Structurally plastic peptide capsules for synthetic antimicrobial viruses

Castelletto et al.

Materials and Methods

Peptide Synthesis and Purification. Capzip triskelion – (RRWTWE)-βA-K(RRWTWE)-K(RRWTWE)-am – was 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 resin. Fmoc-Lys(Mtt)-OH was used to enable orthogonal conjugation via a tri-functional dendritic hub – βA-KKK-am. After post-synthesis cleavage and deprotection (95% TFA, 2.5% TIS, 2.5% water) the peptide was purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The identity of the peptide was confirmed by analytical RP-HPLC and MALDI-ToF. MS $[M + H]^+$: m/z 3090.5 (calc.), 3090.7 (found) (Fig. s1). 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 220 nm (buffer A, 5% and buffer B, 95% aqueous CH_3CN, 0.1% TFA).

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded on a nitrogen-flushed Module B end-station spectrophotometer at B23 beamline (Diamond Light Source, UK). Samples were loaded on a circular Quartz cell (*l* = 0.01 cm), and the data collected with a 1 nm step and 1 second collection time per step is presented as the average of 4 scans. Titration curves were obtained on an Applied Photosystem Chirascan spectropolarimeter using the same experimental conditions. All measurements were taken in ellipticities in mdeg and after baseline correction were converted to mean residue ellipticity by normalising for the concentration of peptide bonds and cuvette path length (MRE = θ/*l*10*xc*, where *l*: path length; *c*: molar concentration; *x*: number of peptide bonds). Peptide solutions (100 μM) were prepared in filtered (0.22 μm) 10 mM aq. MOPS, pH 7.4. Titrations were performed with siRNA titrated (2.5-16 μM) to capzip (1 hour incubations, constant peptide 100 μM). Kd values were calculated using Hills equation.[19]

Fourier Transform Infra-red (FT-IR) 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) peptide samples (100 μM, 10 mM MOPS, pH 7.4) 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.

Small-Angle X-ray Scattering (SAXS). Experiments were performed at the synchrotron SAXS beamline B21 (Diamond Light Source, UK). A few microlitres of samples were injected via an automated sample exchanger at a slow and very reproducible flux into a quartz capillary (1.8 mm internal diameter), which was then placed in front of the X-ray beam. The quartz capillary was enclosed in a vacuum chamber, in order to avoid parasitic scattering. After the sample was injected in the capillary and reached the X-ray beam, the flow was stopped during the SAXS data acquisition. B21 operated with a fixed camera length (4 m) and fixed energy (12.4 keV) allowing data collection for q= 0.004-0.4 $Å^{-1}$ ($q = 4\pi sin\Theta/\lambda$, with Θ: scattering angle and λ= 1 Å). The images were captured using a Pilaturs 2M detector. Data processing (background subtraction, radial averaging) was performed using dedicated beamline software Scatter.
Sasfit software (https://kur.web.psi.ch/sans1/SANSSoft/sasfit.html) was used to fit the SAXS curve for siRNA (Fig 3c) using the form factor for a generalized Gaussian coil with radius of gyration R_G= 18 Å, consistent with the double helix dimensions (R_G= 16.7 Å).

High Resolution Transmission Electron Microscopy (HR-TEM). Micrographs were recorded using a FEI Tecnai 20 twin lens scanning transmission electron microscope, operated at 200 kV. Droplets of solution were placed on glow discharge treated Cu grids coated with pioloform and carbon film, stained with uranyl acetate (1%, wt) for a few seconds and the buffer excess was removed by blotting paper. Grids for samples containing only capzip were placed on a drop of water for a few seconds, after uranyl acetate staining, and before removing excess of sample by blotting paper. High resolution images were recorded using a FEI Eagle 4kx4k CCD.

Cryogenic Scanning Electron Microscopy (cryo-SEM). Micrographs were recorded using a FEI Quanta 400 SEM instrument. An aluminium stub was covered with filter paper and a Whatman membrane (0.05 µM pore size) was glued on the surface of the filter paper. A drop of peptide solution (100 µM) was placed on the membrane surface. The sample was frozen in liquid nitrogen slush and transferred under vacuum to the preparation chamber stage of an Oxford Instruments CT1500HF CryoTrans (cryotransfer) system held below -140 ^oC. The sample was fractured followed by raising the preparation stage temperature to -90 ^oC for 3 minutes to sublime ice and clean the surface. The temperature was then decreased to -110 ^oC, and the sample was coated with a platinum sputter coating and transferred onto a second cryo-stage mounted in the FEI Quanta 400 SEM and held at below -140 ^oC for the duration of the standard high vacuum imaging. The sample was imaged at 5 kV.

