

Hydrazone-modulated peptides for efficient gene transfection

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1. Materials and methods

Polystyrene Fmoc protected Rink Amide resin (load 0.71 mmol/g) and Fmoc-L-Lys(Mtt)-OH were purchased from Iris. Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Leu-OH, glutaric anhydride, *tert*-butyl carbazate, triisopropylsilane (TIS), Diisopropylethyl amine (DIEA) were obtained from Sigma-Aldrich. Trifluoroethanol, 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was purchased from TCI. Egg yolk L- α -phosphatidylcholine was purchased from Avanti Polar Lipids. Peptide synthesis grade *N,N*-dimethylformamide was purchased from Scharlau. All other chemicals were purchased from Sigma-Aldrich, TCI or Fisher.

Lipofectamine 2000 was purchased from Life Technologies, Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate) was purchased from Gibco.

The aldehydes tested were either commercially available or synthesized following reported protocols^[1] from the corresponding alcohols. Boc protected adipic acid monohydrazine **2** was prepared as previously described.^{[2][3]}

A microwave assisted peptide synthesiser (Liberty Lite, CEM) was used to prepare the peptide according to standard methods developed by the manufacturers involving diisopropylcarbodiimide (DIC) 0.5 M in DMF as activator and ethyl(hydroxyimino)cianoacetate (Oxyma) 1 M in DMF as activator base.

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II equipped with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column with Solvent A:Solvent B gradients between 5:95 and 95:5 (Solvent A: H₂O with 0.1 % TFA; Solvent B: CH₃CN with 0.1 % TFA). High-performance liquid chromatography (HPLC) preparative purification was carried out on Waters 1525 provided with a binary pump with a dual wavelength Waters 2489 UV detector using a Phenomenex Luna C18(2) 100A column. A Jasco LC 4000 equipped with a quaternary pump and a photo diode array MD-4015 was used for semi-preparative purification using an Agilent Eclipse XDB-C18 column gradients between 95:5 and 25:75 (Solvent A: H₂O with 0.1 % TFA; Solvent B: CH₃CN with 0.1 % TFA).

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz. Chemical shifts are reported in ppm referenced to the following solvent signals: HOD δ H 4.79 and CDCl₃ δ H 7.26 ppm, δ C 77.0 ppm. Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) with coupling constants (*J*) given in Hz, or multiplet (m). Accurate mass determinations (HR-MS) using ESI-MS were performed on a Bruker MicroTof mass spectrometer.

Circular Dichroism (CD) measurements were performed with a Jasco J-1100 CD Spectrometer equipped with a Jasco MCB-100 Mini Circulation Bath for temperature control. For the acquisition of cell microscopy images an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E epifluorescence microscope was used.

A Tecan Infinite F200Pro microplate reader was used to measure fluorescence of cells expressing eGFP directly in Costar cell culture 96-well plates as well as UV-Vis absorbance for the viability assays.

Flow cytometry was performed on a Guava easyCyteTM cytometer. Data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

Dynamic Light Scattering (DLS) and zeta (ζ) potential were acquired in a Malvern Nano ZS equipped with a HeNe laser (633 nm). Scattered light was detected at an angle of 90°.

2. Abbreviations

Aa: Amino acid; AFM: atomic force microscopy; Arg: Arginine; Boc: tert-Butoxycarbonyl; Calcd: Calculated; CD: circular dichroism; DCM: Dichloromethane; DIC: diisopropylcarbodiimide; DIEA: N,N-Diisopropylethylamine; DLS: dynamic light scattering; DMEM: Dulbecco's Modified Eagle Medium; DMF: N,N-Dimethylformamide; DMSO: Dimethylsulfoxide; ESI: electrospray ionization; Fmoc: N-Fluorenyl-9-methoxycarbonyl; eGFP: enhanced Green Fluorescent Protein; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HKR: HEPES-Krebs-Ringer; HPLC: High-performance liquid chromatography; HRMS: High resolution mass spectrometry; Leu: leucine; Lys: Lysine; Mtt: 4-Methyltrityl; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *N*-HATU: *N*-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; *N*-HBTU: *N*-[(1H-Benzotriazol-1-yl)4(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; PBS: Phosphate buffered saline; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; RP: Reverse Phase; SPPS: Solid Phase Peptide Synthesis; TAE: tris-acetate-ethylenediaminetetraacetic acid; TEM: transmission electron microscopy; TFE: Trifluoroethanol; TIS: Triisopropylsilane.

3. Peptide Synthesis

P1 was synthesised either manually or by using an automated peptide synthesiser. In brief, Rink Amide resin (loading 0.71 mmol/g) was swollen in DMF (peptide synthesis grade, 2 mL) for 20 min in a peptide synthesis vessel. Coupling cycles involved the removal of Fmoc protecting group with a solution of piperidine in DMF (20 %, 2 mL) for 10 min and then the mixture was filtered and the resin was washed with DMF (3 x 2 mL, 1 min). Then, a premixed solution in DMF of Fmoc- α -amino acid (4 equiv), *N*-HBTU (4 equiv) (2 mL) and DIEA (0.195 M solution in DMF, 4 equiv) was added to the resin under nitrogen stream for 15 min. Finally, the resin was washed with DMF (3 x 2 mL, 1 min). The completion of each amino acid coupling and deprotection steps was monitored by TNBS test.^[4]

For automated synthesis, a variant of the previous protocol was used instead, according to manufacturer's recommendations. 0.05 mmol of Rink Amide resin was placed into the peptide synthesiser reaction vessel, swollen in DMF, followed by cycles of Fmoc cleavage with piperidine 20% in DMF, washings (3x), then amino acid (5 equiv 2M amino acid solution in DMF), DIC (10 equiv) and Oxyma (10 equiv) were added into the reaction vessel and microwaved for 5 min under temperature control followed by washings (3x). All steps were performed under nitrogen atmosphere. After the linear peptide was finished the resin was transferred to a different reaction vessel to perform the peptide modification manually.

3.1. Hydrazone peptide modification

Following peptide synthesis, solid phase N-terminus acetylation at 0.05 mmol scale with a mixture of acetic anhydride (Ac₂O) and 2,6-lutidine (1:1, 1 mL) was carried out under

nitrogen stream for 30 min. The resin was washed with DCM (3x) prior to add a mixture of DCM/HFIP/TFE/TIS (6.5/2/1/0.5, 2 mL) to selectively cleave the lysine Mtt protecting groups. The suspension was mechanically stirred for 2 h followed by washings with DCM. After a second deprotection cycle, the resin was washed and subsequently swollen in DMF for 30 min. Then a solution of **2** (5 equiv) in DMF under nitrogen stream, followed by *N*-HATU (5 equiv) in DMF and pure DIEA (8 equiv) was added dropwise. The reaction was stirred under nitrogen stream for 30 min and thoroughly washed with DMF.

