Supporting Information

Model Self-Assembling Arginine-Based Tripeptides Show Selective Activity Against Pseudomonas Bacteria

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Experimental

Materials. Peptides were obtained as TFA salts from Peptide Synthetics (Peptide Protein Research Ltd, Fareham, UK). Molar masses by ES-MSI are as follows: RFR 477.55 g mol^{-1} (477.56 g mol^{-1} expected), RWR 516.59 g mol^{-1} (516.60 g mol^{-1} expected), R2NAlR 527.61 g mol^{-1} (527.62 g mol^{-1} expected) Scheme 1 shows the chemical structure of the peptides. Purities were found (by HPLC) to be > 95%. The native pH of the lipopeptide in water was found to be 6. The mixtures were left to equilibrate for 24 hours before characterization. The solvent used to prepare the solution is only indicated for samples dissolved in PBS (otherwise it is native conditions in ultrapure water).

Nucleotide c-di-GMP was synthesized by Biolog Life Science Institute (Bremen, Germany) and Axxora-Enzo Co (Cat. No. C057). Other reagents were obtained from Sigma-Aldrich.

Fluorescence Assays. The critical aggregation concentrations (*cac*) of the tripeptides were determined using fluorescence spectroscopy. Fluorescence spectra were recorded with a Varian Cary Eclipse fluorescence spectrometer with samples in 4 mm inner width quartz cuvettes. ANS (8-anilinonaphthalene-1-sulfonic acid) was used to probe the aggregation as a probe that is sensitive to hydrophobic environments making it suitable to locate the *cac*.[34–38] ANS assays were performed using a 66.8 μM ANS solution to solubilise the tripeptides. Fluorescence spectra were recorded between 400-650 nm (λ_{ex} = 356nm).

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded using a Chirascan spectropolarimeter (Applied Photophysics, UK). Solutions were placed in a quartz cover slip cuvette (0.1 mm thick). Spectra are presented with absorbance $A < 2$ at any measured point with a 0.5 nm step, 1 nm bandwidth, and 1 s collection time per step. The CD signal from the water background was subtracted from the CD data of the sample solutions.

Determination of Binding Constant from CD Titration Curves. CD was used to study the binding of the peptides to c-di-GMP. CD spectra were measured for the solution containing the mixture of peptide + nucleotide (CDnp), the peptide solution (CDp) and the CD for the nucleotide solution (CDn). The difference spectrum ΔCDnp= CDnp – (CDn+CDp) shows that there is binding, and can be analysed to provide the dissociation constant. Following Foletti *et al*.[1] we used the expression

$$\Delta CD_{np} = A + \frac{\Delta CD_{np,max}[RXR]^{\propto}}{K_d^{\propto} + [RXR]^{\propto}}$$

where [RXR] is the molar concentration of peptide and α is the Hill coefficient, and *A* is a baseline which was found to be necessary to account for the background offset.

Cryogenic-Transmission Electron Microscopy (Cryo-TEM). Imaging was carried out using a field emission cryo-electron microscope (JEOL JEM-3200FSC), operating at 200 kV. Images were taken in bright field mode and using zero loss energy filtering (omega type) with a slit width of 20 eV. Micrographs were recorded using a Gatan Ultrascan 4000 CCD camera. The specimen temperature was maintained at -187 ^{o}C during the imaging. Vitrified specimens were prepared using an automated FEI Vitrobot device using Quantifoil 3.5/1 holey carbon copper grids with a hole size of 3.5 μm. Just prior to use, grids were plasma cleaned using a Gatan Solarus 9500 plasma cleaner and then transferred into the environmental chamber of a FEI Vitrobot at room temperature and 100 % humidity. Thereafter 3 ml of sample solution was applied on the grid and it was blotted twice for 5 seconds and then vitrified in a 1/1 mixture of liquid ethane and propane at temperature of -180 °C. The grids with vitrified sample solution were maintained at liquid nitrogen temperature and then cryo-transferred to the microscope.

