Peptide/Cas9 Nanostructures for Ribonucleoprotein Cell Membrane Transport and Gene Edition

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Supporting information

Abbreviations

CPZ: chlorpromazine; crRNA: CRISPR RNA; DIEA: N,N-Diisopropylethylamine; DMEM: Dulbecco's modified Eagle medium; DMF: N,N-Dimethylformamide; DMSO: Dimethylsulfoxide; EDTA: Ethylenediaminetetraacetic acid; EGFP: enhanced green fluorescent protein; EIPA: 5-(N-Ethyl-N-isopropyl)amiloride; FBS: fetal bovine serum; Fmoc: 9-fluorenylmethoxycarbonyl; GR: glucocorticoid receptor; gRNA: guide RNA; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HKR: HEPES-Krebs-Ringer buffer; HPRT1: hypoxanthine phosphoribosyltransferase 1; hpt: hours post-treatment; IPTG: Isopropyl β-D-1-thiogalactopyranoside; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; MβCD: Methyl-β-Cyclodextrin; *N*-HBTU: N-[(1H-Benzotriazol-1-yl)4-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide; Ox-Dex: Dexamethasone Acid; PAM: protospacer adjacent motif; PBS: Phosphate buffered saline; RNP: ribonucleoprotein; T7E1: T7 endonuclease I; TAE: tris-acetate-ethylenediaminetetraacetic acid; TALENs: transcription activator- like effector nucleases; TAMRA: [5(6)-Carboxytetramethylrhodamine]; TFE: Trifluoroethanol; TIS: Triisopropylsilane; tracrRNA: trans-activating crRNA; ZFNs: Zinc-finger nucleases.

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-Leu-OH, glutaric anhydride, *tert*-butyl carbazate, triisopropylsilane (TIS), Diisopropylethyl amine (DIEA) were obtained from Sigma-Aldrich. Fmoc-L-Arg(Pbf)-OH was purchased from Carbosynth, Trifluoroethanol (TFE) and 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) were purchased from TCI. Peptide synthesis grade *N,N*-dimethylformamide was purchased from Scharlau. All other chemicals were purchased from Sigma-Aldrich, TCI or Fisher. 5 nm Ni-NTA-Nanogold® was purchased from Nanoprobes and used according to the manufacturer recommendations.

The aldehydes tested were either commercially available or synthesized following reported protocols from the corresponding alcohols[1]. 5-(2-(tert-butoxycarbonyl)hydrazineyl)-5-oxopentanoic acid was prepared as previously described[2,3].

Purified Cas9 protein (Alt-R™ S.p. Cas9 Nuclease 3NLS), Alt-R™ CRISPR-Cas9 crRNA, unlabelled and $ATTO_{550}$ labelled Alt-R™ CRISPR-Cas9 tracrRNA, and oligonucleotides for PCR were purchased from Integrated DNA Technologies. GeneArt Genomic Cleavage Detection kit, Lipofectamine 2000, LysoTracker Green DND-26, LysoTracker Deep Red, Hoechst 33342, HisPur Ni-NTA resin and cell culture reagents were purchased from ThermoFisher. Endonuclease T7E1 was from New England Biolabs. Agarose D1 with medium EEO was bought from Laboratorios Conda, Agarose (low melting point) was from Promega and Protogel (30 %; 37.5:1 Acrylamide to Bisacrylamide Stabilized Solution) from National Diagnostics. Wortmannin was purchased from Fluorochem, chlorpromazine hydrochloride from TCI Chemicals, methyl-β-

cyclodextrin from Carbosynth, MTT and heparin from Alfa Aesar; Dynasore and G-418 from EMD Millipore; ethidium bromide, EIPA, ammonium chloride and chloroquine were purchased from Sigma-Aldrich.

A Guava EasyCyte^{TM} cytometer (EMD Millipore) was used for all flow cytometry experiments. Cell microscopy images were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E epifluorescence microscope or with a Leica SP5 confocal microscope. Absorbance of formazan was measured in a microplate reader Tecan Infinite F200Pro. PCR reactions, re-annealing and digestions were done on a T-Personal PCR Thermocycler (Biometra) or on a MJ Mini Thermal Cycler (Bio-Rad). Gels were imaged on a ChemiDoc XRS+ system (Bio-Rad) and analysed with the software ImageLab 5.2.1 (Bio-Rad).

Transmission electron microscopy was performed on a JEOL JEM-2010 microscope operating at 200 kV or on a JEOL JEM-1011 microscope operating at 100 kV. EDX mapping and microanalysis were performed on a JEOL JEM-2010 FEG operating at 200 kV at CACTI building (University of Vigo, Spain). DLS and ζ potential measurements were performed in a Malvern Zetasizer NanoZS using standard disposable cuvettes. All experiments were done in triplicate at 25 ºC.

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II associated with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column with *Solvent A*:*Solvent B* gradients between 5:95 (*Solvent A*: H_2O with 0.1 % TFA; *Solvent B*: CH_3CN with 0.1 % TFA). High-performance liquid chromatography (HPLC) preparative purification was carried out on Waters 1525 composed by a binary pump with a dual Waters 2489 detector with a Phenomenex Luna C18(2) 100A column. An Agilent 1200 with an Agilent Eclipse XDB-C18 column was used for semi-preparative purification using gradients of 5:95 (*Solvent A*: H_2O with 0.1 % TFA; *Solvent B*: CH_3CN with 0.1 % TFA). Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz spectrometer. Chemical shifts are reported in ppm (δ units) referenced to the following solvent signals: D_2O δ H 4.79. Spin multiplicities are reported as a singlet (s), doublet (d), with coupling constants (*J*) given in Hz, or multiplet (m). Accurate mass determinations (HR-MS) using ESI-MS were performed on a Sciex QSTAR Pulsar mass spectrometer and are reported as mass-per-charge ratio (*m/z*).

Peptide Synthesis

Peptides were synthesised as previously described elsewhere[4]. The TAMRA and Ox-Dex *N*-terminated **P** were prepared following a modified protocol.

The *N*-terminal Fmoc protecting group was removed with a solution of piperidine in DMF (20 %, 2 mL) for 15 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 6-(Fmoc-amino)hexanoic acid (4 equiv), *N*-HBTU (4 equiv) (2 mL) and DIEA (6 equiv) was added to the resin under nitrogen stream for 20 min. Finally, the resin was washed with DMF (3 x 2 mL, 1 min) and DCM (3 x 2 mL, 1 min).

The Lys-Mtt protecting group was cleaved by using mild acidic conditions DCM/HFIP/TFE/TIS (6.5/2/1/0.5, 2 x 2 mL) followed by acylation of the Lys δ-amino group with 5-(2-(tert-butoxycarbonyl)hydrazineyl)-5-oxopentanoic acid as previously described[4].

TAMRA terminating peptide (TmP): Fmoc-protecting group of the previously attached Fmoc-aminohexanoic acid was removed by using a solution of piperidine in DMF (20%, 4 mL) for 15 min and the resin was washed with DMF (3 x 3 mL). The coupling was carried out by the addition of a premixed solution of TAMRA (1 equiv), *N*-HBTU (1 equiv) and DIEA (2 equiv) in DMF (2 mL) and the mixture was stirred under nitrogen stream for 30 min followed by washings with DMF (3 x 3 mL) and DCM (3 x 3 mL).