Atomic Force Microscopy on silicon and gold substrates.

Silicon wafers (in-air)

For AMF imaging a drop (5 µL) of peptide solution was placed on a clean silicon wafer and the buffer excess was removed by blotting paper. All measurements were carried out using tapping mode AFM on a Cypher instrument (Asylum research) using super-sharp silicon probes (Nanosensors; resonant frequency ~ 330 kHz, tip radius of curvature < 5 nm, force constant 42 N/m). Images were flattened via line-by-line subtraction of first-order fits to the background, using SPIP software, version 6.0.2.

Gold substrates (in water)

For in-liquid imaging, negatively charged gold substrates were used to restrict the movement of cationic capsules to allow position-fixed imaging. The measurements were done on the same Cypher instrument using a droplet (30 µL) cantilever holder. All images were taken using BioLever mini, Olympus (BL-AC40TS-C2) cantilevers (Resonant frequency ~ 25 kHz in water, tip radius of curvature ~ 8nm and spring constant 0.09 N/m). As seen in the Figure s4a, gold substrates contain granular structures with peak to valley ~ 2.5 nm making it relatively rough compared to silicon wafers. After optimisation and control image acquisition, capzip (5 µL, 100 µM) was introduced in the droplet and images were acquired continuously. Image analysis was carried out in a similar manner as above.

***In-Liquid Atomic Force Microscopy on Supported Lipid Bilayers*.**

Supported lipid bilayers were prepared as described elsewhere.[6] The lipids (Avanti Polar Lipids), 1,2-dilauroylphosphatidylcholine (DLPC) and 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DLPG), 75%/25% (molar ratios) were used for the construction of small unilamellar vesicles (50 nm). Typically, freshly prepared vesicles (5 µL, 4 mg/mL) were added on a cleaved mica disc pre-hydrated with buffer (50 µL, 150 mM NaCl, 20 mM $MgCl_2$ and 20mM of $CaCl_2$, 10mM MOPS, pH 7.4). After incubation (20 min) at room temperature the sample was washed (x10) with buffer containing the same ingredients except $MgCl_2$ and $CaCl_2$ (50 µL). Capzip (2 µL, 100 µM) incubated overnight was added to SLBs to a final concentration of 2 µM. Imaging was performed at room temperature using Peak Force Tapping mode on a MultiMode 8 AFM system applying maximum force of ~85 pN with MSNL-E cantilevers (all Bruker, USA). Images were flattened via line-by-line subtraction of first-order fits to the background using the NanoScope Analysis software v. 1.5.

Cross-Sectional Analysis by Laser Scanning Confocal Microscopy

Capzip (100 µM) was incubated in 10 mM MOPS, pH 7.4 with 25 µM of fluorescein for 2 hours. 20 µL of the solution was deposited onto 8-well glass chamber (Labtek, Nunc). Fluorescence was measured and images acquired at 488 nm using an inverted confocal laser scanning microscope (CLSM) (FV-1000, Olympus) with a 60x objective. Recorded xyz stacks of images were processed using Imaris 6.2 software.

Minimum Inhibitory Concentration Assay. Minimum inhibitory concentrations (MIC) were determined by broth microdilution on *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* K12, *Staphylococcus aureus* ATCC 25723 according to the Clinical and Laboratory Standards Institute. Typically, 50 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 50µL of serial two-fold dilutions of the peptides (from 50 to 0 µM – final concentration) at 37°C on a 3D orbital shaker. The absorbance was measured after peptide addition at 600 nm using a Fluostar plate reader (BMG Labtech). Minimum inhibitory concentrations (MIC's) were defined as the lowest peptide concentration after 24 hours at 37°C. All tests were done in duplicate.

Haemolysis Assay. Haemolysis was determined by incubating 10% (v/v) suspension of bovine erythrocytes (Seralab UK) with peptide. 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 deionized water (fully haemolysed 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 deionized water defined complete haemolysis. The values below correspond to the percentage of haemolysis at tested concentrations. All tests were done in triplicate.