Atomic Force Microscopy with Infrared Nanospectroscopy. AFM-IR infrared nanospectroscopy assays were performed using an Anasys NanoIR2-s AFM microscope as described elsewhere.[2, 3] Solutions containing peptides and nucleotides were mixed to produce complexes containing 0.13 wt% RWR + 0.1 wt% c-di-GMP. Mixtures were prepared at room temperature and samples were left to rest for 30 minutes prior to further analysis. After complexation, droplets of RWR/c-di-GMP solutions were deposited onto the surface of Au-coated silicon substrates and left to rest for about 5 minutes. The substrates were then rinsed with ultrapure water and left to dry overnight in desiccators. The microscope was used in tapping mode and substrates were illuminated by a tunable laser. To collect IR spectra from individual aggregates, the AFM tip was positioned on top of each particle where laser pulses with wavenumbers in the range 1550 – 1800 cm^{-1} illuminated the sample, generating absorbance spectra from the area underneath the tip (diameter ~30 nm). Chemical maps were obtained by fixing the laser source at specific wavenumbers and scanning the surface with the AFM tip. This procedure provided topography images along with spatially-resolved absorbance maps at the corresponding wavenumber.

Cytotoxicity assays. The cytotoxicity of the peptides was examined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using primary human dermal fibroblasts, HDFa (Gibco™).

HDFa cells were cultured in DMEM F12 media supplemented with 5% fetal bovine serum (FBS), 1% antibiotic-antimycotic, and 5 μg/mL insulin. All cells were maintained in a humidified atmosphere at 37 ^{o}C and 5% CO_2.

HDFa cells were seeded into a 96-well plate at a concentration of 4×10^4 cells/mL and left to adhere for 24 hours in 100 μL of complete medium. Peptides RFR, RWR and R2NAlR were dissolved in complete medium, and added to the cells to give a final volume of 200 μL at

concentrations between 0.005 and 0.5 wt%. One well, containing 200 μL of complete medium with no peptide, was used as negative control. Then, the cells were incubated for 67 hours. After this, 20 μL MTT (5 mg/ mL, in PBS) was added to each well plate and allowed to incubate for 5 hours (72 hours total). Following the incubation time, the solution was removed from the wells and replaced with 100 μL DMSO per well to dissolve the formazan crystals. Plates were incubated for 30 minutes, and then analysed using a UV microplate reader (λ = 570 nm). Results are reported as a % cell viability compared to control (untreated) values. MTT assays were performed in triplicate, ANOVA and Bonferroni *post hoc* tests were used to assess statistical significance.

Antimicrobial Survival assays. The antimicrobial assays were performed with 3 species of bacteria: *Staphylococcus aureus, Escherichia coli* (K12), and *Pseudomonas aeruginosa (PA01)*. Stock cultures were stored in -80 ^{o}C in 7% (v/v) DMSO. Prior to experiments all three cultures were streaked out onto Lysogeny Broth (LB) agar and grown overnight at 37 ^{o}C.

From these plates, one colony was then transferred into 5 mL LB, and grown at 37 ^{o}C under agitation at 150 rpm, on an orbital shaker overnight and these cultures were used for ongoing experiments. Cultures were then transferred into a 15 ml Falcon tube and cells were harvested by centrifugation at 9000 rpm and 4 ^{o}C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1.5 mL ice chilled PBS (phosphate buffered saline).

After this, 20 μL of this solution was transferred into 200 μL of 0.5 mg/mL (or 0.05 wt%) of RFR, R2NAlR or RWR in sterile water, or control solutions of 200 μL of sterile water. Solutions were then vortexed for 5 seconds and 3 x 20μL aliquots were taken at times 0 hrs, 2 hrs, and 24 hrs. These samples were then serially diluted in PBS, and 10 μL of each dilution was plated onto LB agar and incubated at 37 ^{o}C overnight before colony counting. The whole

assay was repeated 3 times (n=3) and statistics were calculated using ANOVA and Turkey statistical tests.

Antimicrobial Biofilm Assays.: To form a biofilm, *Pseudomonas aeruginosa* PA01 was grown overnight in LB media. Following this, the culture was diluted 1:100 in M63 minimal medium supplemented with arginine and magnesium sulphate, with and without RFR, RWR or R2NALR, with four technical replicates. After this the plate was incubated for 24 hrs.