Ox-Dex terminating peptide (DexP): the Fmoc-protecting group of the linker was removed by using a solution of piperidine in DMF (20%, 4 mL) during 15 min and the resin was washed with DMF (3 x 3 mL). A solution of Ox-Dex[5] (3 equiv), *N*-HATU (2.9 equiv) and DIEA (0.195 M, 3 equiv) in DMF (2 mL) was added and the mixture was shaken by bubbling nitrogen for 30 min. Finally, the resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

The modified peptides were cleaved from the solid support with removal of the protecting groups under strong acidic standard conditions: TFA/DCM/TIS/H_2O (90/5/2.5/2.5) for 2 h and precipitated in Et_2O. The suspension was centrifuged, the solid residue dissolved in H_2O/CH_3CN (1/1) and purified by preparative C18 reverse-phase HPLC [Phenomenex Luna C18(2) 100A column, H_2O (0.1% TFA)/CH_3CN (0.1% TFA) 95:5 (5 min), 95:5→5:95 (5→35 min)] with a binary gradient of *Solvent A* and *Solvent B*.

TmP: The corresponding fractions were freeze-dried to afford the pure peptide **TmP** as a pink solid (11.2 mg, 11% yield). Purity and characterization were confirmed by analytical HPLC, mass spectrometry and ^{1}H NMR (Figure S10). ^{1}H NMR (300 MHz, D_2O) δ (ppm): 8.31 (s, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.2 Hz, 1H), 7.15-6.91 (m, 2H), 6.90-6.62 (m, 4H), 4.29-3.78 (m, 13H), 3.46-3.19 (m, 2H), 3.11 (s, 12H), 3.08-2.85 (m, 16H), 2.89-2.72 (m, 2H), 2.39-2.01 (m, 8H), 1.89-1.15 (m, 61H), 0.93-0.53 (m, 30H). MS (ESI, H_2O/ CH_3CN): 512 (22, $[M+5H]^{5+}$), 641 (50, $[M+4H]^{4+}$), 669 ($[M+4H+TFA]^{4+}$), 892 ($[M+3H+TFA]^{3+}$), 930 (100, $[M+3H+2TFA]^{3+}$), 968 (38, $[M+3H+3TFA]^{3+}$), 1394 (8, $[M+2H]^{2+}$), 1451 (10, $[M+2H+TFA]^{2+}$), 1508 (5, $[M+2H+2TFA]^{2+}$). HRMS (ESI): Calcd for $C_{119}H_{204}N_{41}O_{22}$ $[M+3H]^{3+}$: 853.2029; found: 853.2032.

DexP: The corresponding fractions were freeze-dried to afford the pure peptide **DexP** as a white solid (7.3 mg, 3 % yield). Purity and characterization were confirmed by analytical HPLC, mass spectrometry and ^{1}H NMR (Figure S11). ^{1}H NMR (300 MHz, D_2O) δ (ppm): 7.38 (d, *J* = 8.9 Hz, 1H), 6.27 (d, *J* = 8.7 Hz, 1H), 6.07 (s, 1H), 4.32-3.79 (m, 15H), 3.33-3.15 (m, 2H), 3.17- 2.93 (m, 17H), 2.91-2.75 (m, 2H), 2.66- 2.49 (m, 2H), 2.35-2.04 (m, 11H), 2.02- 1.08 (m, 71H), 0.99- 0.56 (m, 33H). MS (ESI, H_2O/CH_3CN): 1368 (5, $[M+2H+2TFA]^{2+}$), 950 (25, $[M+3H+3TFA]^{3+}$), 912 (45, $[M+3H+2TFA]^{3+}$), 874 (100, $[M+3H+TFA]^{3+}$), 836 (25, $[M+3H]^{3+}$).

Preparation of amphiphiles

Peptide was mixed with 6 equiv of the aldehyde tail per hydrazide dissolved in DMSO in the presence of 5 % of AcOH and shaked at 60 ºC for 2 h. Concentration of the peptide in the reaction was 1.25 mM. Before incubation with the complexes, reactions were diluted to 50 µM using DMEM or PBS.

Oligonucleotides

The following crRNAs targeting EGFP (Alt-R™ crRNA 5'-CCTCGAACTTCACCTCGGCG-3')[6] and HPRT1 (Alt-R™ crRNA 5'-GGCTTATATCCAACACTTCG-3') were used. For the amplification of the surrounding regions in the genomic DNA, the primers TACGGCAAGCTGACCCTGAA and GTCCATGCCGAGAGTGATCC[6] (Tm = 54 ºC) were used in the case of EGFP and CAAATTATGAGGTGCTGGAAGG and TGGACACATGGGTAGTCAG (Tm = 52 ºC) for HPRT1.

Cell lines and culture

Cervix adenocarcinoma HeLa cell line and human lung cancer A549 cell lines were maintained on Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate) supplemented with 10 % FBS and 1 % Penicillin-Streptomycin-Glutamine Mix (Fisher) at 37 ºC/5 % CO_2 /95 % humidity in an INCO 108 incubator (Memmert).

To generate an EGFP expressing HeLa cell line, HeLa cells were transfected with pEGFP-C1 (Clontech) and stable transfectants were selected with 400 µg/mL of G-418 (Millipore). Green clones were selected by epifluorescence microscopy and tested for stability of EGFP expression through several passages. A clone with a highly stable EGFP expression (~90 % EGFP positive cells) was chosen for further experiments.

A chicken fibroblast cell line (DF-1 EGFP), containing a tetracycline-inducible EGFP gene was a kind gift from Dr. Lisa K. Busch (Molecular Virology group).

RNP delivery

For RNP assembly, crRNA and tracrRNA were mixed to a final concentration of 4 µM in Nuclease-Free Duplex Buffer (30 mM HEPES, 100 mM potassium acetate, pH 7.5), incubated for 5 min at 95 ºC and slowly cooled at room temperature. Cas9 protein was dissolved in DMEM at a final concentration of 4 µM and equal volumes of the RNA complex and Cas9 were mixed and incubated for 5 min at room temperature. The desired amount of RNP was then mixed with the freshly prepared amphiphiles diluted at 50 µM in DMEM and incubated for 20 min to form the peptide/Cas9 complexes. Usually 4 µL of RNP were mixed with 10 µL of the amphiphile at 50 µM. Lipofectamine was used at a concentration of 10 µg/mL to deliver 160 nM of RNP, and this ratio was kept constant in all the experiments.

Cells seeded the day before in a 96 well plate (50.000 cells/mL, 100 µL/well) were washed once with DMEM, and DMEM or DMEM supplemented with FBS (to obtain a final concentration of 5 %) was added to the wells. Peptide/Cas9 complexes were added to a final volume of 50 µL per well and cells were incubated for 4 h before removing the complexes and adding 100 µL of DMEM with 10 % FBS and antibiotics.