***Stain-dead Antimicrobial Assay (biofilm formation*).** Bacterial inocula were prepared in Mueller Hinton broth at A600 = 0.6, then diluted to 1:100 in the pre-warmed medium, and 50 µL of this dilution was added to each well of a Nunc LabTek chambered cover glass slides. Capzip solution (50 µL, 100 µM) was equally distributed between the 8-wells of the chamber (25-50 µM final concentration). Bacterial colonisation (films) formed after 16-h incubations were analysed using a Live/Dead BacLight bacterial viability stain kit (Molecular Probes, UK), and were visualized using a confocal laser scanning microscope (CLSM) (FV-1000, Olympus). Cell counts were done using ImageJ software.

Agarose gel electrophoresis. Peptide-siRNA complexes at different N/P ratios were analyzed using a horizontal agarose gel (1.2 % of agarose in TBE buffer). The 2-Log DNA Ladder, 0.1-10.0 kb, (NEB, USA) was used as molecular weight markers. Ethidium Bromide, used as an intercalating dye, was added directly into the gel. 20 µL of the prepared complexes were mixed with 5 µL of 5xDNA loading buffer, with 12.5 µL subsequently loaded into the gel. The gels were run for 2 hours at 70 V, and were then imaged using a UV trans-illuminator (BioDoc-iT, UVP).

***Transfection assays*.** HeLa cells were maintained in DMEM cell culture medium supplemented with serum growth supplement and antibiotics (gentamicin and amphotericin B) in 25 cm^2 culture flasks, and grown at 37°C, 5% CO_2 for 72 hrs to reach 60% confluency. The cells were then washed (x3) with PBS and trypsinized followed by the addition of trypsin inhibitors to eliminate secondary toxic effects of trypsin. Detached cells were spun down by centrifugation, and the excess solvent was replaced by cell growth media. 10 µL of cell solution was mixed with 10 µL of Trypan blue. The mixture was then placed on a counting plate to count cells ($25x10^3$ cells per well). Before transfection, the cells were washed (x3) with Opti-MEM® serum reduced media. Alexa-labelled siRNA (Eurogentec, UK) at different ratios with capzip were transferred to the wells containing HeLa-GFP cells, and incubated for 48 hours marking different time points. siRNA was incubated with peptide in MOPS (20 µL, 10 mM) at pH 7.4 for 15 minutes before being diluted into Opti-MEM® (200 µL) and added to cells (total siRNA at 75 nM). After incubation the wells were visualised using confocal microscopy. Results were analysed as a function of total fluorescence: green for GFP background fluorescence and red for Alexa-labelled siRNA. Lipofectamine® RNAiMAX and Alexa-labelled siRNA (Invitrogen, UK) were used as a positive and negative (background) controls, respectively.

***Cell viability assay*.** Hela cells were seeded in a 96-well plate at 4,000 cells per well and incubated overnight at 37 °C, 5% CO_2. After incubation, control transfection reagents (loaded with siRNA according to the proprietary protocols) or capzip assembled in 10 mM MOPS (pH 7.4) at different concentrations were added (10 µL), and diluted with Opti-MEM® to a final volume of 100 µL. After 3-hour incubation, 100 µL of DMEM cell culture medium were added to each well, and the plates were incubated for 24 and 48 hours. Alamar Blue® reagent (ThermoFisher Scientific) is supplied as a 10× solution and added to each well by diluting (1×) in the culture medium. The cells were incubated for 2 hours at 37 °C in 100 µL of the reagent. The fluorescence of each well was measured with a microplate reader (BMG Labtech, Germany), with 544 nm excitation and 590 nm emission filters. Standard calibration curves (200−20,000 cells) were generated by plotting measured fluorescence values versus cell numbers. Total viable cell counts are expressed in percentage after subtracting the total cell counts measured for samples without transfection reagents (control). All measurements were done in triplicate.

Gene knockdown assays. mRNA concentrations in cells transfected with capzip-siRNA were measured according to the MIQE guidelines.[20] The knockdown assay was performed using two recommended housekeeping genes ACTB (targeted) and GAPDH (reference). Lipofectamine® RNAiMAX and N-TER® were used as positive controls and prepared according to the proprietary protocols. siRNA alone was used as a negative (background) control. Proprietary primers (design optimised for PCR), RNA extraction and RT-qPCR kits together with method development protocols were adapted to limit assay optimisation. All measurements were done in triplicate.