Following incubation, the supernatant was discarded and then gently washed in water several times to decrease background staining. After this, 125 μL of 0.1 wt% Crystal violet solution was added to each well of the microtiter plate, and incubated for 10 minutes. Following this, the plate was washed several times and left to dry overnight.

After this, 125 μl of 30% acetic acid in water was added, to solubilise the biofilm, and the plate was incubated for a further 10 minutes to allow solubilisation. Absorbance was measured using a plate reader at 550 nm. The assay was repeated 3 times, and significance was assessed using Anova and Tukey statistical tests.

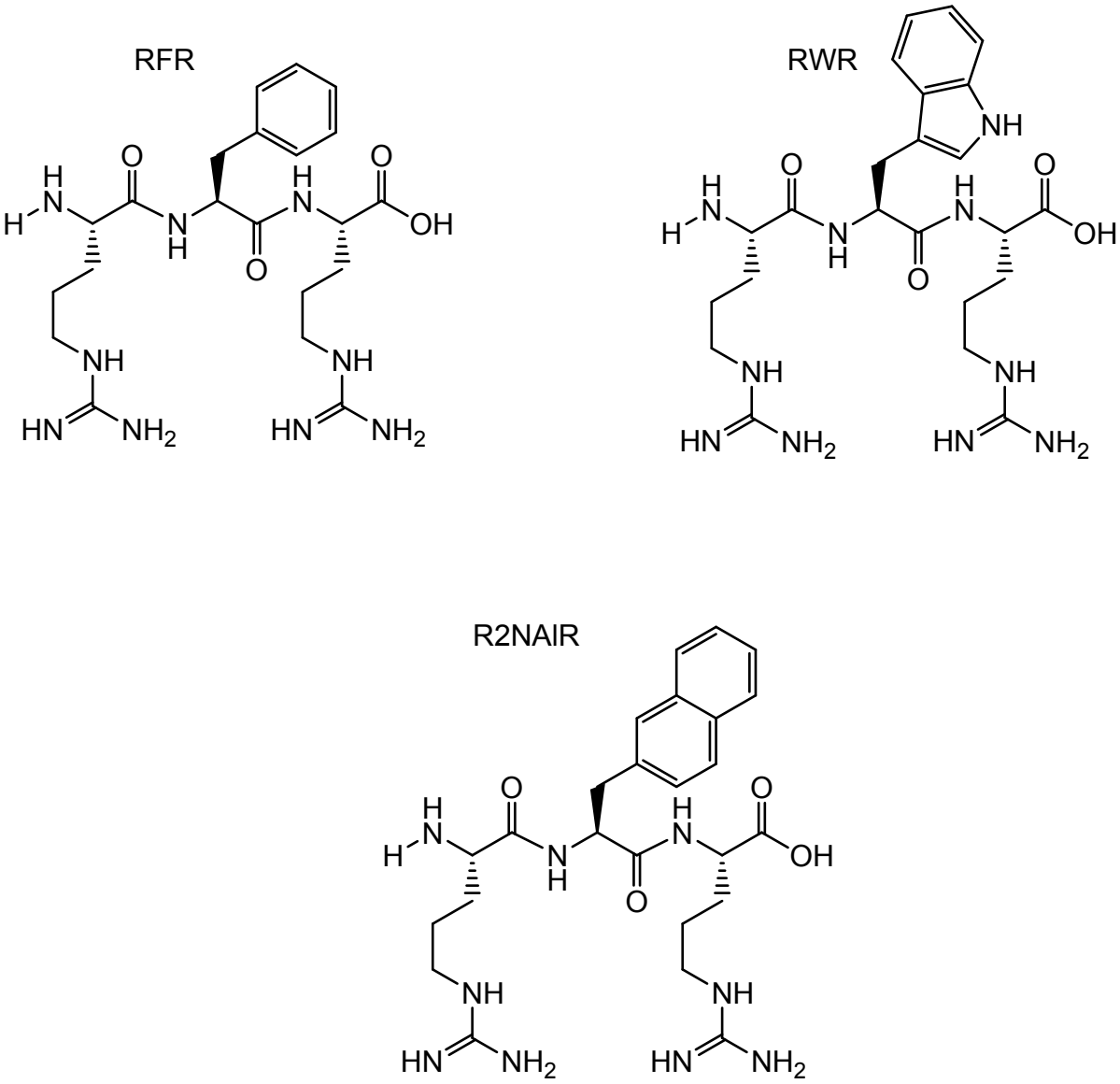
Congo red Measurements of c-di-GMP Levels. *Pseudomonas aeruginosa* strain PA01 was grown overnight in LB media. After this, the culture was diluted 1:100 in 1 ml of M63 minimal medium supplemented with 0.4 % L-arginine and 1 mM magnesium sulphate, with 40 μg/mL Congo red with and without RFR, RWR or R2NAlR. Control solutions were also prepared using the described medium above with no bacterial cells present. Congo red provides an indirect measure of c-di-GMP levels as it binds to the matrix of the biofilm, production of which is correlated to c-di-GMP levels.[4] Samples were shaken at 200 rpm overnight in a shaking incubator. After this, 200 μL of supernatant was added in triplicate to a 96 well place, and measured using a plate reader at 490 nm. Results are reported as (Control solution OD–

