B, 95% aqueous $CH_3CN$, 0.1% TFA).

**Ψ-capsid assembly:** triskelions were assembled overnight at the concentrations stated in the text (100-400 μM) in filtered (0.22 μm), 10 mM MOPS, phosphate or PBS buffers, pH 7.4, at room temperature.

**Circular dichroism spectroscopy:** CD spectra were recorded on a JASCO J-810 spectropolarimeter fitted with a Peltier temperature controller. The measurements were taken in ellipticities in mdeg and converted to molar ellipticities ([$\theta$], deg $cm^2$ $dmol^{-1}$ $res^{-1}$) by normalizing for the concentration of peptide bonds. The data was collected with a 1 nm step, 1 sec collection time per step, and is presented as the average of 4 scans.

**Fourier transform infra-red spectroscopy:** FT-IR spectra were recorded using a Tensor-27 series FTIR spectrometer equipped with a BioATR II unit (Bruker Optics), as the sampling platform, and a photovoltaic mercury cadmium telluride (MCT) detector and a Bruker Optics workstation equipped with OPUS software. Low-volume (20 μL) capsid samples (100 μM) were placed in a circular sampling area of radius 2 mm with a path length of 6 μm. FTIR spectra was recorded with resolution 4 $cm^{-1}$, scanner velocity 20 kHz, 256 scans, phase resolution 32 and zero filling factor 4. Spectra deconvolutions were performed by Gaussian peak fitting using the proprietary software.

**Molecular dynamics simulations:** MD simulations were performed using the GROMACS 2016 software with the GROMOS96 53A6 force field (atomistic) and the MARTINI force field (coarse grain) (*32*, *33*). A SPC water model was used for atomistic simulations and standard MARTINI water for coarse grain simulations (*33*). In all simulations chloride and sodium ions were added up to a 150 mM concentration. Additional ions were placed if required for charge neutralization. The initial Ψ-capsid configuration was constructed geometrically using the editconf tool from GROMACS. The DLPC/DLPG (3:1) membrane was constructed with the PACKMOL software with dimensions of 12 × 12 nm (atomistic) or 30 × 30 nm (coarse grain) (*39*). Periodic boundary conditions were imposed, setting the minimal distances between the protein and the box boundaries to 3 nm. All simulations are run in the NPT ensemble maintaining the temperature at 303K with the velocity rescale thermostat, and the pressure at 1 bar using the Parrinello-Rahman barostat, with either an isotropic or semi-isotropic coupling; simulations without and with membranes, respectively. For atomistic simulations of the Ψ-capsid in solution, multiple restrained equilibration runs (65 ns overall) precede the 100 ns production, which were run in three replicates. Similarly, for coarse grain simulations a 10 ns equilibration precedes the 1 μs production, which were run in three replicates. For simulations including membranes, the lipid patches were equilibrated for 500 ns (1 μs for coarse grain) before placing the peptide close in contact with the lipid surface (atomistic) or at the minimal distance of 1 nm (coarse grain). Production was then carried out for 500 ns, and 10 μs for coarse grain. Atomistic simulations with membranes were run with an electric

field of 130 mV/nm in the direction perpendicular to the membrane, with the higher potential region on the side of the bilayer where the peptide rests.

**Transmission electron microscopy:** micrographs of the Ψ-capsids were recorded using a JEOL 1010 transmission electron microscope equipped with an Orius SC1000 (Gatan Inc.) CCD camera, operated at 80 keV. Droplets of capsid solution were placed on glow discharged Cu finder grids coated with carbon film (mesh 200), stained with uranyl acetate (aq. 2%, w/v) for a few seconds and buffer excess was removed by blotting paper. Micrographs of *E. coli* were recorded using FEI Tecnai T12 transmission electron microscope equipped with a Morada G2 (Olympus Inc.) camera, operated at 80 keV. Samples were fixed with glutaraldehyde (aq. 5%, w/v), treated with a post-fixation agent osmium tetroxide (1%, w/v, 100 mM PIPES, pH 7.2) and stained with uranyl acetate (aq. 2%, w/v). The resulting samples were embedded in a Spurr resin, and were left to solidify at 60 ºC, over 24 hrs. The resin was then microtomed to ultrathin sections that were placed on Formvar carbon coated grids (mesh 200), post-stained using lead citrate (aq. 5%, w/v) and imaged.

**Preparation of unilamellar phospholipid vesicles for AFM imaging**: 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) with 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DLPG) lipids used for vesicle construction were from Avanti Polar Lipids (Alabaster, USA). DLPC were used as mammalian model membranes, and DLPC/DLPG (3:1, molar ratios) were used as bacterial model membranes. The lipids were weighted up, dissolved in chloroform-methanol (2:1, vol/vol), dried under a nitrogen stream and then under vacuum to form a thin film. The film was hydrated in 10 mM phosphate buffer (pH 7.2) with shaking (1 h, 220 rpm) and bath sonicated for 30 min. The obtained suspension was extruded using a hand-held extruder (Avanti Polar lipids) (twenty nine times, polycarbonate filter, 0.05 µm) to give a clear solution of small unilamellar vesicles, whose sizes (50 nm) were confirmed by photon correlation spectroscopy.