Transfection

β-actin siRNA was used for transfection with peptide concentration adjusted to the desired N/P ratios. Preparations with siRNA added into peptide after or before the assembly were similar. Capzip-siRNA complexes or controls were added to cells and the cells were then centrifuged (5 min, 500 x g) and incubated for three hours. After incubation the cells were supplemented with complete DMEM media (20%, 200 µL, total siRNA at 75 nM). For RNA extraction cells were harvested as described above and RNA was prepared using RNeasy mini® prep kit, cDNA was prepared from the RNA using the QuantiTect reverse transcription kit and quantified using two-step RT-qPCR dual hybridization with a QuantiFast probe assay kit (all from Qiagen, USA). The PCR hydrolysis probes for β-actin and GAPDH labelled with FAM and MAX dyes were used as per the proprietary protocols (Qiagen).

RT-qPCR

Cells harvested from a single well were lysed (10^5 cells in 350 µL of lysis buffer) and total RNA was purified (RNeasy mini®). RT was performed (QuantiTect®) in PCR 0.2 mL tubes on a GeneAmp PCR system 2700 (Applied Biosystems, UK) using 30-60 ng of total RNA according to the proprietary protocols. qPCR was performed on a SmartCycler® using Software v2.0d (Cephid). PCR of the diluted cDNA product (1-10 ng) was monitored over 45 cycles with the quantification cycle (C_q) determined using a manual threshold of 30 fluor units. PCR titrations

of both genes resulted in co-linear amplification. Control samples of no template control (contamination during qPCR) and no RT control (contamination by genomic DNA during RT) were negative (C_q > 35 cycles) indicating no measureable DNA contamination. Fitness levels were calculated and expressed as the normalised function of cells treated with siRNA alone (negative control) and against the total counts of viable cells:

$$= \frac{\Delta\Delta C_T}{(^{cell\ count}/_{cell\ count\ for\ siRNA\ only\ control})}$$

where $\Delta\Delta C_T$ is the knockdown efficiency of ACTB

(i) relative to reference GAPDH gene for each transfection vector (ΔC_T (vector))

ΔC_T (vector) = C_T (ACTB) – C_T (GAPDH), where the threshold cycle (C_T) is calculated from the PCR thermal cycle.

and

(ii) to the siRNA only control (ΔC_T (siRNA only control);

$\Delta\Delta C_T$ = ΔC_T (vector) – ΔC_T (siRNA only control).

Table, Figures and Video

Table S1. Biological activities of capzip in comparison.

Biological cell	MIC, μM		
	capzip	ampicillin	gramicidin S
E. coli (K12)	20	9	22
*P. aeruginosa (*ATCC27853)	18	>200	43
*S. aureus (*ATCC6538)	30	5	22
	LC_{50}		
Bovine erythrocytes	>>100	UD*	≥25

*undetectable (>>10^3)