bacteria-containing solution OD) [OD: optical density]. T-Tests were used to assess significance.

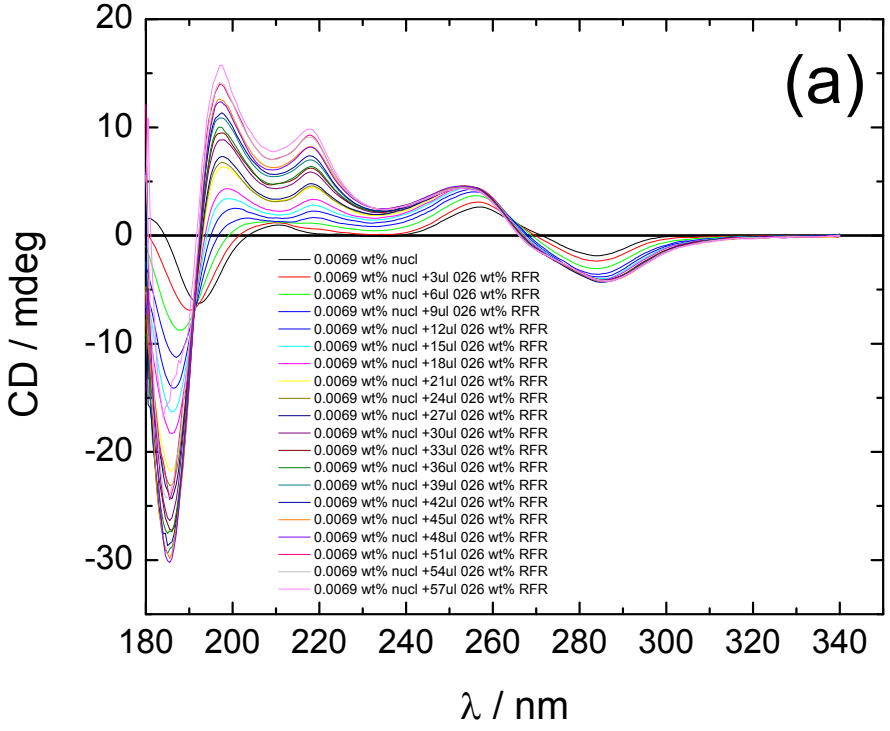
Table, Scheme and Figures

SI Table 1. Coefficients from the Hill equation binding analysis

Peptide	**K_d** [μM]	α	**A** [mdeg]	$\Delta CD_{np,max}$ [mdeg]
R2NalR	94.2±6.5	4.4±1.1	0.061±0.01	1.35±0.01
RFR	125.4±10.2	5.2±1.7	0.62±0.1	1.07±0.08
RWR	262.9±8.7	7.6±1.6	0.33±0.08	1.28±0.04



SI Scheme 1. Molecular structures of peptides studied.