**In-liquid atomic force microscopy on supported lipid bilayers:** supported lipid bilayers were formed using a vesicle fusion method as described elsewhere (*38*). Freshly prepared vesicles (1.5 µL, 3 mg/mL) were added to cleaved mica a pre-hydrated with buffer (70 µL, 120 mM NaCl, 20 mM MOPS, 20 mM $MgCl_2$). Following adsorption and incubation for 45 min, the samples were washed to remove unfused vesicles and resulting SLBs were checked to confirm they were defect free. Peptides were then introduced into a 100-µL fluid cell (Bruker AXS, USA) where they were diluted to the final concentrations of 0.1-0.8 µM. All imaging was performed using Peak Force Tapping™ mode on a Multimode 8 AFM system (Bruker AXS, USA) using MSNL-E cantilevers (Bruker AFM probes, USA). Images were taken at PeakForce frequency of 2 kHz, PeakForce amplitude of 10 nm and PeakForce set-point of 10-30 mV (<100 pN). Images were processed using

Nanoscope analysis software (Bruker AXS, USA) or Gwyddion (http://gwyddion.net) for first order line-by-line background subtraction (flattening) and first-order plane fitting.

**Photon correlation spectroscopy.** Prepared phospholipid vesicles for AFM imaging were re-suspended to a final concentration of 1 mg/mL and were analysed on a Zetasizer Nano (ZEN3600; Malvern Instruments). 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 manufacturer's Dispersion Technology Software (version 5.10).

**Minimum inhibitory concentrations assay:** minimum inhibitory concentrations were determined by broth microdilution on *P. aeruginosa*, *E. coli*, *S. aureus*, *M. luteus*, *B. subtilis*, *S. typhimurium* and *K. pneumoniae* according to the Clinical and Laboratory Standards Institute. Typically, 100 μL of 0.5–1 × $10^6$ CFU per ml of each bacterium in Mueller Hinton media broth (Oxoid) were incubated in 96-well microtiter plates with 100 μL of serial two-fold dilutions of the capsids (from 100 to 0 μM) at 37 °C on a 3D orbital shaker. The absorbance was measured after capsid addition at 600 nm using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices). MICs were defined as the lowest peptide concentration that inhibited visible bacterial growth after 24 h at 37 °C. All tests were done at least in triplicate and results are summarized in Table S1. The values that are given without standard deviations are those for which no variations were found within triplicates.

**Hemolysis assay:** hemolysis was determined using human erythrocytes sourced commercially from Cambridge Bioscience Ltd. and used within two days. 10% (vol/vol) suspensions of human erythrocytes were incubated with the capsids. The cells were rinsed four times in 10 mM phosphate buffer saline (PBS, Gibco™), pH 7.2, by repeated centrifugation and re-suspension (3 min at 3000 × g). The cells were then incubated at room temperature for 1 h in either deionized water (fully hemolysed control), PBS, or with capsid in PBS. After centrifugation at 10,000 × g for 5 min, the supernatant was separated from the pellet, and the absorbance was measured at 550 nm using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices). Absorbance of the suspension treated with deionized water defined complete hemolysis. All tests were done in triplicate and results are shown in Table S1. The values given correspond to concentrations needed to kill a half of the sample population (50% lysis of erythrocytes) and are expressed as median lethal concentrations, $LC_{50}$.

**Bacterial culture for single-cell antimicrobial kinetics measurements:** lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract, and 0.5 g/L NaCl, Formedium) and LB agar plates (LB with 15 g/L agar) were used for planktonic and plate bacteria growth, respectively. *E. coli* BW25113 was purchased from Dharmacon (GE Healthcare). Single colonies were picked from a streak plate and

were incubated at 37 °C, over 17 hours in fresh LB medium (100 mL) with shaking (200 rpm). The prepared culture was span down (10 min, 3000 g and 20 ºC). The supernatant was filtered twice (Medical Millex-GS Filter, 0.22 µm, Millipore Corp.) and used to re-suspend the bacteria pellet to an $OD_{595}$ of 50. The resulting highly concentrated bacterial suspension was used in high throughput single-cell kinetic measurements, together with M9 minimal medium (7 g/L $Na_2HPO_4$, 3 g/L $KH_2PO_4$, 1 g/L $NH_4Cl$, 0.5 g/L NaCl, 1 mM thiamine hydrochloride) (Sigma Aldrich), ampicillin (Sigma Aldrich) and propidium iodide (PI) (Thermo Fisher Scientific), as described below.

**High throughput single-cell kinetic measurements:** antimicrobial efficacy with a single-cell resolution was measured using a multi-channel microfluidics device as reported elsewhere (*40*, *41*). The device used was a polydimethylsiloxane (PDMS, Dow Corning) replica of the original mold kindly provided by Suckjoon Jun (*40*). The device is an array of dead-end microfluidic channels 1.5 µm in diameter and a 25 µm in length. Each channel can host 1-4 bacterial cells in a single file. The channels are connected to a main microchamber (25x100 µm) that is continuously supplied with antimicrobial agents, fresh LB or propidium iodide (see below). The device was permanently attached to a glass coverslip and functionalized with a solution of bovine serum albumin (50 mg/mL). 2 µl of the highly concentrated bacterial suspension, prepared as described above, was injected in the device and individual bacteria were allowed to diffuse into the lateral channels for 30 min. The device was then completed with fluorinated ethylene propylene inlet and outlet tubing (1/32"×0.008") connected to a flow-rate measuring system (Flow Unit S, Fluigent, France) with the applied pressure controlled by a computerized pressure-based flow control system (MFCS-4C, Fluigent). The completed device was mounted on an inverted microscope (IX73 Olympus, Tokyo, Japan) equipped with a 60×, 1.2 N.A. objective (UPLSAPO60XW, Olympus) and a sCMOS camera (Zyla 4.2, Andor, Belfast, UK) used to acquire bright-field images (exposure time 0.03s) of thousand channels per hour for the duration of each experiment (24 hrs). After acquiring the first set of images, the microfluidic environment was changed by flowing M9:LB (9:1 v:v) containing antimicrobial agents (D/Ψ-capsids or ampicillin) at 25×MIC at 100 µL/h over three hours and then by LB for a further 21 hrs. At 24 h PI (30 µM) was delivered into the microfluidic device for 10 min to identify dead cells with compromised cell membranes. The device allowed to track each bacterial cell and its progeny throughout the assay, with the images obtained in each channel loaded to ImageJ.