The modified peptide was cleaved from the solid support together with removal of protecting groups under strong acidic standard conditions: TFA/DCM/TIS/H₂O (90/5/2.5/2.5) for 2 h and precipitated in Et₂O. The suspension was centrifuged, the solid residue dissolved in H₂O/CH₃CN (1/1) and purified by preparative C18 reverse-phase HPLC [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 95:5 (5 min), 95:5→25:75 (5→35 min)] with a binary gradient of *Solvent A* and *Solvent B*. The corresponding fractions were freeze-dried to afford the pure peptide **P1** as a white solid (11.2 mg, 11% yield). Purity and characterization were confirmed by analytical HPLC, mass spectrometry and ¹H NMR (Figure S1). ¹H NMR (300 MHz, D₂O) δ (ppm): 4.50-4.13 (m, 13H), 3.35-2.90 (m, 16H), 2.41-2.24 (m, 8H), 2.06 (s, 3H), 2.00-1.27 (m, 55H), 1.00-0.84 (m, 30H). MS (ESI, H₂O/CH₃CN): 1209 (12, [M+2H+3TFA]²⁺), 1152 (13, [M+2H+2TFA]²⁺), 1095 (11, [M+2H+TFA]²⁺), 768 (37, [M+3H+2TFA]³⁺), 730 (100, [M+3H+TFA]³⁺), 692 (65, [M+3H]³⁺), 548 (19, [M+4H+TFA]⁴⁺), 519 (26, [M+4H]⁴⁺). HRMS (ESI): Calcd for C₉₀H₁₇₄N₃₈O₁₈ [M+2H]²⁺: 1037.6929; found: 1037.6933.

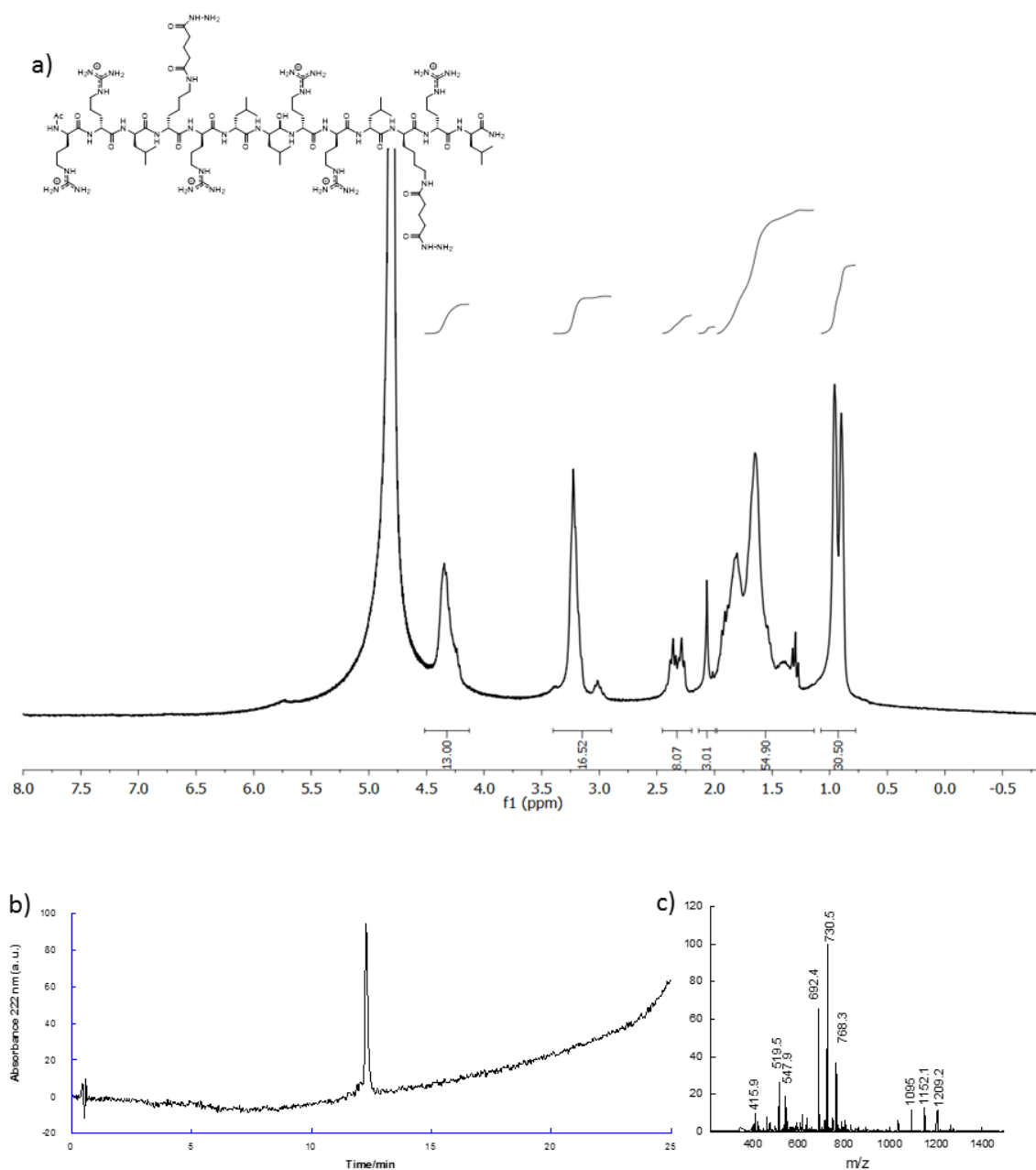


Figure S1. ¹H NMR spectrum (D₂O, 300 MHz) of **P1** (a), analytical RP-HPLC chromatogram: *R*_t 12.3 min [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→25:75 (0→21 min)] (b), ESI-MS traces of **P1**. 1209 (12, [M+2H+3TFA]²⁺), 1152 (13, [M+2H+2TFA]²⁺), 1095 (11, [M+2H+TFA]²⁺), 768 (37, [M+3H+2TFA]³⁺), 730 (100, [M+3H+TFA]³⁺), 692 (65, [M+3H]³⁺), 548 (19, [M+4H+TFA]⁴⁺), 519 (26, [M+4H]⁴⁺).