T7 Endonuclease I assay

For T7E1 assays, cells were harvested at 48 hpt by trypsinization and centrifugation. Genomic DNA from the cellular pellet was extracted and amplified using the GeneArt Genomic Cleavage Detection kit (ThermoFisher) following the manufacturer's instructions. 2 µL of the PCR product were re-annealed and digested using 1 U of T7E1 (NEB) at 37 ºC for 1.5 h. DNA fragments were resolved in a 5 % polyacrylamide gel for 35 min at 150 V in TAE buffer. Gels were stained with ethidium bromide before imaging on a ChemiDoc XRS+. Band intensity was measured with the software ImageLab 5.2.1 (Bio-Rad) and cleavage efficiency (C. e.) was calculated using the following equation:

$$C.\ e. = 1\ - [(1 - \frac{sum\ of\ cleaved\ bands\ intensities}{sum\ of\ cleaved\ and\ parental\ band\ intensities})^{1/2}]$$

Results were expressed as a percentage.

Flow cytometry

For EGFP gene disruption studies, HeLa-EGFP cells were incubated with Peptide/RNP complexes targeting the EGFP gene. At 72 hpt, cells were washed with PBS and trypsinized with 100 µL of Trypsin-EDTA for 10 min at 37 ºC. After neutralizing trypsin by the addition of 100 µL of 2 % FBS and 5 mM EDTA in PBS, cells were analysed on a Guava EasyCyte™ cytometer. EGFP fluorescence was measured 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 with low EGFP fluorescence (lower than the main peak of the untreated control) were considered as EGFP negative for further calculations.

To assess the state of the lysosomal compartment, HeLa cells were incubated in DMEM with the peptide/RNP complexes in the presence of 50 nM Lysotracker Green DND-26. After 1 h of incubation at 37 ºC, cells were washed three times with DMEM and trypsinized for 10 min at 37 ºC. Trypsin was neutralized by the addition of 2 % FBS and 5 mM EDTA in PBS and samples were analysed on a Guava EasyCyte™ cytometer. Lysotracker Green DND-26 fluorescence was measured by excitation at 488 nm and detection at 512/18 nm. After gating to discard debris, median fluorescence intensity (MFI) of each sample was calculated. MFI value from unlabelled cells was subtracted from all samples. Values were normalized to the MFI of LysoTracker in the untreated control (100 %).

To study the cellular uptake mechanisms, HeLa cells growing in a 96 well plate were treated for 30 min with the following compounds diluted in DMEM without serum or antibiotics: Dynasore (80 µM), chlorpromazine

(30 μM), methyl-β-cyclodextrin (5 mM), Wortmannin (200 nM), EIPA (50 μM), heparin (5 μg/mL), chloroquine (100 μM) or ammonium chloride (50 mM). For the incubation at low temperature, another plate was incubated on ice and ice-cold solutions were used for the washes and incubations. Cells were then incubated with the complexes (prepared with the TAMRA-labelled peptide conjugated with oleic aldehyde) in the presence of the same amount of inhibitors in DMEM for 1 h at 37 ºC. Cells were washed twice with HKR and 0.1 mg/mL of heparin in HKR and trypsinized. Trypsin was neutralized with 2 % FBS in PBS with 5 mM EDTA and cell fluorescence was measured on a Guava EasyCyte™ cytometer using a green laser (532 nm) and collecting the emission at 575/25 nm. Cells with typical FSC and SSC parameters were selected and the median fluorescence intensity calculated for each sample (MFI). Each condition was done in triplicate. Fluorescence values were normalized to the uptake of the untreated control (100 %) after blank subtraction.

In all cases, data analysis was performed with InCyte software included in GuavaSoft 3.2 (Millipore).

Live cell microscopy

HeLa cells grown in four chamber glass bottom dishes (CellVis) were stained with 1 μM Hoechst 33342 for 20 min. After nuclear staining, cells were washed once with DMEM and incubated with the peptide/RNP complexes (containing **$TmPT_{24}$** and the RNP, or $ATTO_{550}$-labelled RNP with unlabelled **PT_{24}**) for 1 h in DMEM without FBS or antibiotics (unless otherwise stated). For lysosomal staining, 50 nM of LysoTracker Deep Red was added to the culture. Cells were then washed twice with 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) and twice for 5 min with 0.1 mg/mL of heparin in HKR buffer and examined under an epifluorescence microscope.

Glucocorticoid induced GFP translocation assay

Endosomal escape was studied using a glucocorticoid induced GFP translocation assay (GIGT)[7]. HeLa cells were transfected with the plasmid pK7-GR-GFP (a gift from Ian Macara[8], Addgene plasmid #15534) using Lipofectamine 2000 the day before the treatments. Cells were then incubated for 30 min with 1 μM Hoechst 33342 in DMEM and 50 μM EIPA or 5 % FBS where indicated. This media was removed and cells were incubated for 1 h with 1 μM dexamethasone (positive control), 10 μM **$DexPT_{24}$**, or left untreated (negative control) diluted in DMEM with or without EIPA or 5 % FBS, as indicated. Twenty to thirty images of each sample were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E microscope at 60x magnification and the translocation ratio (the ratio of the median intensities of GFP in the nucleus and in the 2 μm wide surrounding region) was calculated with CellProfiler[9] as follows. Nuclei were identified as Hoechst stained objects using the three-class thresholding Otsu method and the cytoplasmic region was defined as the 2 μm surrounding area. To ensure a better separation of the cytoplasmic and nuclear region, nuclei were shrunk 0.5 μm before measuring object intensity. Cells falling below the 15 % of the maximum intensity of the image were considered untransfected and discarded for the analysis. A total of 40 to 80 cells were analyzed for each sample.

Statistical analysis of the data was performed with R software[10]. Results were subjected to pairwise two-tailed Student's t-test and p-values were adjusted using Bonferroni's correction.

Viability assay

Cell viability was determined using a MTT assay, which relies on the ability of cells to reduce the water-soluble tetrazolium salt to the insoluble formazan. HeLa cells incubated with the complexes as previously described were incubated for 48 h at 37 ºC before performing the assay. Cell culture media was removed and 100 µL of fresh DMEM containing FBS, antibiotics and supplemented with 0.5 mg/mL of MTT was added. As a blank control, 3 wells were incubated with DMEM and MTT in the presence of 1 % Triton X-100. Cells were incubated for 4 h at 37 ºC before carefully removing the supernatant and dissolving the formazan crystals with DMSO (100 µL/well). Absorbance was measured at 570 nm using a microplate reader (Infinite F200pro, Tecan). Data points were collected in triplicate and values were normalized for untreated control cells (100%) after blank subtraction.