**Time-lapse structured illumination microscopy of *E. coli* and *S. Aureus*:** bacteria (*E. coli* ATCC 15597 and *S. aureus* ATCC 6538) were inoculated into Mueller Hinton broth, grown overnight at 37 °C, 150 rpm, re-inoculated and grown to $OD_{600}$ of 0.5-0.8. The cells were then pelleted by centrifugation (4000 rpm, 10 mins), and after removing the supernatant, were re-

suspended in 1 mL PBS (10 mM) and transferred to an Eppendorf tube. FM4-64 (1 mg/mL, 5 μL) was added to each tube followed by rotation on a rotating disk for 5 mins. The cells were then centrifuged (7000 rpm, 3 mins), the supernatant was removed, and the cell pellet was re-suspended in PBS (1 mL). Bacteria was then diluted to $OD_{600}$ of 0.16 and 0.08 for *E. coli* and *S. aureus*, respectively. 100 μL of each suspension was added to different wells of a chambered microscope coverslip (Ibidi μ-Slide 8 Well) and left for 30 min to allow cells to settle onto the substrate. Immediately prior to each imaging experiment a 100-μL solution of Ψ-capsids assembled from caroxyfluorescein-labelled and unlabelled triskelions at 1:100 molar ratios were added to the well to a final concentration of 50 μM (total peptide). For control datasets, 100 μL of PBS was added prior to imaging. Time lapse imaging was performed at 5-min intervals using a custom-built structured illumination microscope (*50*). Briefly, sinusoidal excitation patterns were generated by projecting a spatially filtered image of a spatial light modulator (SLM), configured to display a series of binary phase gratings, into the focal plane of the microscope objective lens (UPLSAPO 60x/1.3, Olympus). Sample images were acquired using a scientific CMOS camera (ORCA-Flash4.0, Hamamatsu Photonics), with the global exposure period of the camera's rolling shutter synchronised to the pattern displayed on the SLM. At each time point FM4-64 and carboxyfluorescein-labelled capsids were imaged sequentially, using excitation at 561 nm with a 655 nm long pass emission filter and excitation at 488 nm with a 525/50 bandpass emission filter respectively. Images were reconstructed as described elsewhere (*51*) and lateral drift and chromatic offsets in the reconstructed images were corrected using ImageJ. The FM4-64 colour channel was then corrected for photobleaching using an exponential fit to the image intensity within a suitable region of interest. Manual background subtraction and colour balancing was performed to optimise image contrast.

**In vivo MRSA clearance assay**: *Galleria mellonella* larvae assay was performed as described elsewhere (*53*, *54*). Wax moth larvae were purchased in bulk from Livefood UK, stored at 4°C upon arrival and kept at 37°C during the course of the assay. Ψ-capsid samples were prepared from stock solutions in Milli-Q water to working concentrations in sterile phosphate buffered saline (PBS). Typically, a single bacterial colony was picked to inoculate 5 mL of tryptic soy broth (TSB), and the resulting culture was left to grow overnight (~16 hours) at 37°C with shaking (200 rpm). The culture was then diluted (100x) into a fresh TSB (5 mL) and incubated over 4 hours at 37°C with shaking (200 rpm). After centrifugation (2500g) for 10 min, pellets were obtained and re-suspended in sterile PBS to an $OD_{595}$ of 0.2, giving ~$10^8$ CFU/mL.) The re-suspended culture (10 μL aliquots; ~$1.2 \times 10^6$ CFU) was administered to larvae (seven groups; n=10 in each group) behind the rear thoracic segments using a Tridak Stepper Pipette Dispenser (Dymax, UK). The infected larvae were then treated by injection with 10 μL of L/Ψ-capsid (12.5 μM), D/Ψ-capsid, (12.5 μM),

magainin 2 (12.5 μM), cecropin B (12.5 μM), LL37 (12.5 μM), vancomycin (40 μM) or PBS at stated time points after inoculation. The toxicity of Ψ-capsid was assessed using two groups of larvae (n=10 in each group). These were mock-infected with PBS and treated with 10 μL of L or D/Ψ-capsid (25 μM) at stated time points after inoculation. For the delayed treatment assay, three groups of infected larvae (~$10^6$ CFU) were treated with 10 μL of L/Ψ-capsid (12.5 μM), vancomycin (40 μM) or PBS at stated time points after inoculation. All experiments were carried out as blind studies, and the treatment identities were not revealed until each experiment was completed. Larvae were considered dead when they did not respond to touch to the head. Survival curves were generated and analysed using GraphPad Prism 6 software.

## Table, Figures and Movies

**Table S1.** Biological activities of Ψ-capsids in comparison with other antimicrobial agents.

| Cell | Antimicrobial agent | | | | | |
|---|---|---|---|---|---|---|
| | Ψ-capsids | | ampicillin | melittin | polymyxin B | cecropin B |
| | L-form | D-form | | | | |
| | Minimum Inhibitory Concentration, μM | | | | | |
| *E. coli* | 7.8 ± 2.7 | 6.6 ± 3.9 | >25 | 2.3 ± 1.1 | 1.3 ± 0.3 | <1 |
| *S. aureus* | 8.3 ± 2.9 | 17.2 ± 8.1 | <1 | <1 | 25 | 50 |
| *P. aeruginosa* | 9.4 ± 3.1 | >25 | >25 | 9.3 ± 3.1 | 1.5 | 25 |
| *S. typhimurium* | 12.5 | 9.4 ± 5.4 | 8.3 ± 2.9 | 3.1 | 1.5 | <1 |
| *K. pneumoniae* | >25 | 8.2 ± 4.6 | 12.5 | 3.1 | 2.6 ± 0.7 | 1.5 |
| *B. subtilis* | 2.3 ± 0.8 | 5.2 ± 1.5 | 25 | 3.1 | 3.1 | >50 |
| *M. luteus* | 1.2 ± 0.4 | 2.3 ± 0.8 | 1 ± 0.4 | <1 | 2 ± 0.7 | <1 |
| | $LC_{50}$,[a] μM | | | | | |
| *Human erythrocytes* | >250 | >250 | n.d. | <10 | >250 | >250 |