(a)
CD / mdeg
λ / nm
20
10
0
-10
-20
-30
180
200
220
240
260
280
300
320
340
0.0069 wt% nucl
0.0069 wt% nucl +3ul 026 wt% RFR
0.0069 wt% nucl +6ul 026 wt% RFR
0.0069 wt% nucl +9ul 026 wt% RFR
0.0069 wt% nucl +12ul 026 wt% RFR
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0.0069 wt% nucl +42ul 026 wt% RFR
0.0069 wt% nucl +45ul 026 wt% RFR
0.0069 wt% nucl +48ul 026 wt% RFR
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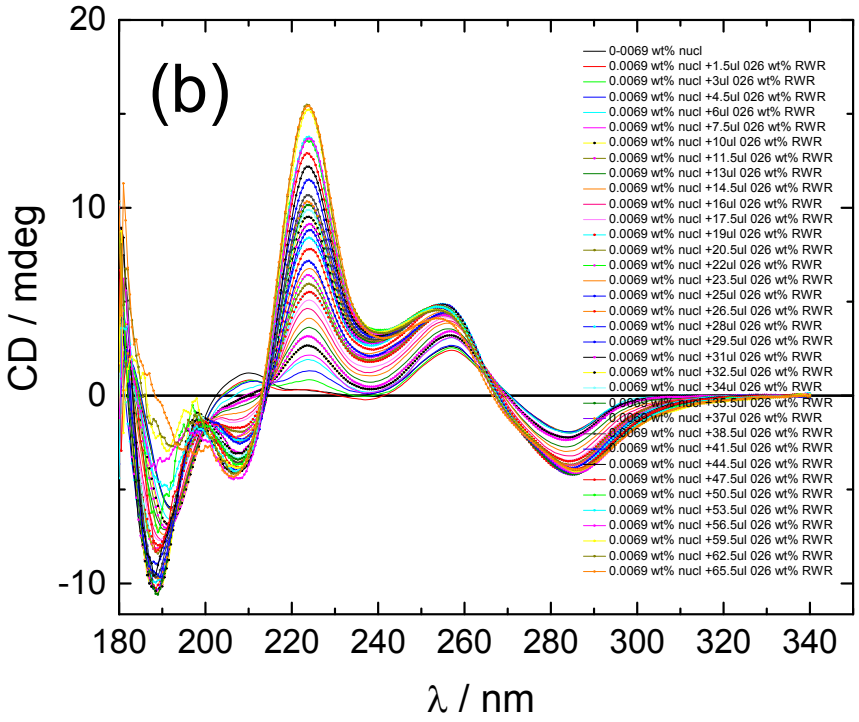
0.0069 wt% nucl +57ul 026 wt% RFR