Protein expression and purification

Escherichia coli BL21(DE3)-Codon Plus cells (Stratagene) were transformed with the plasmid pET-Cas9-NLS-6xHis (a gift from David Liu[6], Addgene plasmid #62933). A bacterial preculture grown overnight at 37 ºC was diluted 1:100 in LB medium supplemented with 100 µg/mL Ampicillin. Cultures were incubated at 37 ºC until OD_{600} ~ 0.6, cooled to 20 ºC and induced with 0.5 mM IPTG for 16 h at 20 ºC. Cells were harvested by centrifugation, pellets were resuspended in 1:10 volume of lysis buffer (220 mM NaCl, 4.3 mM KCl, 12.8 mM Na_2HPO_4, 2.4 mM KH_2PO_4, 20 % glycerol, 0.1 % Triton X-100, 10 mM imidazole, 1 mg/mL lysozyme supplemented with protease inhibitors without EDTA) and sonicated 15 x 10 s (Cycle 0.5, Amplitude 70 %) with a UP200S ultrasonic processor (Hielscher). Cellular debris was removed by centrifugation at 16.000 g 15 min and the supernatant was incubated with HisPur Ni-NTA resin (ThermoFisher) for 1 h at 4 ºC, washed 3 times with wash buffer (220 mM NaCl, 4.3 mM KCl, 12.8 mM Na_2HPO_4, 2.4 mM KH_2PO_4, 10 mM imidazole) and then the protein was eluted with several incubations of the same buffer containing 300 mM imidazole. Imidazole from the fractions containing the protein of interest was removed by several washes on an Amicon UFC (Millipore), and finally, protein was concentrated in storage buffer (20 mM HEPES pH 7.5, 500 mM KCl, 20 % of glycerol). Aliquots were snap-frozen and stored at -80 ºC.

Gel retardation assay

For gel retardation assay, the RNA complex was prepared by mixing an $ATTO_{550}$-labelled tracrRNA with the crRNA. Cas9 was diluted in DMEM without phenol red and incubated for 5 min at room temperature with the RNA complex to form the RNP. 4 µL of 2 µM RNP were mixed with different amounts of the freshly prepared amphiphile and incubated for 20 min to allow for the complexes to form. As controls, 4 µL of 2 µM

RNP or 4 μL of 2 μM gRNA were mixed with 10 μL of DMEM and incubated for 20 min. Samples were resolved in an 2 % agarose gel, for 25 min at 80 V in TAE buffer and imaged in a ChemiDoc.

Confocal microscopy

The RNP was prepared mixing $ATTO_{550}$-labelled tracrRNA, crRNA and Cas9 protein as in the gel retardation experiments. For the peptide/Cas9 complexes formation, 4 μL of 2 μM RNP were mixed with 10 μL of 50 μM **PT_{24}** and incubated for 20 min at room temperature. These complexes were diluted to a final concentration of 160 nM RNP and 10 μM **PT_{24}** using DMEM without phenol red. As a control, RNP alone was diluted to a final concentration of 160 nM. Samples were imaged on a Leica SP5 confocal microscope.

TEM

General: RNP was prepared at 2 μM as for a delivery experiment. Amphiphiles were resuspended at a concentration of 50 μM in DMEM and 4 μL of the RNP were mixed with 10 μL of the amphiphile and incubated for 20 min at room temperature before adsorption on the TEM grids. The resulting 14 μL of mixture were pipetted onto a carbon coated TEM grid (300 mesh) for 10 min, the excess was removed by using filter paper. The grids were washed with ultrapure water prior to stain the samples with 10 μL of a 2% solution in water of phosphotungstic acid or either uranyl acetate for 1 min. The grids were washed again with ultrapure water and dried at atmospheric conditions.

To prepare the complexes incorporating gold nanoparticles: Freshly prepared amphiphiles (6 equiv of oleic aldehyde) were incubated with the ribonucleoprotein for 20 min as previously described in this section. Subsequently, 2.3 μL of 100-fold diluted gold nanoparticle suspension from 5 nm Ni-NTA Nanogold (commercial stock 0.5 μM) were added to the suspension and incubated for 30 min. The mixture was then placed onto a TEM grid. After 10 min, the excess of solution was removed by using a filter paper. The grids were washed with ultrapure water prior to stain the samples with 10 μL of a 2% uranyl acetate solution in water for 1 min. The grids were washed again with ultrapure water and dried at atmospheric conditions.

To prepare the complexes embedded in agarose: Low melting point agarose (2% in PBS) was dissolved on a hot plate at 90 ºC and 3-4 drops were added to freshly prepared complexes (14 μL) and manually agitated to obtain a homogeneous suspension. Samples were incubated at 4 ºC to allow the agarose to solidify (3–4 h). The sample was removed from the tube and the agar excess was removed by using a razor blade. The agarose block was cut into tissue-size pieces for embedding. Sample was stained with osmium tetroxide, dehydrated and embedded in epoxy resin prior to visualization.

DLS

RNP at a concentration of 2 μM was prepared by mixing equal amounts of the tracrRNA:crRNA complexes and Cas9 produced in bacteria at 4 μM. Amphiphiles were resuspended at a concentration of 50 μM in DMEM and 80 μL of the RNP were mixed with 200 μL of the amphiphile and incubated for 20 min at room temperature. The mixtures were diluted in ultrapure water or in PBS up to 10 μM prior to be transferred to a standard disposable cuvette for size or ζ potential measurements. All experiments were done in triplicate at 25 ^{o}C.

Supporting figures

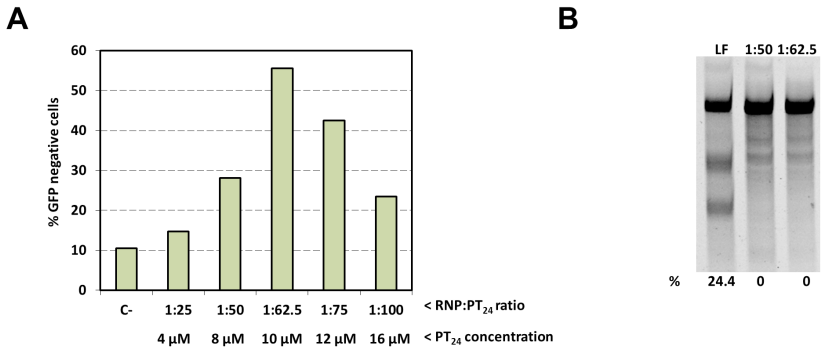


Figure S1. Optimization of peptide/Cas9 RNP delivery conditions. A) To find the optimal transfection ratio, HeLa-EGFP cells were incubated with a fixed amount of RNP targeting EGFP (160 nM) and different concentrations of **PT_{24}**, as indicated on the figure. At 72 hpt the number of EGFP negative cells was measured by flow cytometry. As the optimization was done using oleic aldehyde, it is possible that some of the amphiphiles generated with other aldehydes may be active under different conditions. B) Aldehyde alone is not able to deliver Cas9 RNP. HeLa cells were incubated with the equivalent amount of linoleic aldehyde that is present in a mixture with the peptide at the indicated ratios. LF is the positive control with Lipofectamine. [Cas9] = 80 nM.