[a]concentration required to achieve 50% cell death compared to untreated cells

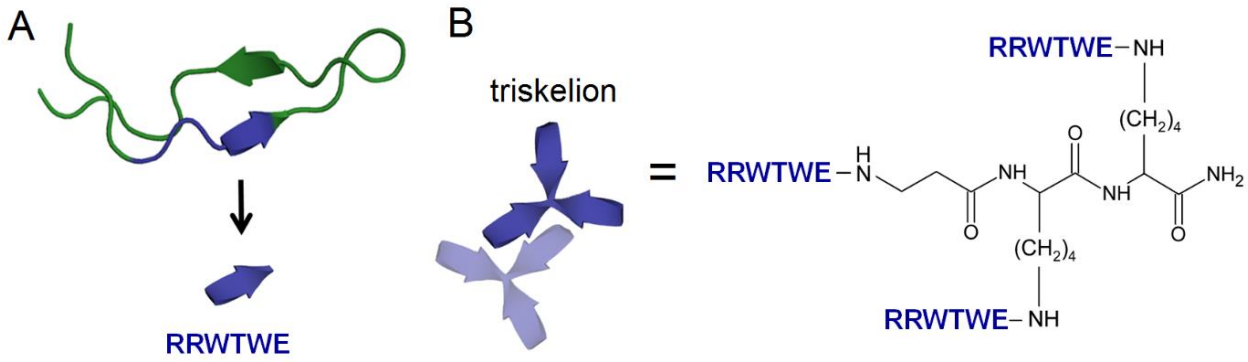


**Figure S1. Triskelion design.** (a) N-terminal domain of lactoferrin (PDB entry 1LFC rendered by PyMol). The core sequence, RRWQWR, in the domain is highlighted in blue, and converted to a self-complementary β-strand, RRWTWE. (B) Three copies of RRWTWE are conjugated into a β-strand triskelion. Triskelions interact via β-strand arms. For clarity only two triskelions forming a β-sheet are shown and highlighted in different colours. (C) The chemical structure of the triskelal RRWTWE conjugate. Note: the schematics are the same for the D-form of the triskelion, with all amino acids being D-epimers.