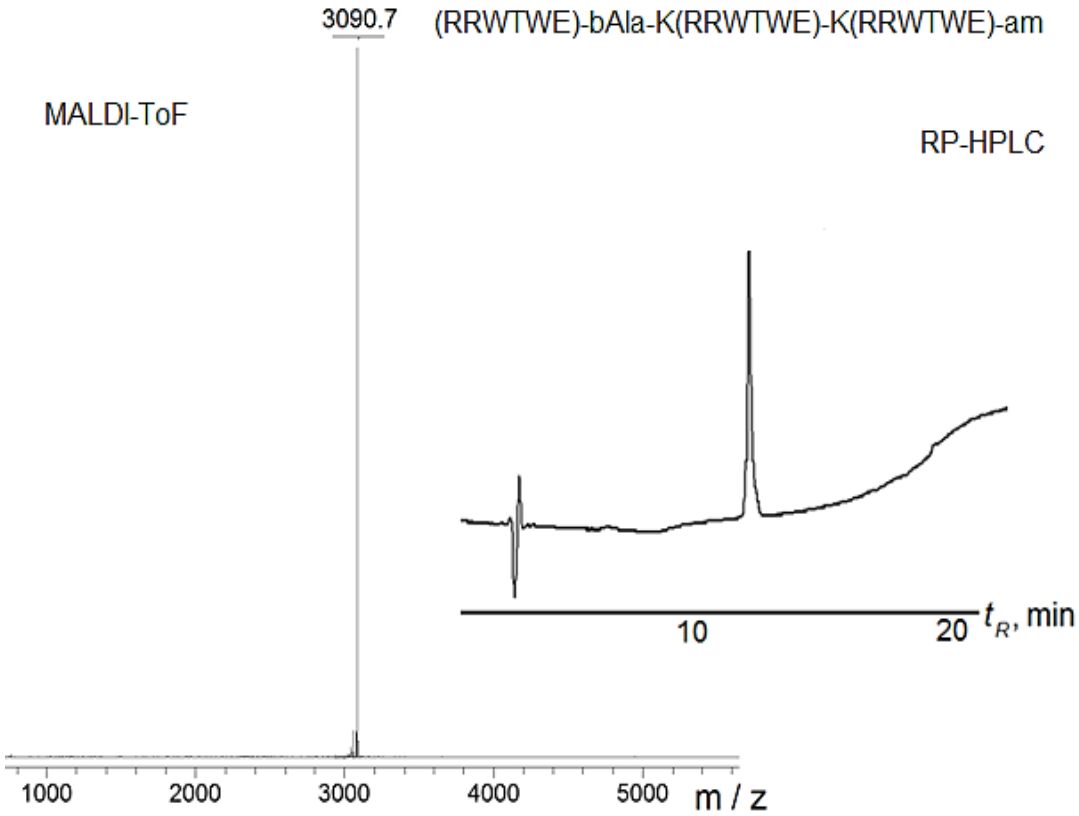


Fig S1. Capzip synthesis. MALDI-ToF and RP-HPLC spectra for purified capzip conjugate.

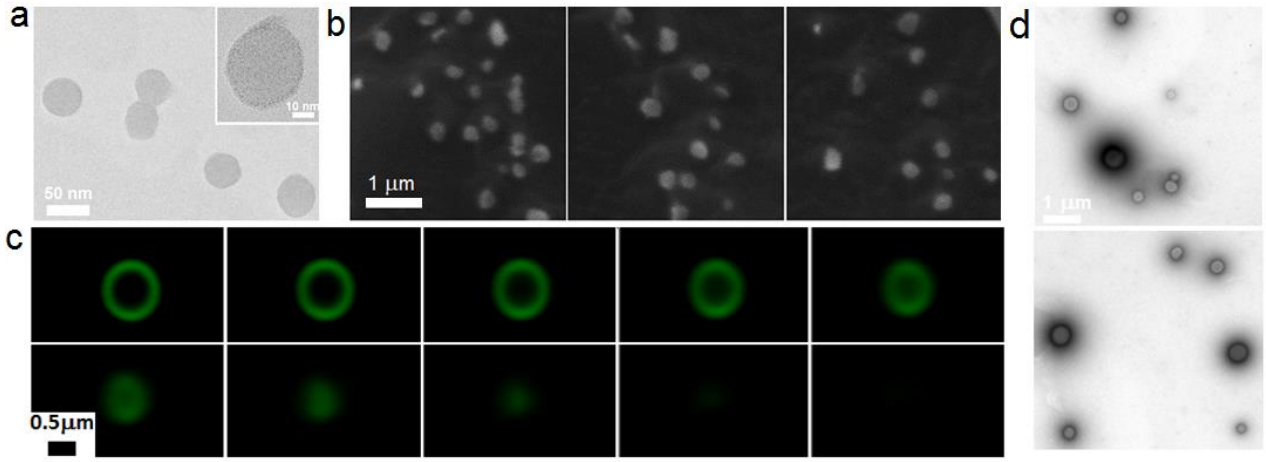


Fig S2. Capzip assembly. TEM (a) and cryo-SEM (b) images of assembled capsules. (c) Cross-section fluorescence micrographs of a large capsule with gradual depth changes (0.1-0.2 µm). (d) TEM images of capsules assembled with siRNA. Assembly conditions: 1/5 N/P (siRNA-peptide), pH 7.4, 10 mM MOPS, 20 °C, overnight.