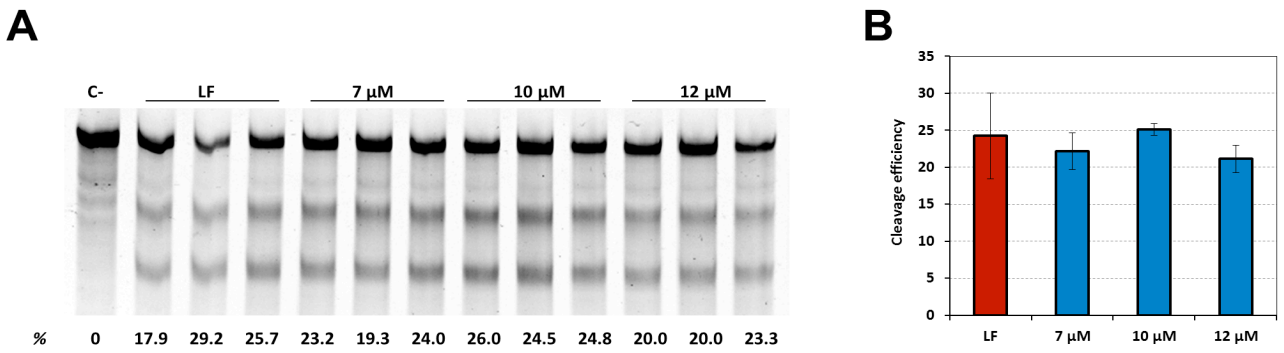


Figure S2. Incubation with peptide/RNP in the presence of 5 % serum. HeLa cells were incubated with 160 nM RNP targeting the HPRT1 gene and different concentrations of peptide **PT_{24}** (7, 10 or 12 µM) or with Lipofectamine 2000 (10 µg/mL). A) T7E1 assay done at 48 hpt. Cleavage efficiency is indicated below the gel. B) Average cleavage efficiency for each concentration. Error bars indicate standard deviation.

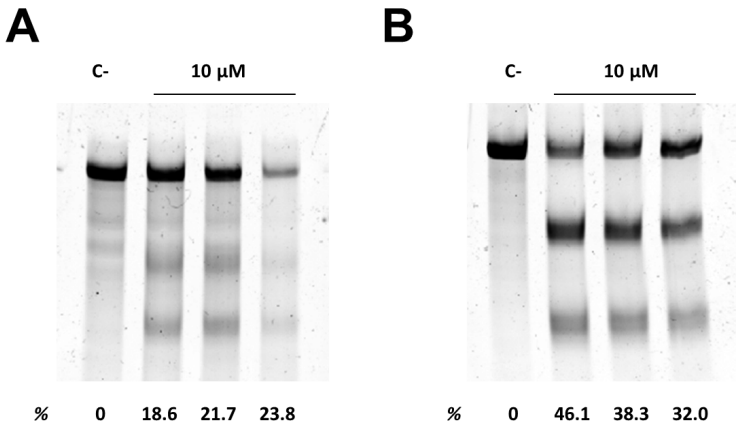


Figure S3. Gene edition of other cell lines using $\mathbf{PT_{24}}$. A) Human lung cancer A549 cells incubated with 10 µM $\mathbf{PT_{24}}$/160 nM of RNP against HPRT1. B) Chicken fibroblast DF-1 EGFP cell line incubated with 10 µM $\mathbf{PT_{24}}$/160 nM of RNP against EGFP. Cleavage efficiency is indicated below each lane.

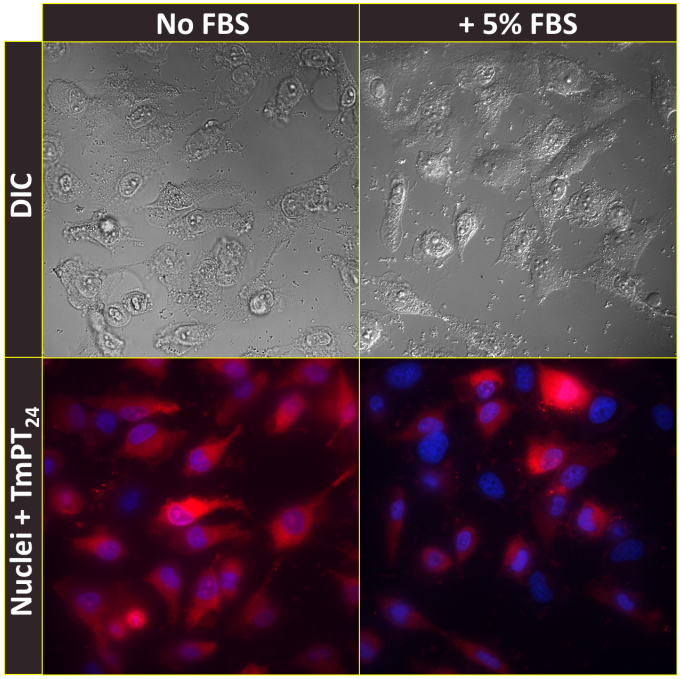


Figure S4. Increased toxicity of the peptide $\mathbf{TmPT_{24}}$. HeLa cells were incubated with the complexes (10 µM peptide/160 nM RNP) for 1 h. Nuclei were counterstained blue. The presence of the fluorophore increases the toxicity of the peptide, because at 10 µM membrane damage can be seen. Hence, the lower concentration of 5 µM was chosen for all the experiments that used the labelled peptide.

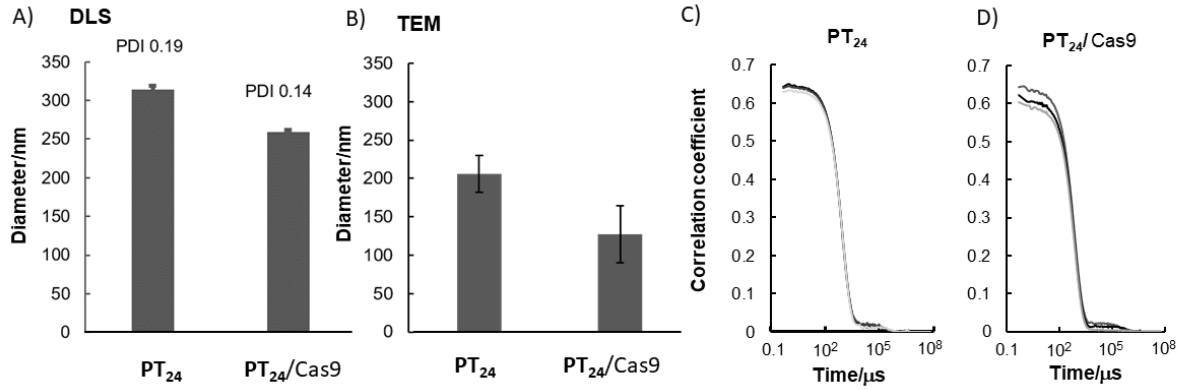


Figure S5. A) **PT_{24}** and **PT_{24}**/Cas9 RNP complex size diameters measured by DLS in DMEM/H_2O 1:4. Polydispersity indexes (PDI) are also shown above the corresponding columns. B) **PT_{24}** and **PT_{24}**/Cas9 RNP complex size diameters measured by TEM. Average and standard deviations (error bars) from 10 independent particles each are depicted. C) and D) Raw correlation data for the three DLS measurements of **PT_{24}** and **PT_{24}**/Cas9 RNP complex respectively. Data shows a slight decrease in size upon complexation with the RNP. All measurements were performed in triplicate. Error bars represent standard deviation.