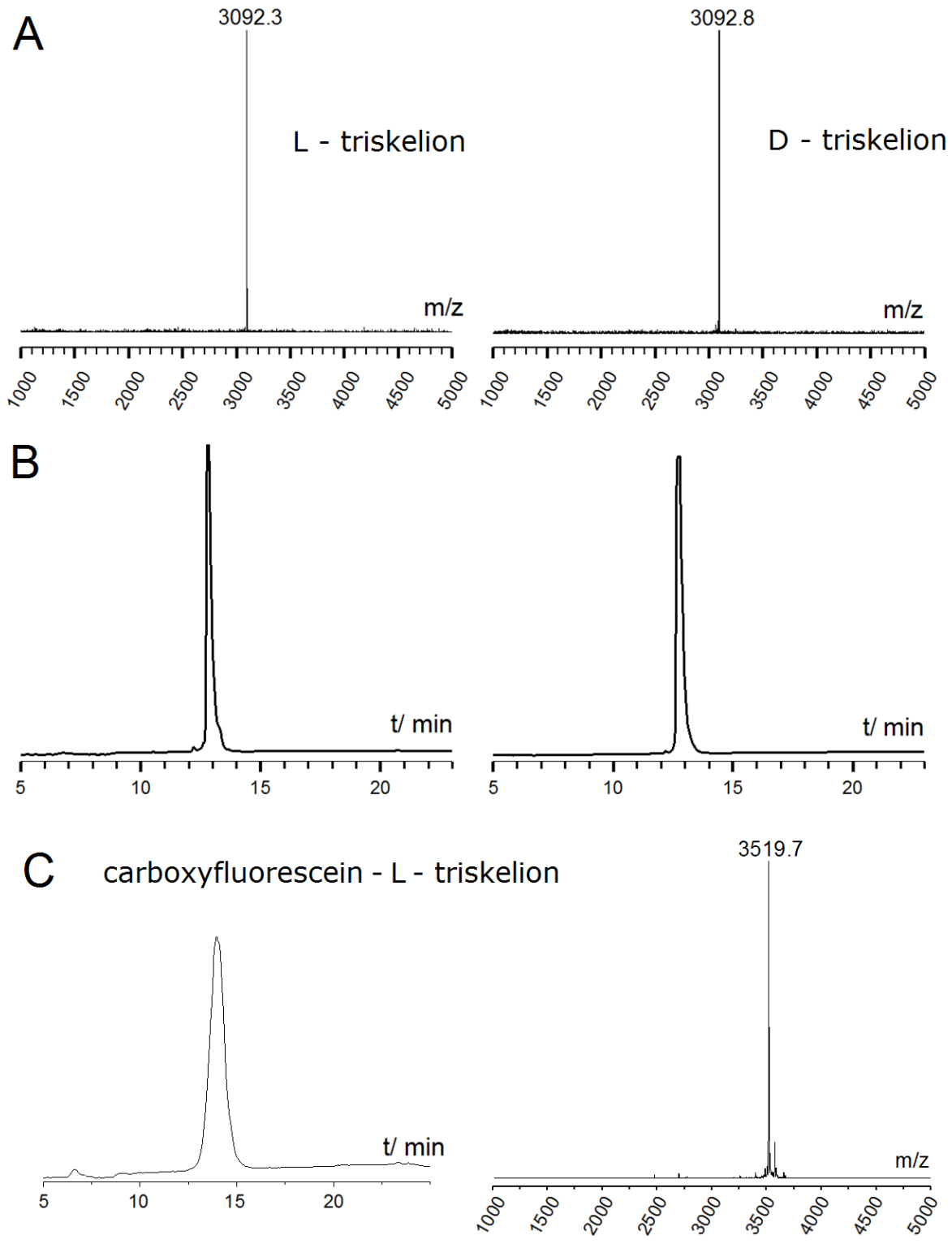


**Figure S2. Triskelion synthesis.** MALDI-ToF spectra (A) and RP-HPLC profiles (B) for purified L- and D-triskelions. (C) RP-HPLC (left) and MALDI-ToF (right) traces for the purified L-triskelion modified with carboxyfluorescein at the N-terminus of one arm.

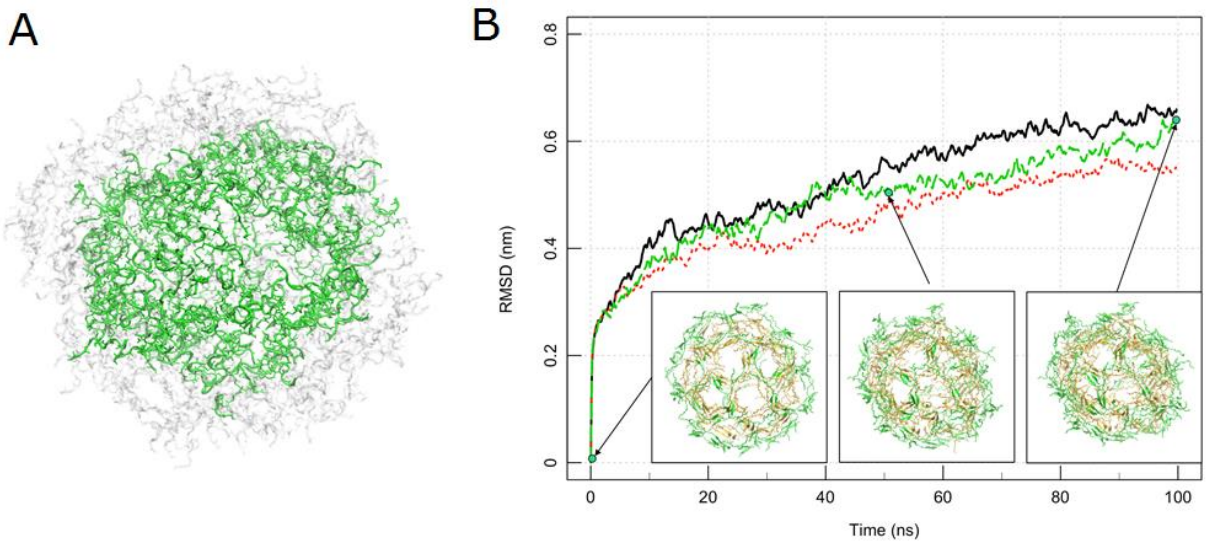


**Figure S3. Pseudo-capsid shells**. (A) Final snapshot (1 μs) of a coarse grain MD simulations of a β-sheet single-walled shell templated on a truncated icosahedron (green, peptide backbone only). For comparison, a final configuration for double-walled shell is shown in grey (peptide backbone only). (B) Root mean square deviations (RMSD) of double-walled shells versus their initial configurations as a function of time. Initial, mid-time and final configurations are shown in insets. See also Movie S1.

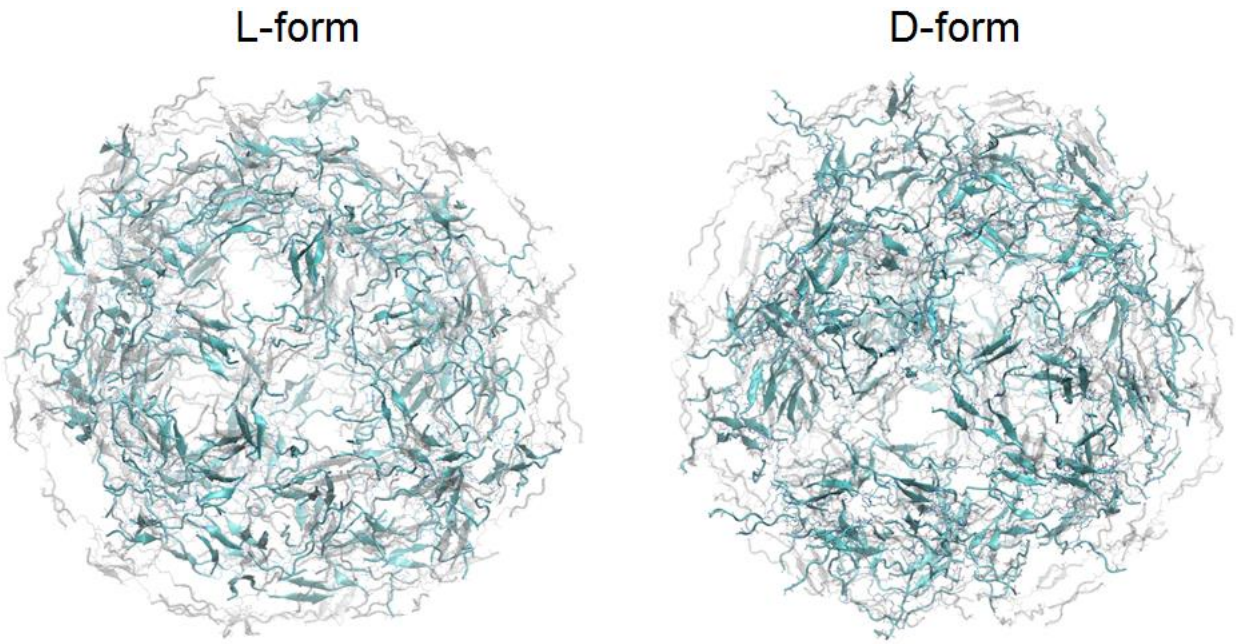


**Figure S4. Double-walled pseudo-capsid shell.** Final configurations of L- and D-forms of the triskelion assembly templated on a truncated isosahedron after 100 ns of atomistic simulations. Initial configurations (peptide backbone only) are shown in grey.