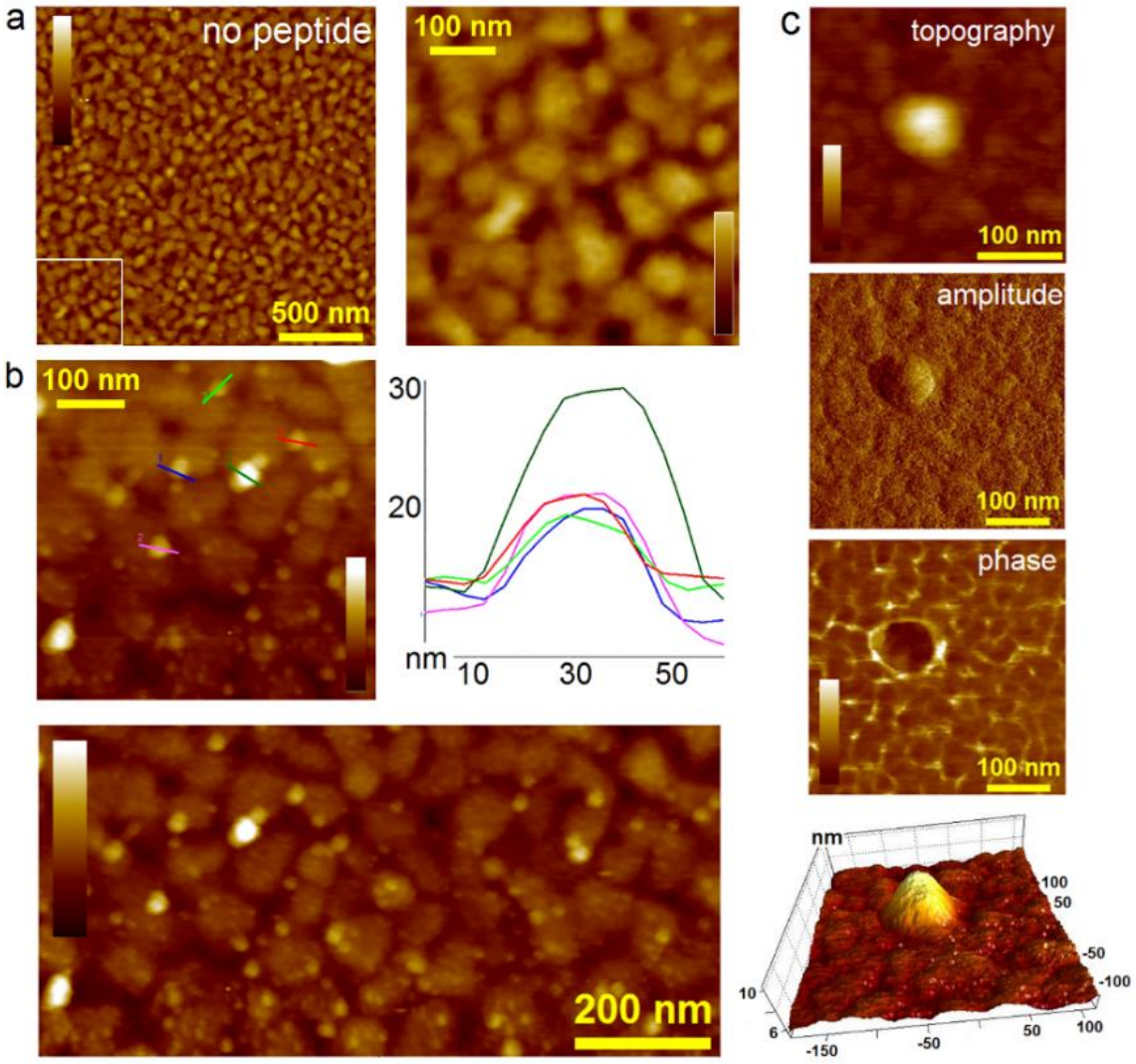


Fig S3. Capzip assembly. AFM images in water of (a) bare gold substrates (b) capzip without siRNA, (c) capzip with siRNA. Color scales: (a) 15 nm (left) and 12 nm (right), (b) 30 nm (upper) and 25 nm (lower), (c) 5 nm (topography) and 70-100 deg (phase).