4. Preparation of amphiphiles

P1 was mixed with 4 equiv of aldehyde tail (2 equiv per hydrazide) in a mixture of DMSO/ultrapure water/AcOH (4.75/4.75/0.5) and shaken at 60 °C for 2h. For instance, 25 μl of **P1** (2.5 mM in ultrapure water) were mixed with 25 μl of 10 mM hydrophobic aldehyde tail in DMSO containing 10% AcOH. The mixture was stirred at 60 °C for 2 h, and the resulting dihydrazones **P1T_x** were then used for DNA activation experiments. Amphiphile

formation was checked by HPLC, and confirmed by ESI-MS (Figure S2). Unless otherwise stated, reactions proceeded at 1.25 mM peptide final concentration in all cases before further dilutions.

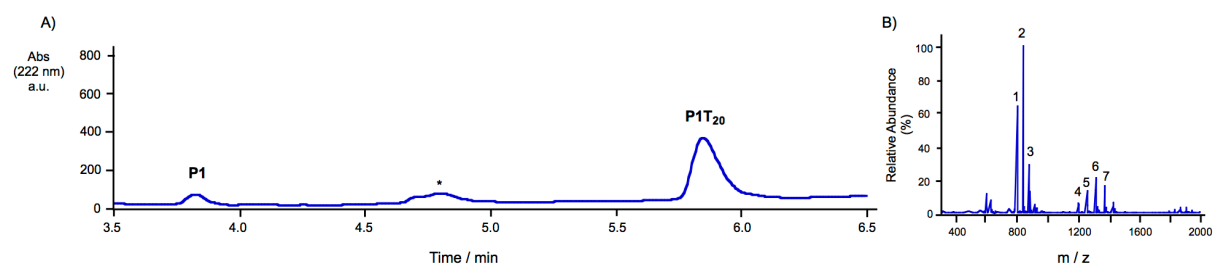


Figure S2. Analytical RP-HPLC chromatogram of crude **P1T₂₀** R_t 5.9 min [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 5:95 (1 min) (a). Residual signals corresponding to **P1** (R_t 3.8 min) and monofunctionalised peptide (marked with an asterisk, R_t 4.8 min) are also depicted. ESI-MS traces of **P1T₂₀**. 803 (**1**, 64, [M+3H]³⁺), 841 (**2**, 100, [M+3H+TFA]³⁺), 879 (**3**, 29, [M+3H+2TFA]³⁺), 1204 (**4**, 6, [M+2H]²⁺), 1261 (**5**, 14, [M+2H+TFA]²⁺), 1318 (**6**, 21, [M+2H+2TFA]²⁺), 1375 (**7**, 16, [M+2H+3TFA]²⁺).

Due to signal saturation, DMSO was substituted by CH₃CN for CD experiments and the acetic acid concentration was reduced to a 1% total volume.

5. DNA transfection screening

In a 96 well plate, HeLa cells seeded one day before (50000 cells/mL, 100 μ L/well) and incubated at 37 $^{\circ}$ C/ 5% CO₂/ 95% humidity in an INCO 108 incubator (Memmert) with Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate), supplemented with 10% foetal bovine serum and 1% of Penicillin-Streptomycin-Glutamine Mix (Fisher).

Freshly prepared peptide amphiphiles were diluted with Dulbecco's Modified Eagle's Medium (DMEM) until concentrations of 50, 25, 5 and 2.5 μ M. Plasmid encoding for eGFP (pEGFP-C1, Clontech) containing 4731 bp (320 μ g/mL in water) was diluted with DMEM until 10 μ g/mL. Described peptide amphiphile solutions were mixed with equal volumes of plasmid solution (typically 15 μ L each) in triplicate in a 96 well plate and incubated at room temperature for 30 min, reaching the charge ratios (+/-) of 10, 5, 1, 0.5. Lipofectamine 2000 was also incubated with the plasmid for the same time. Then, 20 μ L of every peptide amphiphile/plasmid mixture was added to HeLa cells previously covered with 30 μ L of DMEM without FBS or antibiotics. Final peptide amphiphile concentrations were 10, 5, 1 and 0.5 μ M and every well contained 2 ng/ μ L (100 ng/well) of plasmid (except control untreated cells). Positive controls contained 330 ng of Lipofectamine 2000 per well. Cells were incubated for 4 h prior to exchange the medium for DMEM containing 10% bovine foetal serum and 1% of Penicillin-Streptomycin-Glutamine Mix.

After 72 h, the medium was replaced by PBS (137 mM NaCl; 2.7 mM KCl; 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) buffer and the fluorescence intensity was recorded with a Tecan plate reader ($\lambda_{exc}=485$, $\lambda_{em}=535$ nm). Four measurements per well were averaged. Fluorescent cells were also visualised and confirmed by epifluorescence microscope.

6. Dose-response experiments

Optimal peptide amphiphile/plasmid charge ratio (10 or 5) for the hit candidates obtained from transfection screening were used for dose-response experiments.

Freshly prepared peptide amphiphiles were diluted with Dulbecco's Modified Eagles Medium (DMEM) until concentrations of 50 μ M. Plasmid was diluted with DMEM until 40 or 20 μ g/mL (to reach 5 and 10 +/- charge ratio respectively). Peptide amphiphile solutions were mixed with equal volumes of plasmid solution (typically 100 μ L each) and incubated for 30 min at room temperature prior to prepare the following dilutions with DMEM: 25, 12.5, 6.25, 2.5 μ M of peptide amphiphiles. 20 μ L of these solutions were added to HeLa cells previously covered with 30 μ L of DMEM. Final peptide amphiphile concentrations were 10, 5, 2.5 and 1 μ M. The cells were incubated for 4 h before exchanging the medium for DMEM containing 10% bovine foetal serum and 1% of Penicillin-Streptomycin-Glutamine Mix.

After 72 h, the medium was replaced by PBS buffer and the fluorescence intensity was recorded with a Tecan plate reader ($\lambda_{exc}=485$, $\lambda_{em}=535$ nm). Four measurements per well were averaged. Fluorescent cells were also visualised and confirmed by epifluorescence microscope.