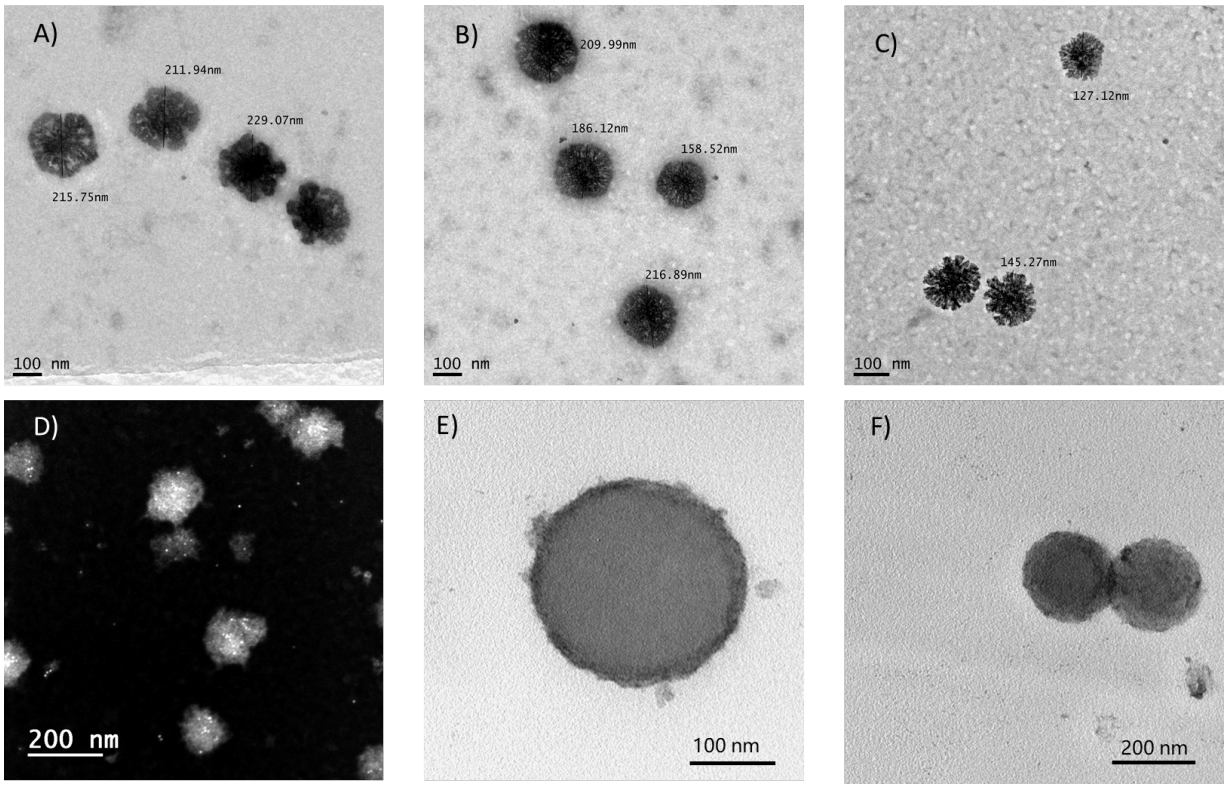


Figure S6. Exemplary TEM micrographs of A) **PT_{24}** in DMEM, B) and C) **PT_{24}**/Cas9 complex in DMEM. D) STEM micrograph of **PT_{24}**/Cas9 complex incubated with Ni-NTA Nanogold. E) and F) TEM micrographs of **PT_{24}**/Cas9 complex embedded in agarose.