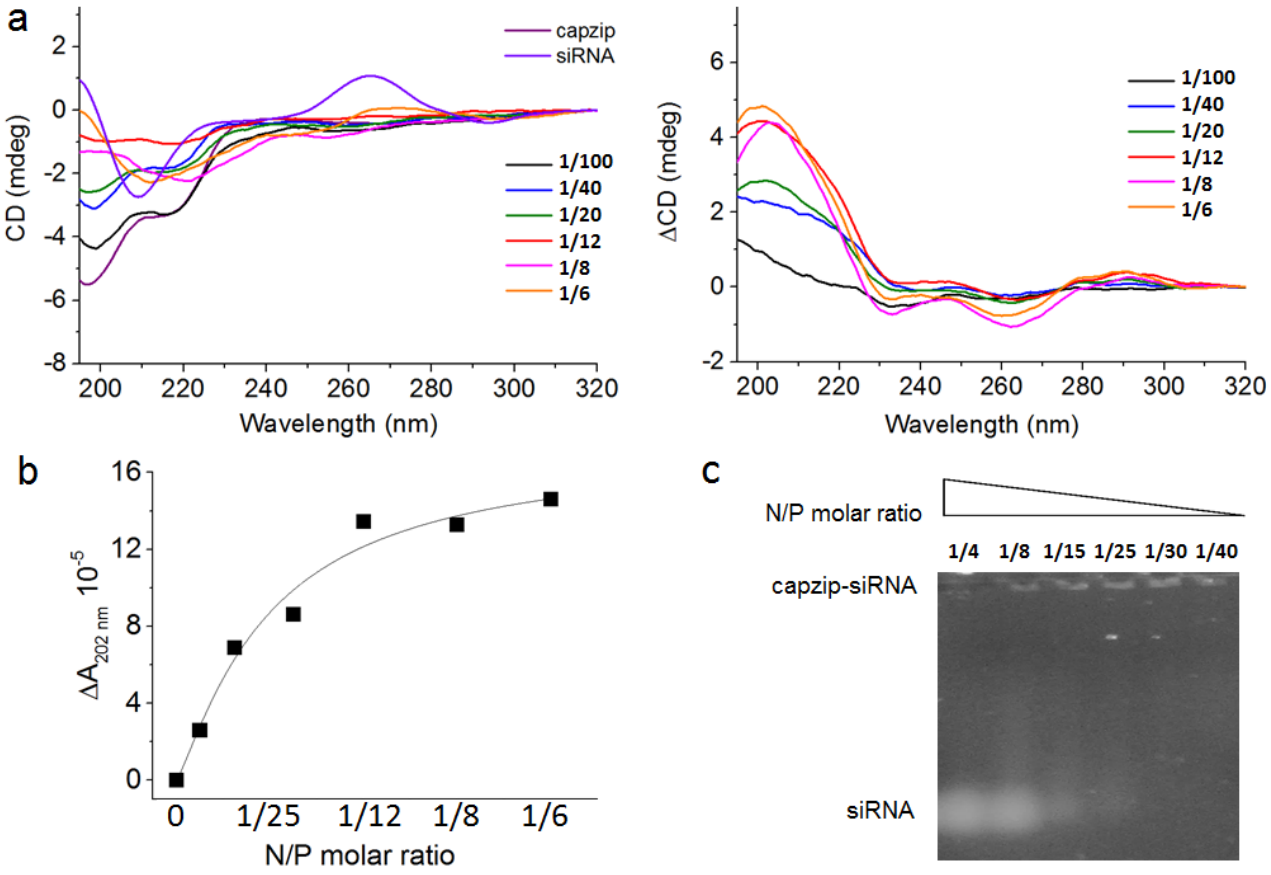


Fig S4. Capzip folding. (a) CD spectra for capzip-siRNA at different N/P molar ratios. Residual spectra (right) after subtracting capzip (100 µM) and siRNA signals from raw CD titration curves (left). (b) Differential absorbance (ΔA) at 202 nm versus molar N/P ratio derived from CD titration spectra for capzip (100 µM), pH 7.4, 10 mM MOPS, 20 °C. (c) Gel electrophoresis for capzip with siRNA at different N/P molar ratios.