7. Flow cytometry

HeLa cells were transfected with pEGFP-C1 plasmid by mixing equal volumes of 25 μ M of **P1T₂₅** freshly prepared and plasmid diluted until 20 μ g/mL in DMEM. 20 μ L of this solution was added to HeLa cells with 30 μ L of DMEM (final concentration 5 μ M, charge ratio 5 charge +/- ratio. Controls with only plasmid (200 ng/well) or with Lipofectamine 2000 (330 ng of Lipofectamine 2000 and 200 ng/well) were also prepared. After 4 h, transfection mixtures were replaced by 100 μ L of DMEM supplemented with 10 % FBS and 1 % Penicillin-Streptomycin-Glutamine Mix. Three days after transfection, cells were washed with PBS, trypsinised with 50 μ L of Trypsin-EDTA (Gibco) for 10 min at 37 °C, and trypsin neutralized with 150 μ L of 5 % FBS in PBS with 5 mM EDTA. Cell clumps were broken by pipetting before analyzing on a Guava easyCyteTM cytometer. GFP levels were determined by excitation at 488 nm and detection at 512/18 nm. For the analysis, cells with typical FSC and SSC parameters were selected, and cells were considered GFP positive when fluorescence signal was higher than that of the untreated control. Data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

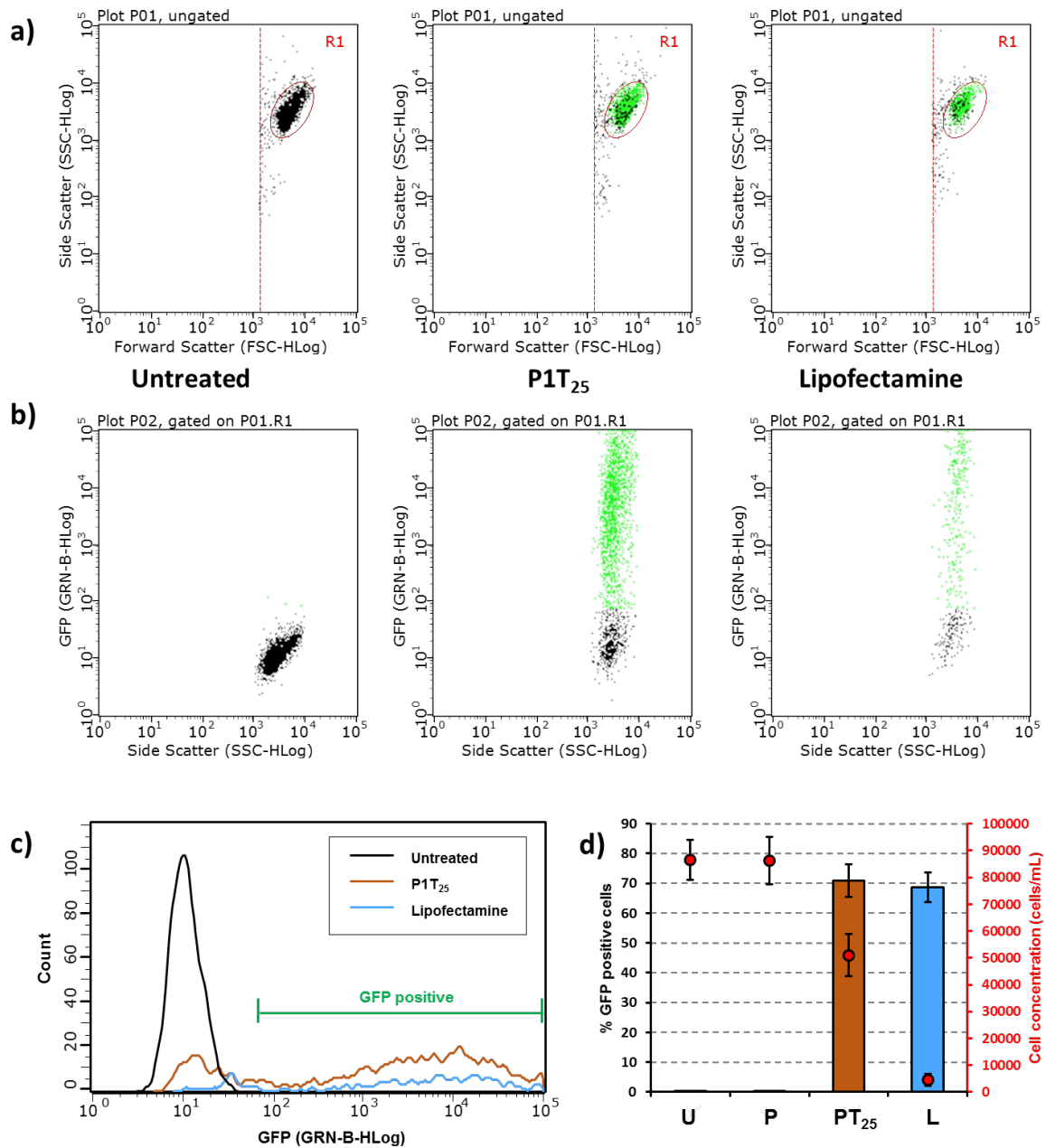


Figure S3. Flow cytometry analysis of transfected cells. Dot plots showing forward and side-scatter channels of untreated cells, cells transfected with pEGFP-C1 using 5 μ M **P1T₂₅**, or cells transfected with Lipofectamine 2000 (a). The region gated for further analysis is shown in red (R1), and GFP positive cells are shown as green dots. Dot plots of the same samples, plotting GFP fluorescence versus the side-scatter channel (b). Histogram of GFP fluorescence of the same samples, showing the region selected as GFP-positive cells for the analysis (c). Percent of GFP positive cells in untreated cells (U), cells incubated with the plasmid alone (P), and cells transfected with peptide P1 conjugated with oleic aldehyde (**P1T₂₅**) or Lipofectamine (L). Red dots indicate the cell concentration of the samples. The mean and SD of three replicates are shown (d).

8. DLS and ζ potential

Freshly prepared peptide amphiphiles **P1T₂₀** and **P1T₂₅** were mixed with the plasmid at 10 charge +/- ratio with filtered (Nylon, 0.45 μM) ultrapure water at an amphiphile concentration of 25 μM . The mixture was incubated for 30 min at room temperature prior to be transferred to a standard disposable cuvette for size measurements. ζ potential measurements were performed in Malvern disposable cuvettes for ζ potential applying the Smoluchovsky model by the instrument software. All experiments were done in triplicate at 25 $^{\circ}\text{C}$ (Figure S4).