(b)
CD / mdeg
λ / nm
20
10
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-10
180
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280
300
320
340
0-0069 wt% nucl
0.0069 wt% nucl +1.5ul 026 wt% RWR
0.0069 wt% nucl +3ul 026 wt% RWR
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0.0069 wt% nucl +29.5ul 026 wt% RWR
0.0069 wt% nucl +31ul 026 wt% RWR
0.0069 wt% nucl +32.5ul 026 wt% RWR
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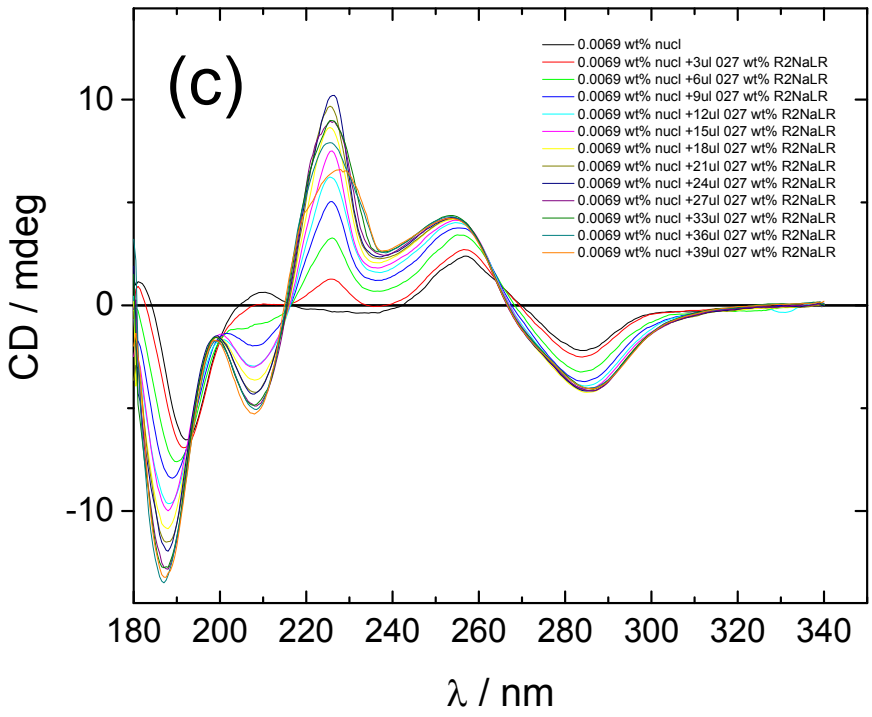
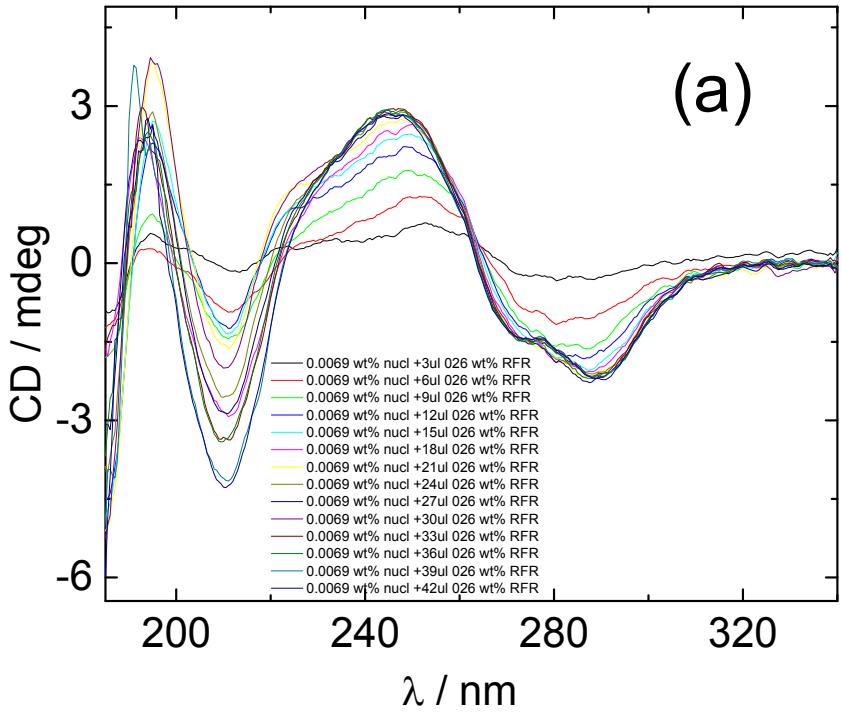


SI Figure S1. CD spectra measured for the titration of 0.26 wt% (a) RFR, (b) RWR, (c) R2NAlR into a 0.0069 wt% nucleotide (100 uM) solution.