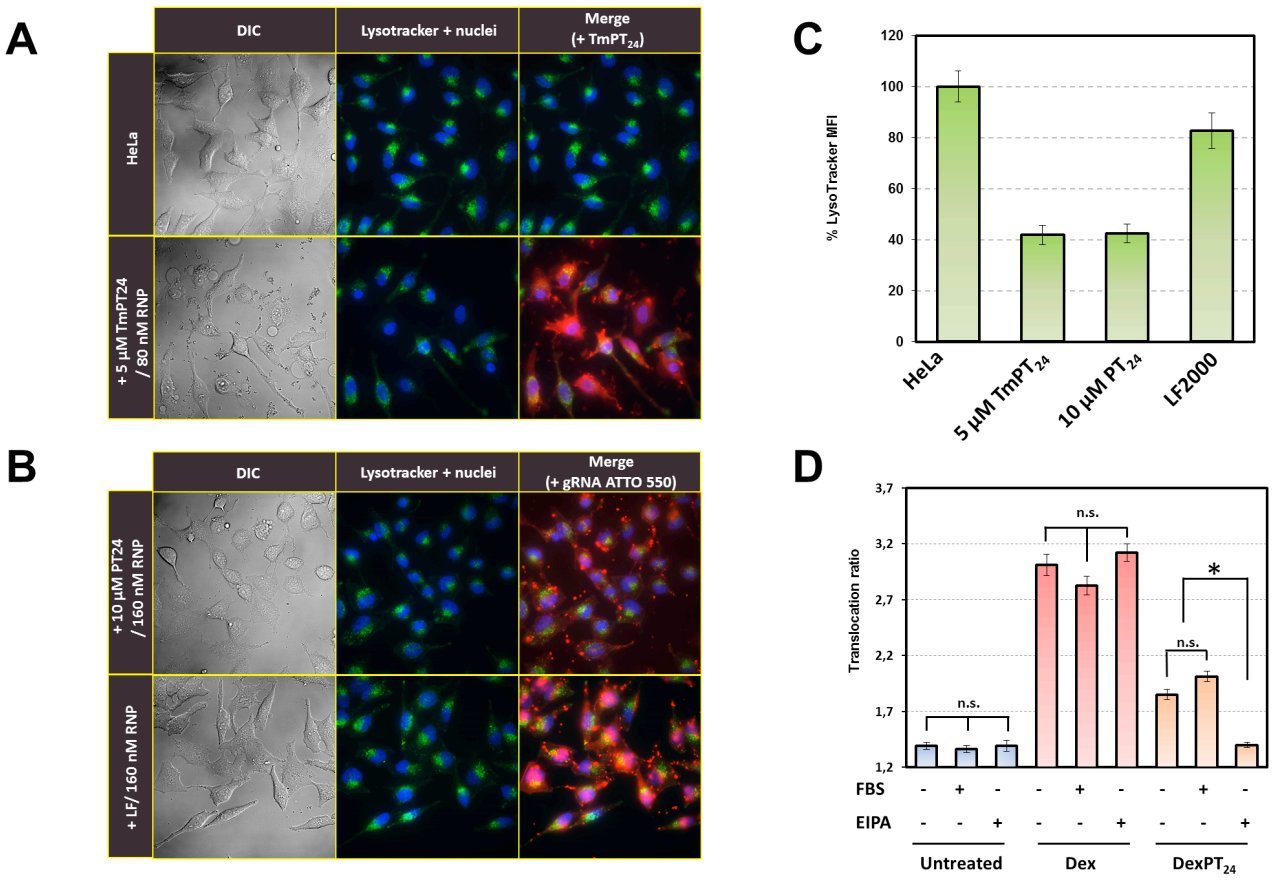


Figure S7. Lysosomal disruption. A) HeLa cells were incubated with 5 µM of **TmPT$_{24}$** and 80 nM of RNP in the presence of 50 nM LysoTracker Deep Red (green). After 1 h of incubation, cells were washed to remove the excess of LysoTracker and imaged. Fluorescence of the peptide is shown in red and nuclei are stained blue with Hoechst 33342. B) HeLa cells were incubated with 160 nM of ATTO$_{550}$-labelled RNP (red) using 10 µM **PT$_{24}$** or 10 µg/mL Lipofectamine 2000 in the presence of 50 nM LysoTracker Deep Red (shown in green). After 1 h, cells were washed and imaged. Nuclei are stained blue. C) Flow cytometry quantitation of lysosomal staining. Cells were incubated with the same complexes used in A and B, in the presence of 50 nM LysoTracker Green DND-26 for 1 h; washed and trypsinized to measure LysoTracker Green fluorescence by flow cytometry. D) GIGT assay in HeLa cells expressing GR-GFP (see methods). Incubations were performed in DMEM stock, DMEM with 5 % of FBS or DMEM containing 50 µM EIPA (a macropinocytosis inhibitor). Cells were left untreated (blue bars), incubated with 1 µM dexamethasone (red bars) or incubated with 10 µM of **DexPT$_{24}$** (orange bars) for 1 h. Asterisk indicates a p-value < 0.001, n.s. = not significant (p > 0.05); error bars indicate SEM.

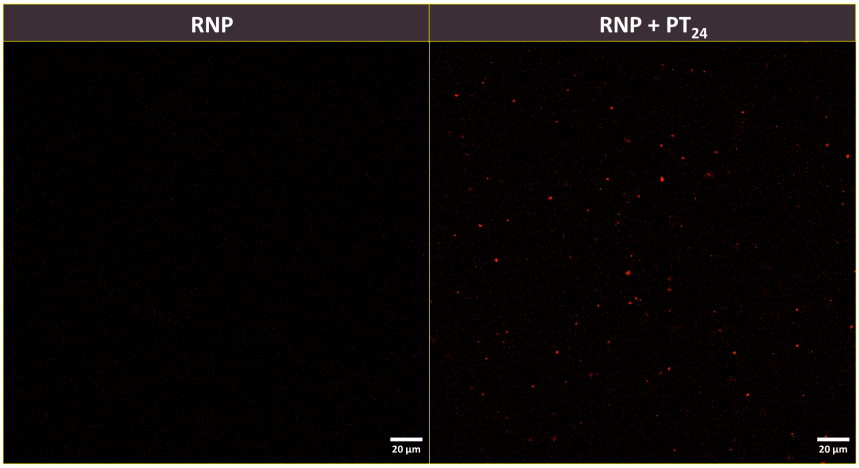


Figure S8. Confocal images of peptide/RNP complexes. RNP was prepared using $ATTO_{550}$-labelled gRNA (red) and diluted to a final concentration of 160 nM (RNP) or complexed with **PT_{24}** and then diluted to a final concentration of 10 µM **PT_{24}** and 160 nM RNP. Dilutions were done in DMEM without phenol red. Samples were imaged with a confocal microscope.

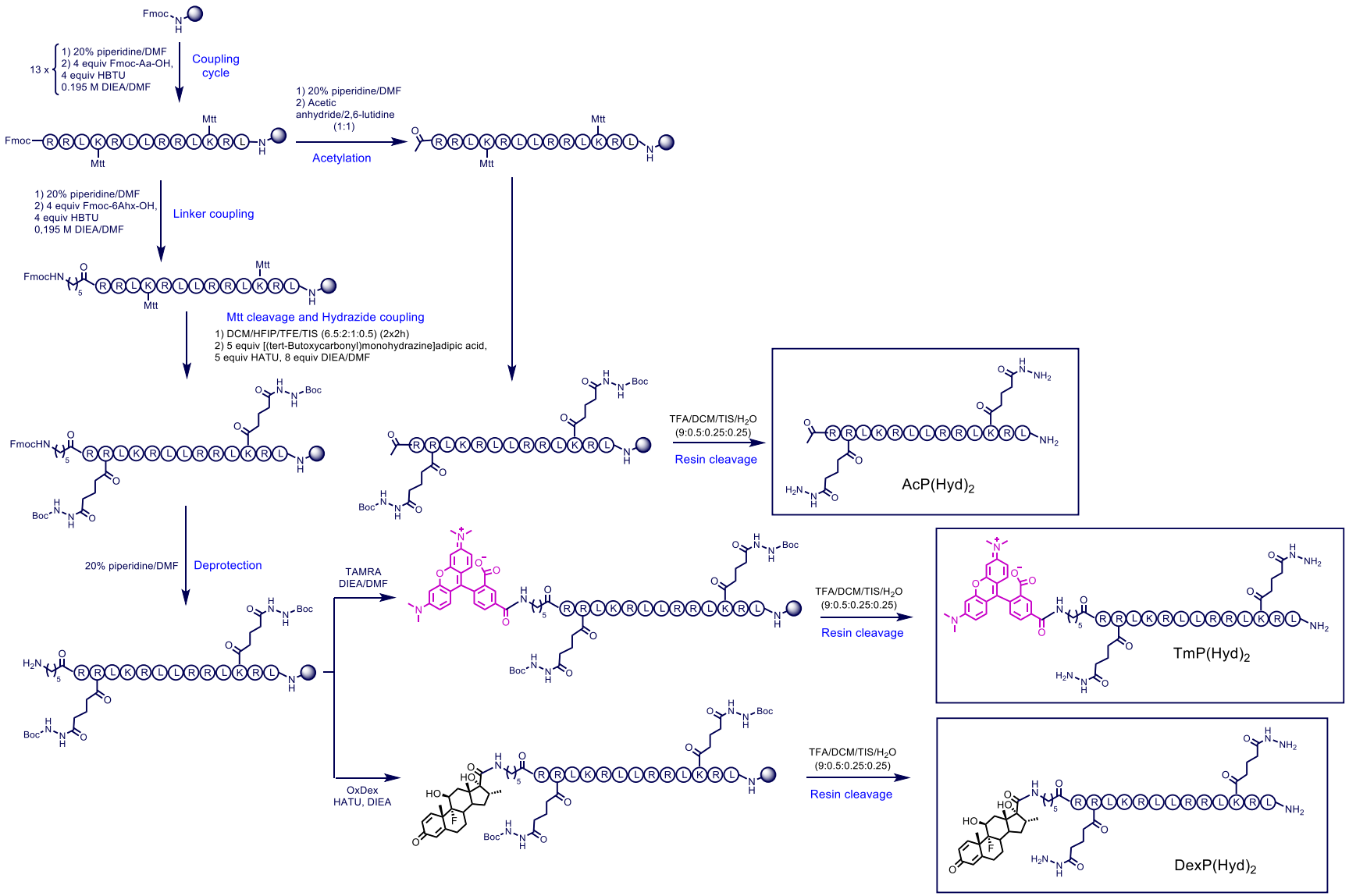
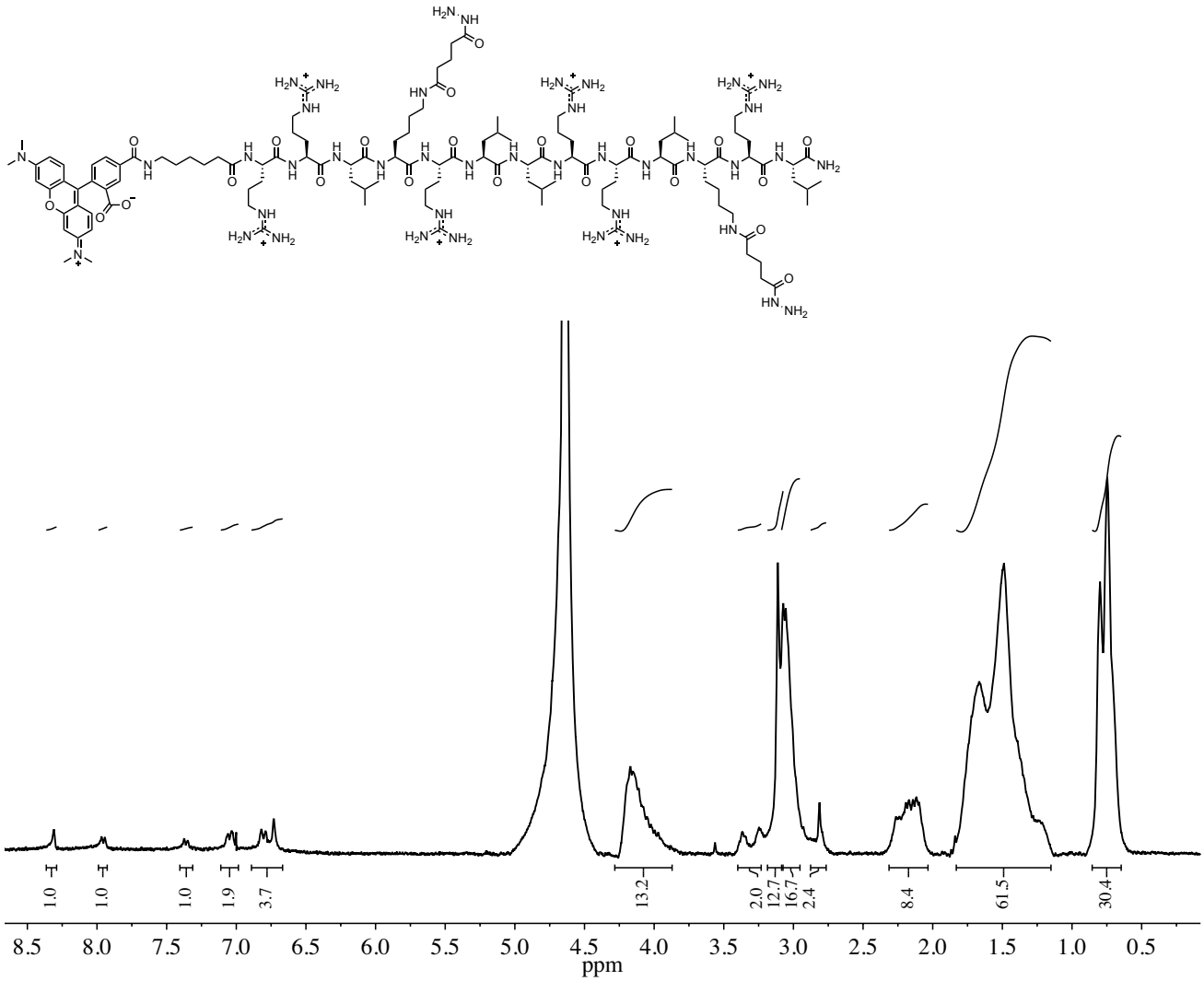