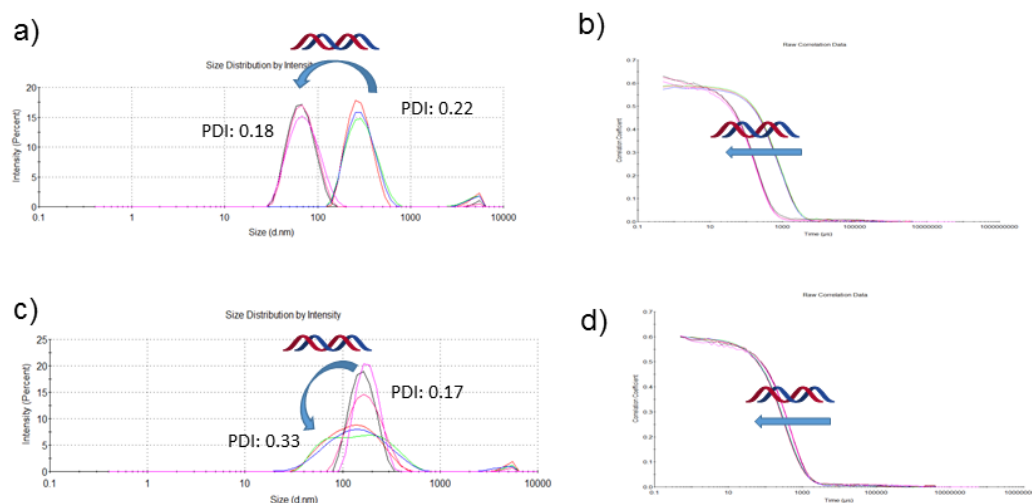


Figure S4. Hydrodynamic diameter size distribution and correlation curves for **P1T₂₅** (a, b) and **P1T₂₀** (c, d) in the presence and in the absence of DNA plasmid. In both cases the hydrodynamic diameter is decreased in the presence of plasmid DNA and **P1T₂₀** complex shows a broader size distribution. All experiments are depicted in triplicate. PDI stands for polydispersity index.

A variant of the previous experiment was performed as assessment of complex stability: A solution of **P1** in ultrapure water and freshly prepared peptide amphiphiles **P1T₂₀** and **P1T₂₅** were mixed with the plasmid at 10 charge +/- ratio with filtered (Nylon, 0.45 μM) PBS pH 7.2 or citrate buffer 100 mM pH 5.9 at a peptide/amphiphile concentration of 25 μM . After 30 min of incubation at room temperature, the mixtures were transferred to standard disposable cuvettes for size measurements. Measurements were performed in triplicate at the times indicated in Figure S5.

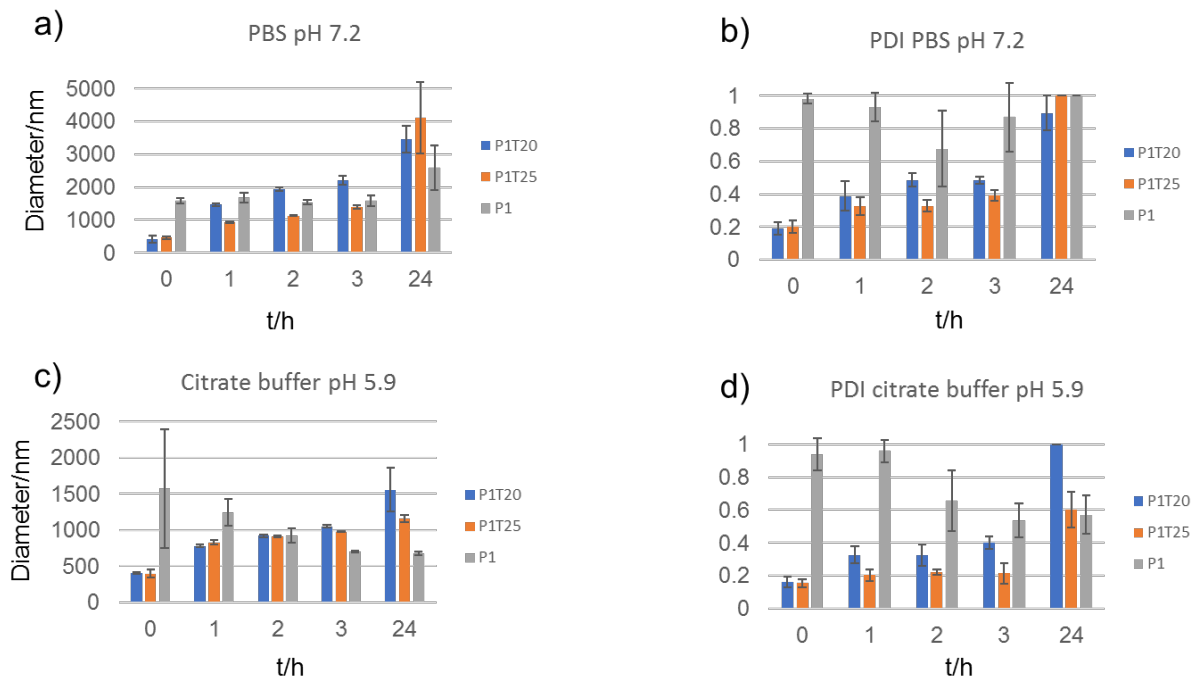


Figure S5. Lipoplex size diameters against the time for **P1T₂₀**, **P1T₂₅** and the parent peptide **P1** in PBS pH 7.2 (a) and citrate buffer 100 mM, pH 5.9 (c). The respective polydispersity indexes (PDI) are also depicted (b and d respectively). Data shows an increased aggregation of material for **P1T₂₀**, **P1T₂₅** within time together with a loss of particle definition (increase of PDI). Inactive **P1** particles do not show the same behaviour. All measurements were performed in triplicate. Error bars represent standard deviation.

9. Atomic Force Microscopy (AFM)

Freshly prepared peptide amphiphiles **P1T₂₀** and **P1T₂₅** were mixed with the plasmid at 10 charge +/- ratio at an amphiphile concentration of 25 μ M in PBS. The mixture was incubated for 30 min and subsequently pipetted onto freshly cleaved mica discs (Grade V-1 Muscovite). After 10 min, the excess was removed, the mica surface thoroughly washed with ultrapure water, and dried under a nitrogen flow. Standard AFM measurements were conducted under ambient atmosphere at room temperature using a XE-100 instrument (Park Systems Corporation) in non-contact mode by using ACTA cantilevers (300 kHz nominal frequency and 37 N/m nominal spring constant). 10x10 μ m images were acquired at a scanning speed of 0.5 Hz (Figure S6).