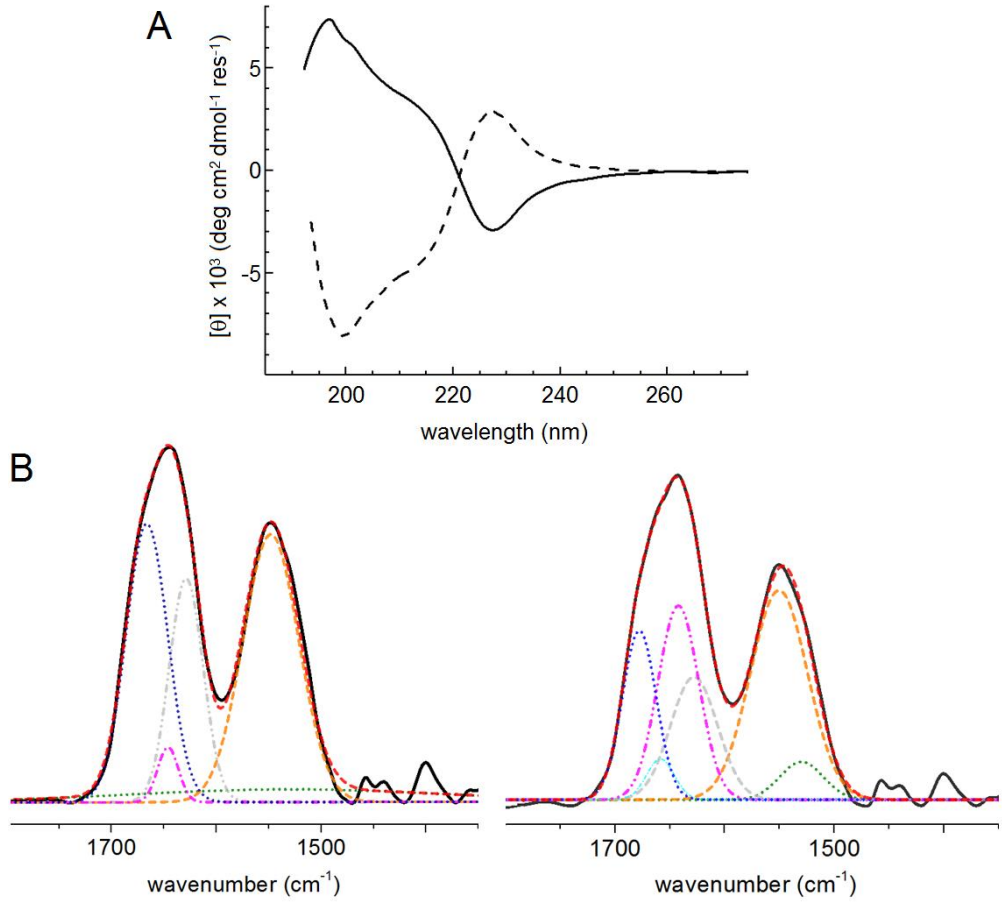


**Figure S5. Pseudo-capsid folding.** (A) CD spectra for L/Ψ-capsids (dashed line) and D/Ψ-capsids (solid line). (B) FT-IR spectra for L/Ψ-capsids (right) and D/Ψ-capsids (left), including raw spectra (black line), cumulative fit (dashed red line), deconvoluted amide spectra (other coloured lines). Folding conditions: 100 μM peptide, pH 7.4, 10 mM MOPS, 20˚C.

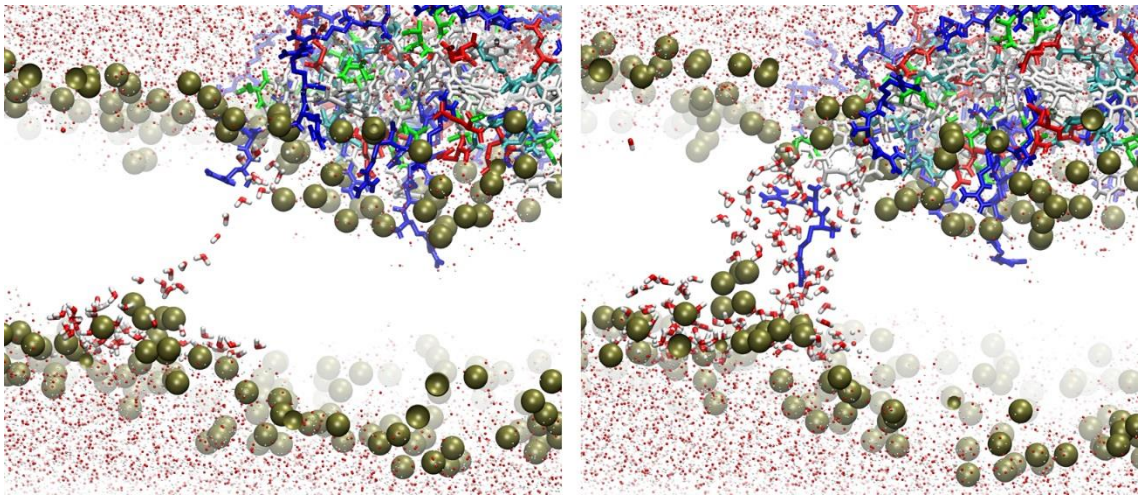

**Figure S6. A rudimentary pore.** Snapshots taken at 62 ns (left) and 72 ns (right) of 100-ns atomistic simulations of a rudimentary pore in phospholipid bilayers. Key: for clarity, only phosphate groups (golden beads) are shown for the bilayer. See also Movies S2 and S3.