(a)
CD / mdeg
λ / nm
0.0069 wt% nucl +3ul 026 wt% RFR
0.0069 wt% nucl +6ul 026 wt% RFR
0.0069 wt% nucl +9ul 026 wt% RFR
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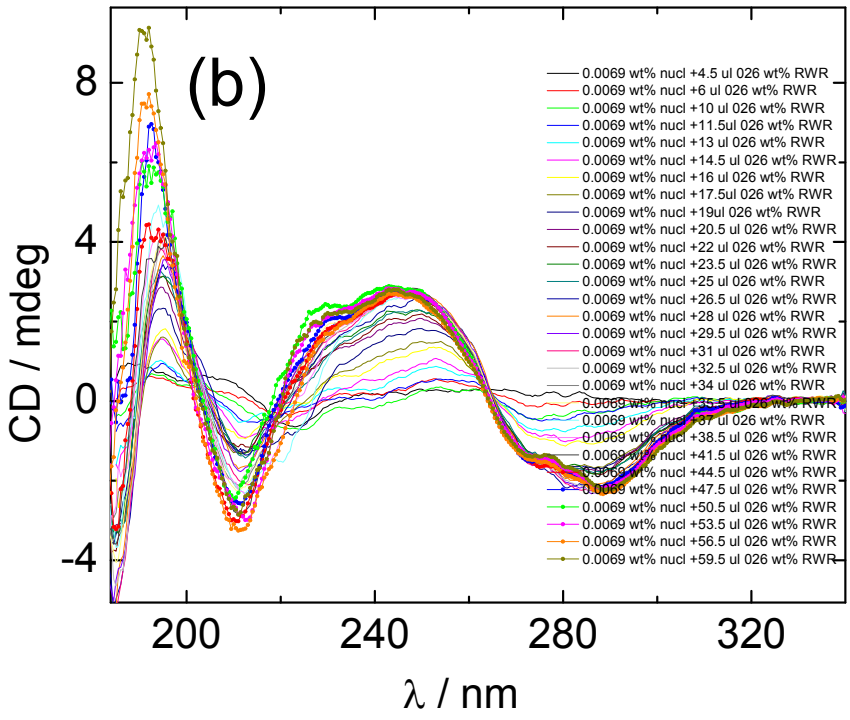
0.0069 wt% nucl +42ul 026 wt% RFR