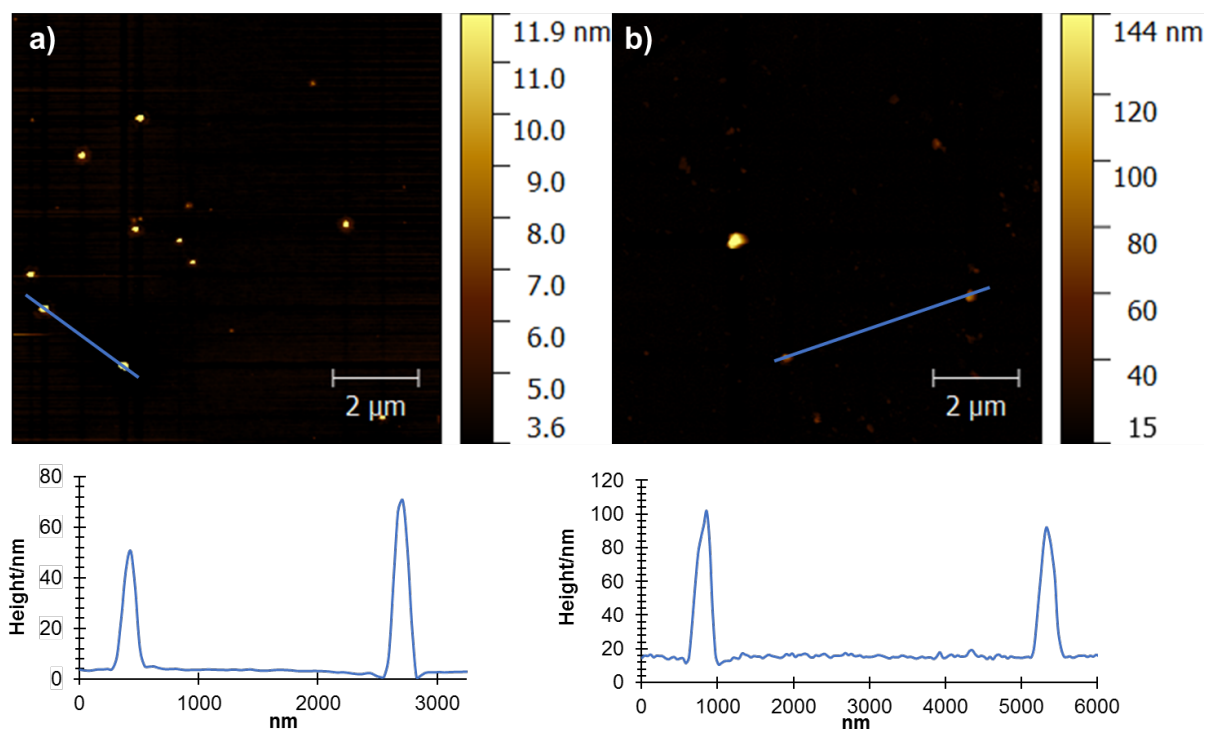


Figure S6. AFM images, including topographic profiles (blue lines) of **P1T₂₅** (a) and **P1T₂₀** (b) lipoplexes with eGFP plasmid on mica (25 mM). Measured diameters were 57.8 ± 0.9 and 101.2 ± 36.4 nm respectively, in close agreement with DLS measurements.

10. Transmission Electron Microscopy (TEM)

Freshly prepared peptide amphiphiles **P1T₂₀** and **P1T₂₅** were mixed with the plasmid at 10 charge +/- ratio at an amphiphile concentration of 25 μ M in PBS. The mixture was incubated for 30 min and subsequently pipetted onto carbon coated copper grids. After 10 min, the remaining solution was removed with filter paper, thoroughly washed with ultrapure water. Staining was carried out by dispensing 10 μ L of a solution of phosphotungstic acid (2% in water) on top of the grids, removed after 1 min and thoroughly washed with ultrapure water. Micrographs were acquired on a JEOL JEM 2010 transmission electron microscope operating at 200 kV accelerating voltage (Fig. S7).

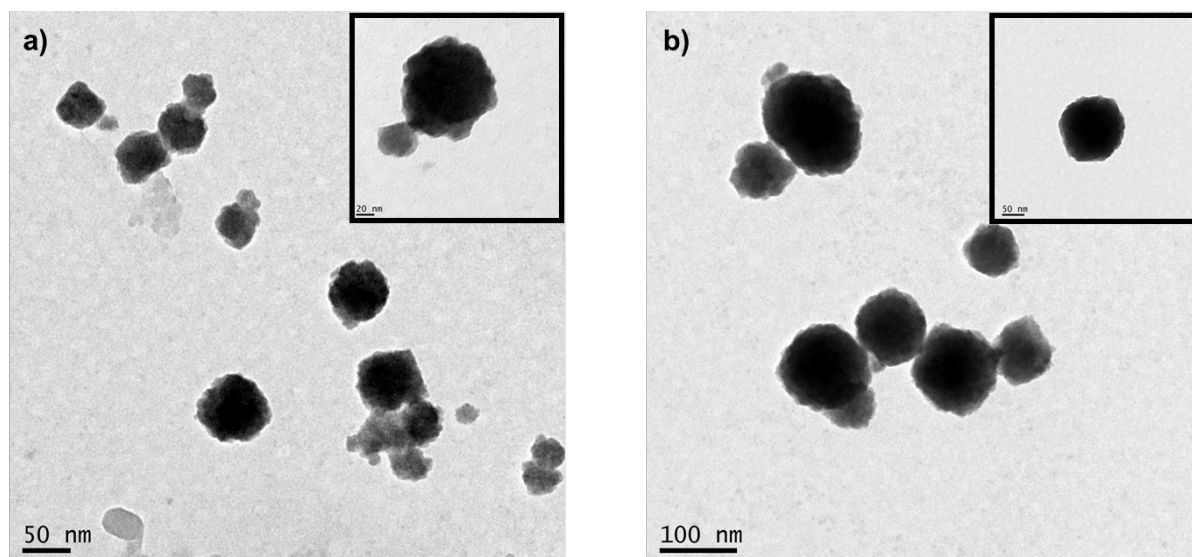


Figure S7. TEM micrographs of **P1T₂₅** (a) and **P1T₂₀** (b) lipoplexes with eGFP plasmid (25 mM). Details of individual complexes are depicted in the insets.

11. Gel retardation assay

Freshly prepared peptide amphiphiles **P1T₂₀** and **P1T₂₅** were mixed with the plasmid at 10, 5, 1, 0.5, 0.05, 0.01 charge +/- ratio (fixed plasmid concentration of 5 ng/ μ L) and incubated at room temperature for 30 min. The mixtures, including a solution of the unmodified plasmid at the same concentration, were loaded into a 0.8% agarose gel containing ethidium bromide with tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. Electrophoresis was performed at 100 V for 15 min.

12. Viability assays

In a 96 well plate, HeLa cells (50000 cells/mL, 100 μ L/well) were incubated at 37 $^{\circ}$ C/ 5% CO₂/ 95 % humidity with Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate), supplemented with 10% foetal bovine serum and 1% of Penicillin-Streptomycin-Glutamine Mix.