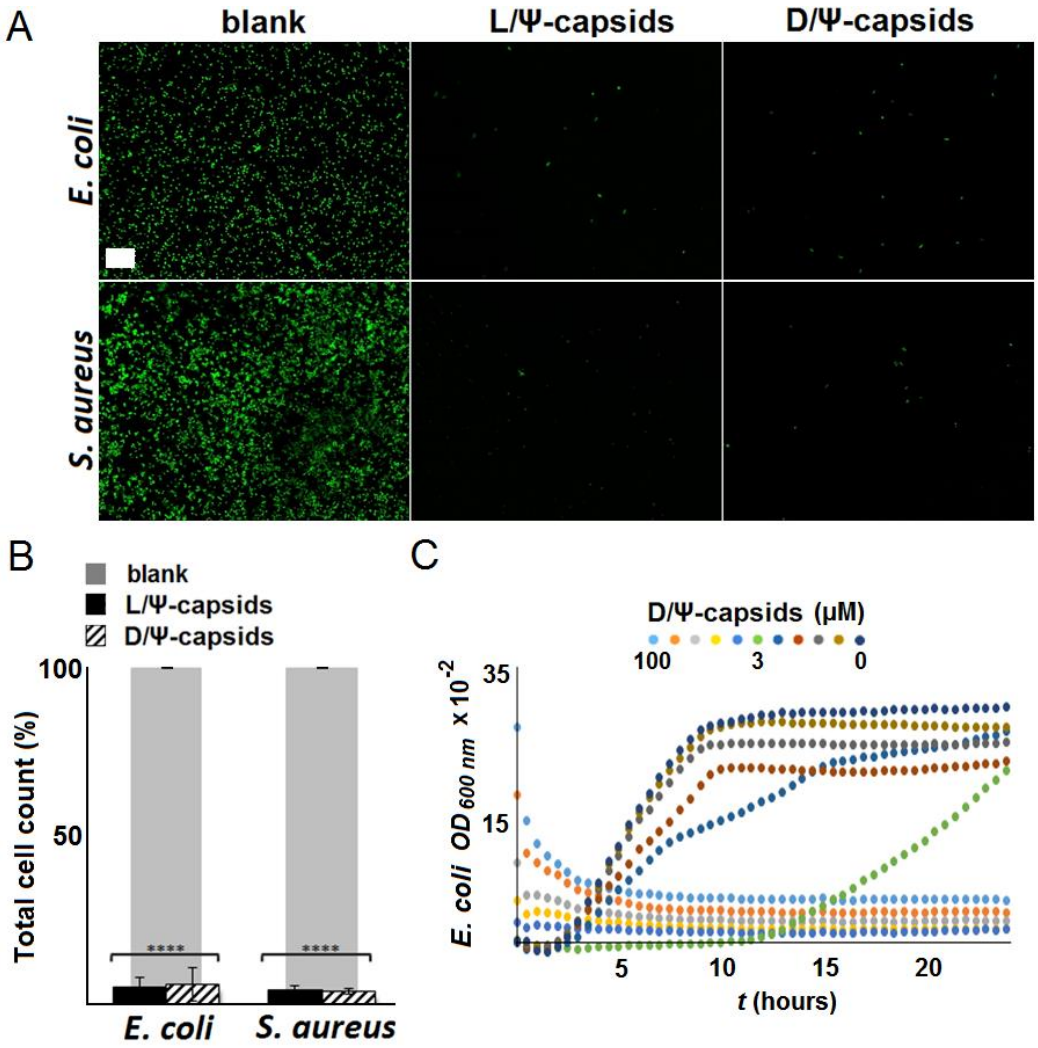


**Figure S7. Antimicrobial kinetics of Ψ-capsids**. (A) Fluorescence micrographs of bacterial cells after 16-hour incubations without (blank) and with Ψ-capsids. The scale bar is 20 μm. (B) Total cell counts for samples after 16-hour incubations without (blank), taken as 100%, and with Ψ-capsids. Total cell counts for samples treated with the capsids were significantly lower than those for un-treated cells ($p$ <0.001). (C) Representative bacteria growth curves (*E. coli*) recorded for serial peptide dilutions (100 μM to 0 μM) as a function of time. Individual data points are collected at 30 min intervals.