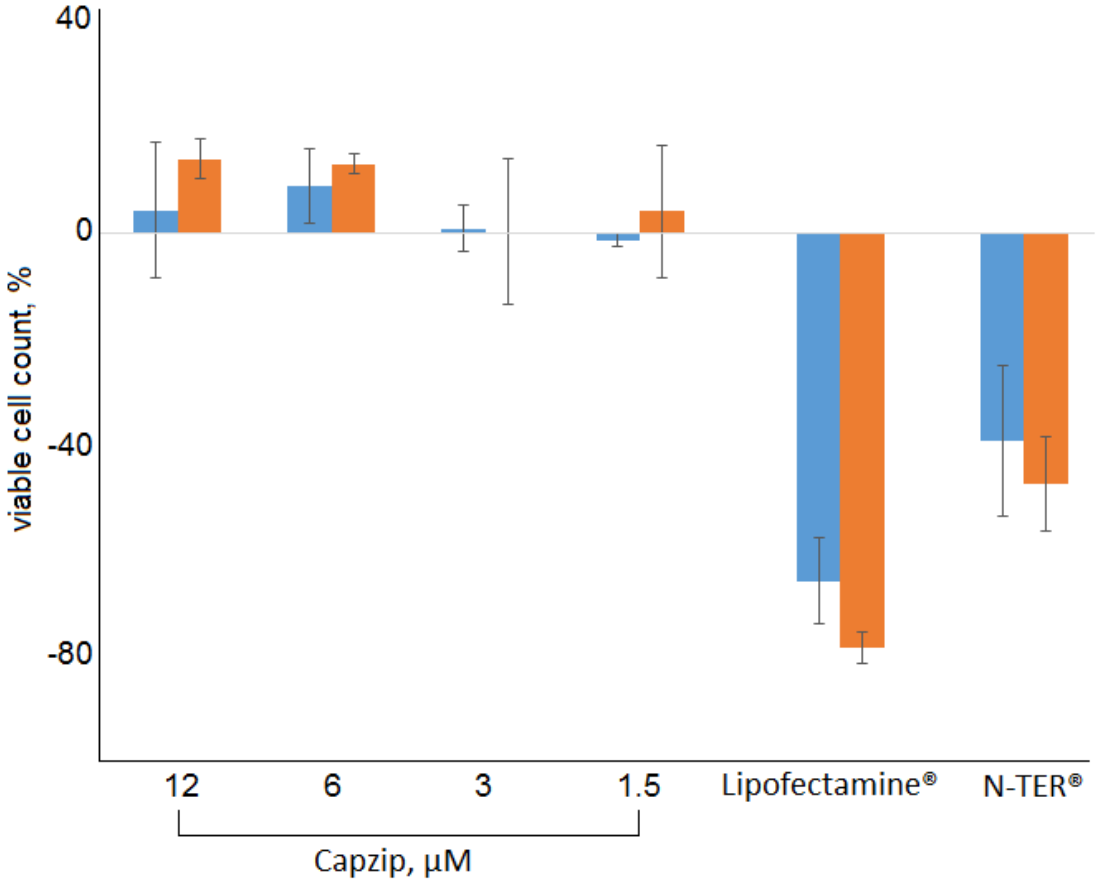
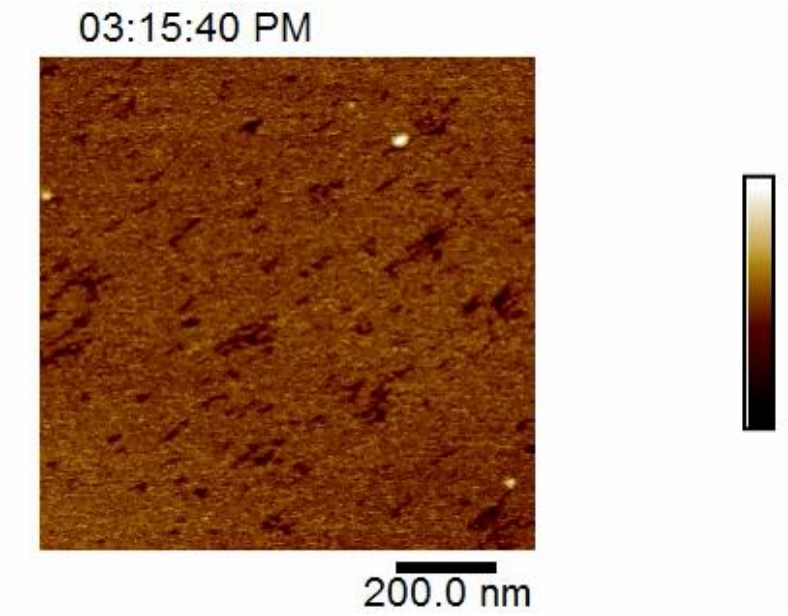


Fig S5. Cell viability expressed as a total count of viable cells after subtracting total viable cell counts in the absence of transfection reagents (background viability), which was taken as 100%. Negative values indicate decreased cell viability.



Video S1. A snapshot of the time-lapse AFM topography of supported lipid bilayers during incubation with capzip in solution. Colour scale is 6 nm.