Freshly prepared peptide amphiphiles were diluted with DMEM until concentrations of 50 μ M. The procedure followed for Dose-response experiments (see section 7) was reproduced. The cells were incubated with the complexes for 4 h prior to exchange the medium by DMEM containing 10% bovine foetal serum and 1% of Penicillin-Streptomycin-Glutamine Mix.

After 72 h, the medium was replaced by fresh medium (100 μ L) and 10 μ L of the water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide^[5] (MTT) reagent (5 mg/mL stock solution in PBS) was used to assess cell viability. Viable cells are capable of reducing MTT to insoluble formazan by their metabolic activity. After incubation at 37 $^{\circ}$ C for 4 h, the medium was aspirated and the purple precipitate was dissolved with DMSO (100

$\mu\text{L}/\text{well}$). Absorbance at 570 nm was measured in a Tecan plate reader. Values were normalised against untreated cells.

13. Circular Dichroism

Spectra were acquired in a Jasco 1100 at 100 μM peptide amphiphile concentration both in trifluoroethanol or aqueous HEPES-Krebs-Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl_2 , 1.8 mM CaCl_2 , pH 7.4). The acquisition range was 300-190 nm, at a scanning speed of 200 nm/min (1.0 nm band width, 1 nm data pitch, 3 accumulations). Measurements were done from 10 $^\circ\text{C}$ to 60 $^\circ\text{C}$ at 10 $^\circ\text{C}$ intervals in a quartz cell of 0.2 cm path length (Figure S8).

For the measurements in liposomes, samples were prepared by mixing egg yolk *L*-phosphatidylcholine (100 mg/mL solution in CHCl_3) with the peptide (100 μM) in TFE to obtain a lipid/peptide ratio of 18:1 in a 10 mL round bottomed flask. The mixture was concentrated in a rotary evaporator to dryness, forming a film. Lipid-peptide mixture was suspended in HKR buffer to a final peptide concentration of 100 μM followed by sonication for 45 min.

The results expressed as the mean residue molar ellipticity $[\theta]_{\text{MRt}}$ with units of $\text{mdeg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ were calculated using the following equation (Eq. S1).

$$[\theta]_{\text{MRt}} = \frac{100 \times \theta}{C \times l \times N} \quad \text{Eq S1}$$

where θ is the ellipticity (mdeg), C is the peptide concentration (M), l is the cell path length (cm) and N is the number of residues.

The percentage of α -helix was calculated using the mean residue molar ellipticity $[\theta]_{\text{MRt}}$ at 222 nm as follows (Eq. S2).

$$\% \alpha \text{ helix} = \left(\frac{[\theta]_{222} - 2340}{30300} \right) \times 100 \quad \text{Eq S2}$$

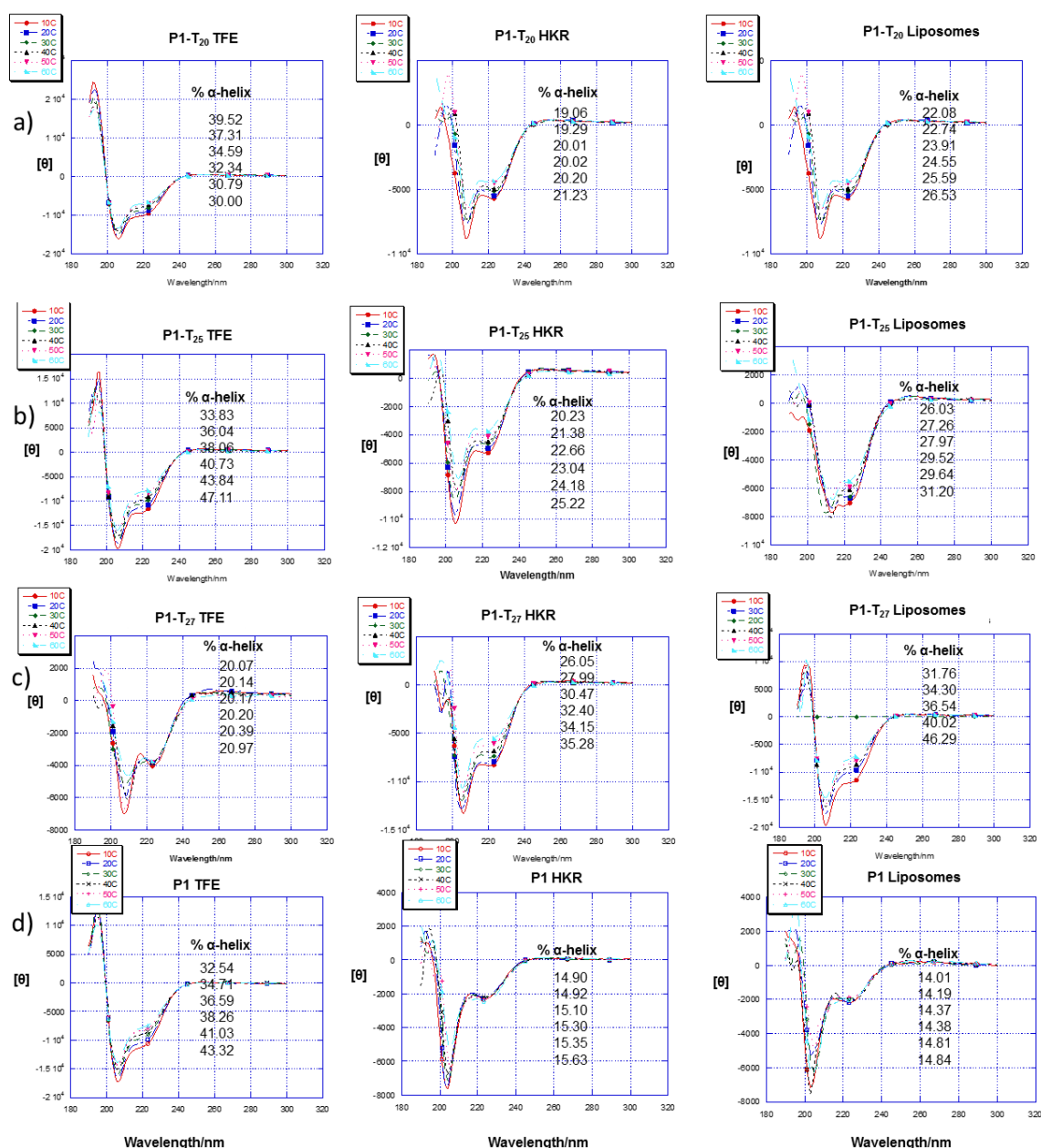


Figure S8. Circular Dichroism spectra at different temperatures of **P1T₂₀** (a), **P1T₂₅** (b), **P1T₂₇** (c) and **P1** (d) in TFE (left), HKR buffer (middle) and in the presence of liposomes (d). The percentage of α -helix was calculated at every temperature by applying Eq S2 and results are presented from higher to lower temperature.