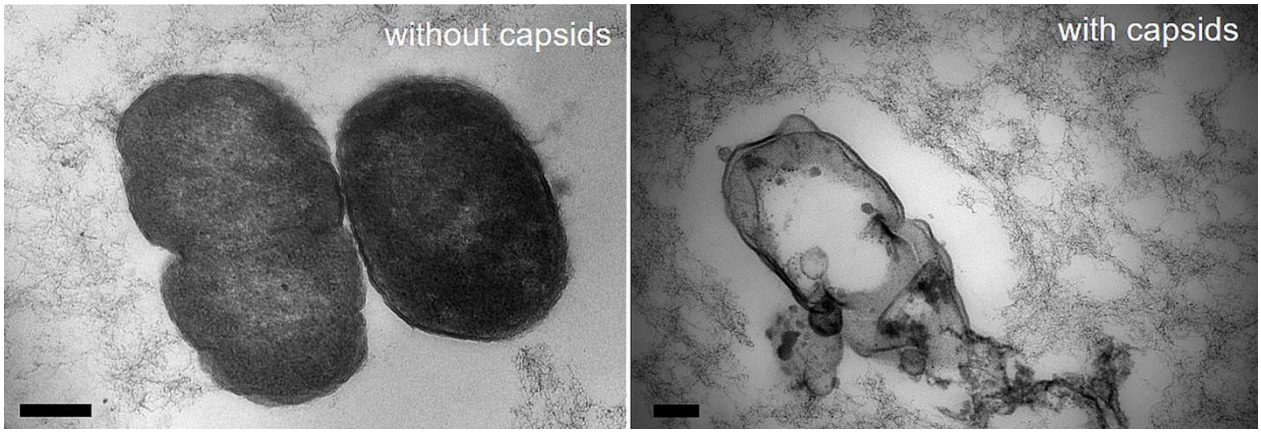


**Figure S8. Single-cell antimicrobial kinetics of Ψ-capsids**. Electron micrographs of microtomed *E. coli* cells without and after treatment with D/Ψ-capsids. The scale bars are 200 nm.

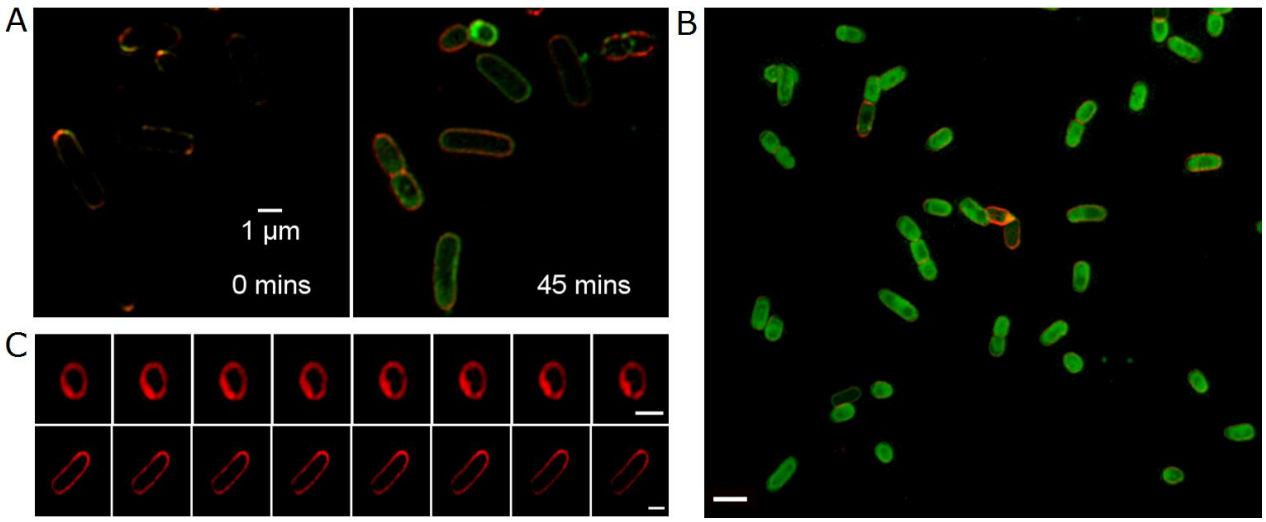


**Figure S9. Time-kill kinetics of pseudo-capsids in vitro**. (A) SIM images of *E. coli* cells immediately after the addition of L/Ψ-capsids and after 45 min of the treatment. The capsids are labelled with carboxyfluorescein (green). Cell membranes are stained with FM4-64FX (red). (B) A larger area of *E. coli* cells imaged at 45 min of the treatment. (C) Control SIM images recorded at 5-min intervals for *S. aureus* (upper) and *E. coli* (lower) without the capsids. Scale bars are 1 µm.

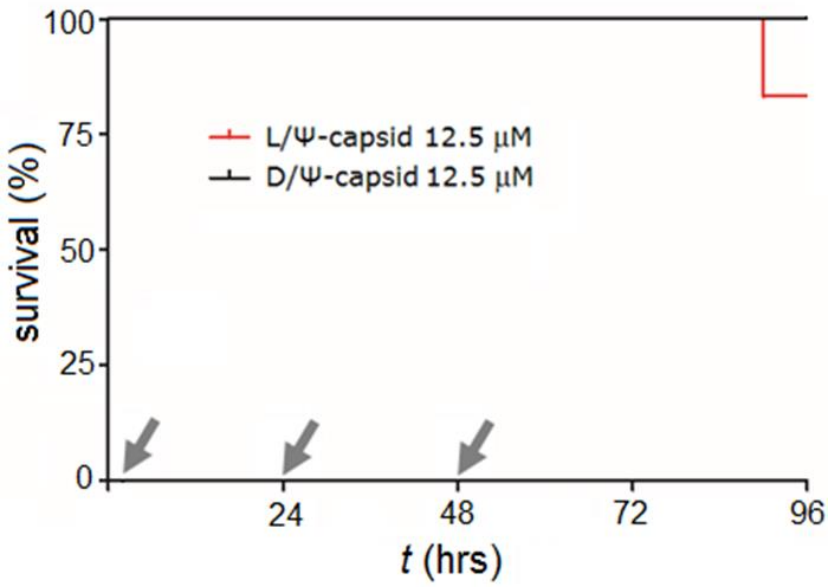


**Figure S10. Time-kill kinetics of pseudo-capsids in vivo.** Survival of *G. mellonella* larvae treated with Ψ-capsids. Inoculations were done straight after the initiation of infection (first 2 hrs) with two subsequent treatments at 24 and 48 hrs.