(b)
CD / mdeg
λ / nm
0.0069 wt% nucl +4.5 ul 026 wt% RWR
0.0069 wt% nucl +6 ul 026 wt% RWR
0.0069 wt% nucl +10 ul 026 wt% RWR
0.0069 wt% nucl +11.5ul 026 wt% RWR
0.0069 wt% nucl +13 ul 026 wt% RWR
0.0069 wt% nucl +14.5 ul 026 wt% RWR
0.0069 wt% nucl +16 ul 026 wt% RWR
0.0069 wt% nucl +17.5ul 026 wt% RWR
0.0069 wt% nucl +19ul 026 wt% RWR
0.0069 wt% nucl +20.5 ul 026 wt% RWR
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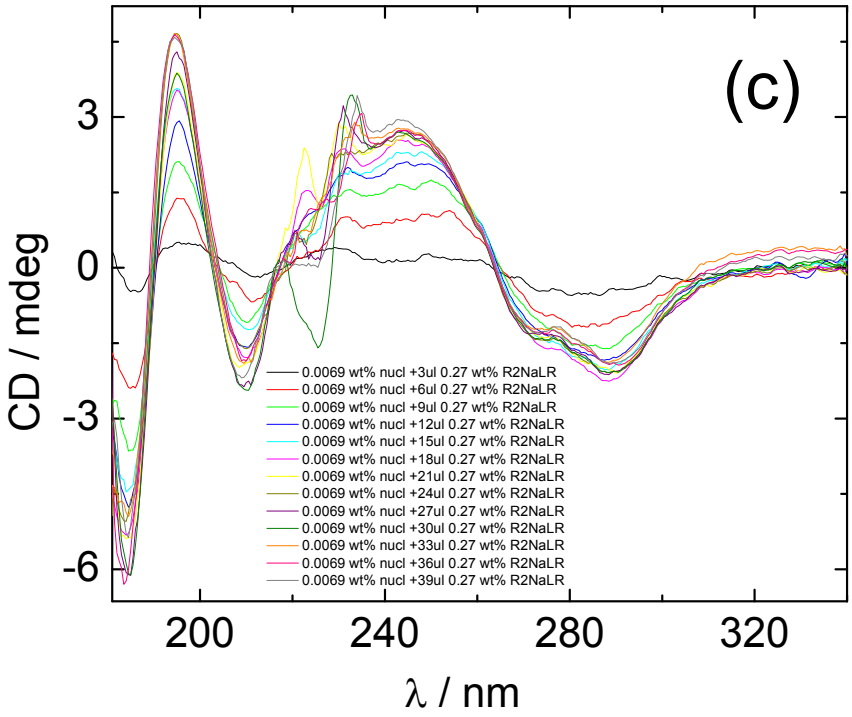
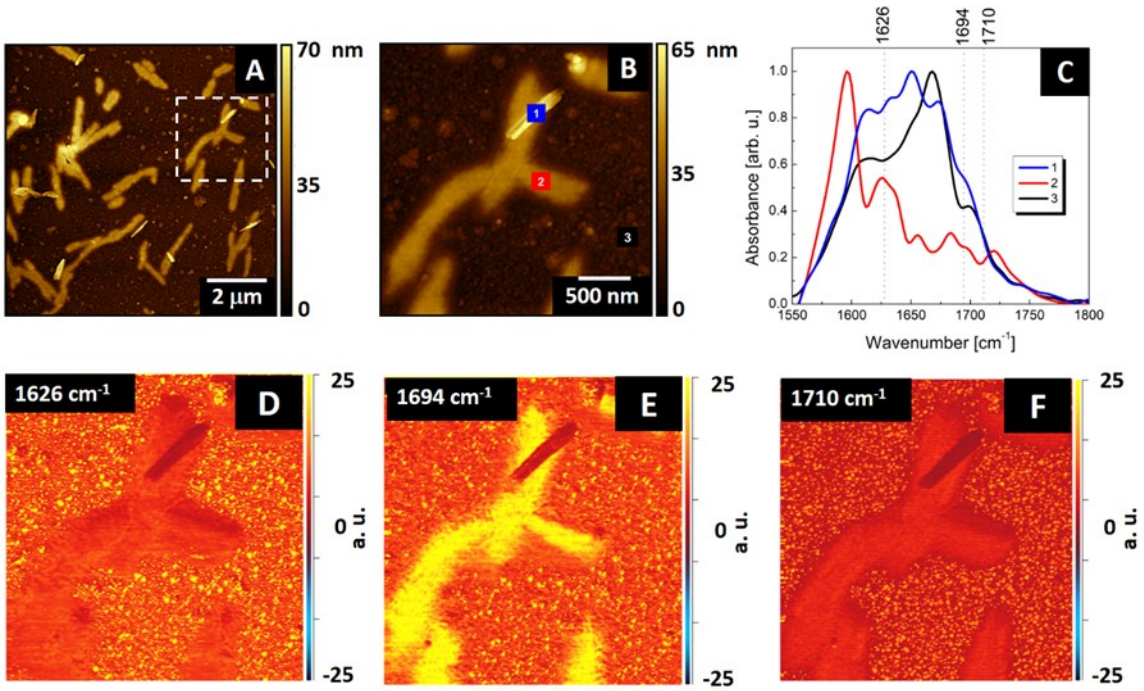


SI Figure S2. CD spectra corresponding to the difference between the spectra plotted in Figure S1 and those for the nucleotide and peptide measured separately. (a) RFR, (b) RWR, (c) R2NAlR. The CD signal at 257 nm was used to calculate the binding curves plotted in Figure 2a.



SI. Figure S3. AFM combined with infrared nanospectroscopy data. (a) and (b) topography images from tape-like assemblies obtained from solutions containing RWR and c-di-GMP. (c) Infrared spectra from regions (~30 nm diameter) indicated by coloured circles in (b). (d-f) Spatially-resolved infrared absorbance maps at the wavenumbers indicated.

References

1. C. Foletti, R. A. Kramer, H. Mauser, U. Jenal, K. H. Bleicher and H. Wennemers, *Angew. Chem. Int. Ed. Engl.* 2018, **57**, 7729-7733. We noted an error in the equation of the specific binding with Hill slope equation in Section SI 5.2 of this paper.
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4. C. J. Jones and D. J. Wozniak, in *c-DI-GMP signaling*, ed. K. Sauer, Humana Press, New York, 2018, ch. 12, pp. 147-156.