14. Computational methods

The starting geometry of the helical natural peptide **P1** was constructed with Avogadro package^[6,7]. The non-standard oleic containing residue was added to the .rtp file in Gromos96 54a7 force field using the parameters obtained from the ATB server^[8] and the natural peptide **P1** was directly mutated to **P1T₂₅** using GROMACS utilities. Both peptides were simulated

in water and in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer using the same force field. The simulations were performed with the GROMACS 5.0^[9] Molecular Dynamics program. After neutralizing the systems adding Cl⁻ ions, several simulations were carried out: i) **P1T₂₅** in a water box during 30 ns (Figure 7A), ii) **P1** and **P1T₂₅** inserted into a POPC bilayer (50ns, Figures 7B, 7E, 7F) and iii) the structure of **P1T₂₅** obtained from simulation i) horizontally oriented with respect to the POPC bilayer, during 50 ns in presence and absence of an electric membrane z-potential (0.01 V/nm), (Figure 7C). The simulations in the membrane were carried out in presence of 0.1 M NaCl concentration. All systems were partially optimized, thermalized, and equilibrated, followed by unrestrained simulations (time step = 2 fs) for each of the systems studied. The constant pressure and temperature NPT ensemble was employed with a pressure of 1 bar controlled using a isotropic (simulations in water) or semi-isotropic (simulations in the membrane) Parrinello–Rahman barostat^[10], and a temperature of 300 K imposed by a V-rescale thermostat temperature coupling using velocity rescaling with a stochastic term^[11] The LINCS^[12] algorithm was employed to remove the bond vibrations. The Particle Mesh Ewald method^[13] coupled to periodic boundary conditions was used to treat the long-range electrostatics using a direct-space cut-off of 1.0 nm. The van der Waals interactions were computed using PBC coupled to a spherical cut-off of 1.4 nm. Data were analysed using GROMACS and locally written code. Molecular graphic images were prepared using visual molecular dynamics (VMD).^[14]

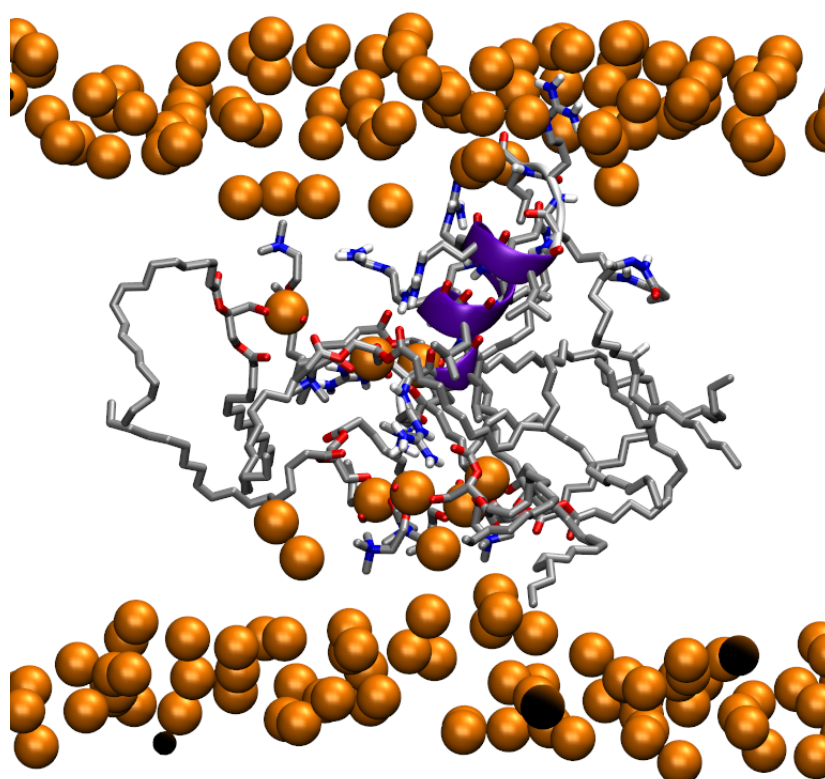


Figure S9: Detail of the lipids around the **P1T₂₅** from its simulation inserted into a POPC bilayer, after 50ns.

15. Supporting References

- [1] C. Gehin, J. Montenegro, E.-K. Bang, A. Cajaraville, S. Takayama, H. Hirose, S. Futaki, S. Matile, H. Riezman, *J. Am. Chem. Soc.* **2013**, *135*, 9295.
- [2] Q. Chen, D. A. Sowa, J. Cai, R. Gabathuler, *Synth. Commun.* **2003**, *33*, 2377.
- [3] M. A. Cole, S. E. Tully, A. W. Dodds, J. N. Arnold, G. E. Boldt, R. B. Sim, J. Offer, P. Wentworth, *ChemBioChem* **2009**, *10*, 1340.
- [4] C. Kay, O. E. Lorthioir, N. J. Parr, M. Congreve, S. C. McKeown, J. J. Scicinski, S. V. Ley, *Biotechnol. Bioeng.* **2000**, *71*, 110.
- [5] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.
- [6] A. an open-source molecular builder and visualization tool. V. 1.XX., **n.d.**, <http://avogadro.openmolecules.net/>.
- [7] M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek, G. R. Hutchison, *J. Cheminform.* **2012**, *4*, 17.
- [8] N. Schmid, A. P. Eichenberger, A. Choutko, S. Riniker, M. Winger, A. E. Mark, W. F. van Gunsteren, *Eur. Biophys. J.* **2011**, *40*, 843.
- [9] M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J. C. Smith, B. Hess, E. Lindahl, *SoftwareX* **2015**, *1–2*, 19.
- [10] M. Parrinello, A. Rahman, *J. Appl. Phys.* **1981**, *52*, 7182.
- [11] G. Bussi, D. Donadio, M. Parrinello, *J. Chem. Phys.* **2007**, *126*, 14101.
- [12] B. Hess, H. Bekker, H. J. C. Berendsen, J. G. E. M. Fraaije, *J. Comput. Chem.* **1997**, *18*, 1463.
- [13] U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, *J. Chem. Phys.* **1995**, *103*, 8577.
- [14] W. Humphrey, A. Dalke, K. Schulten, *J Molec Graph.* **1996**, *14*, 33.