Figure S9. General synthetic scheme for the solid phase peptide synthesis (SPPS).



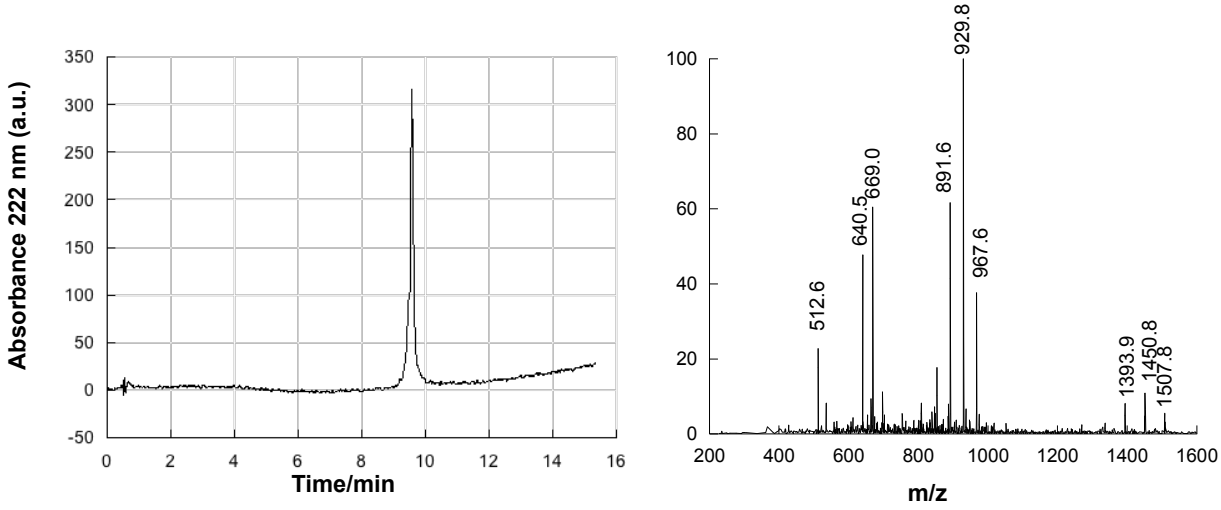
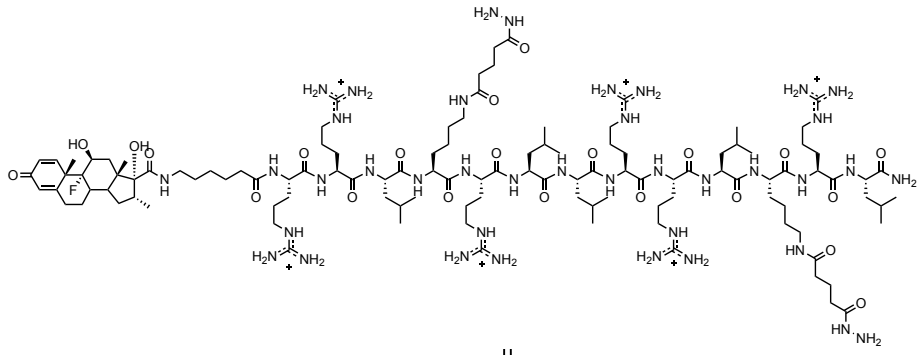
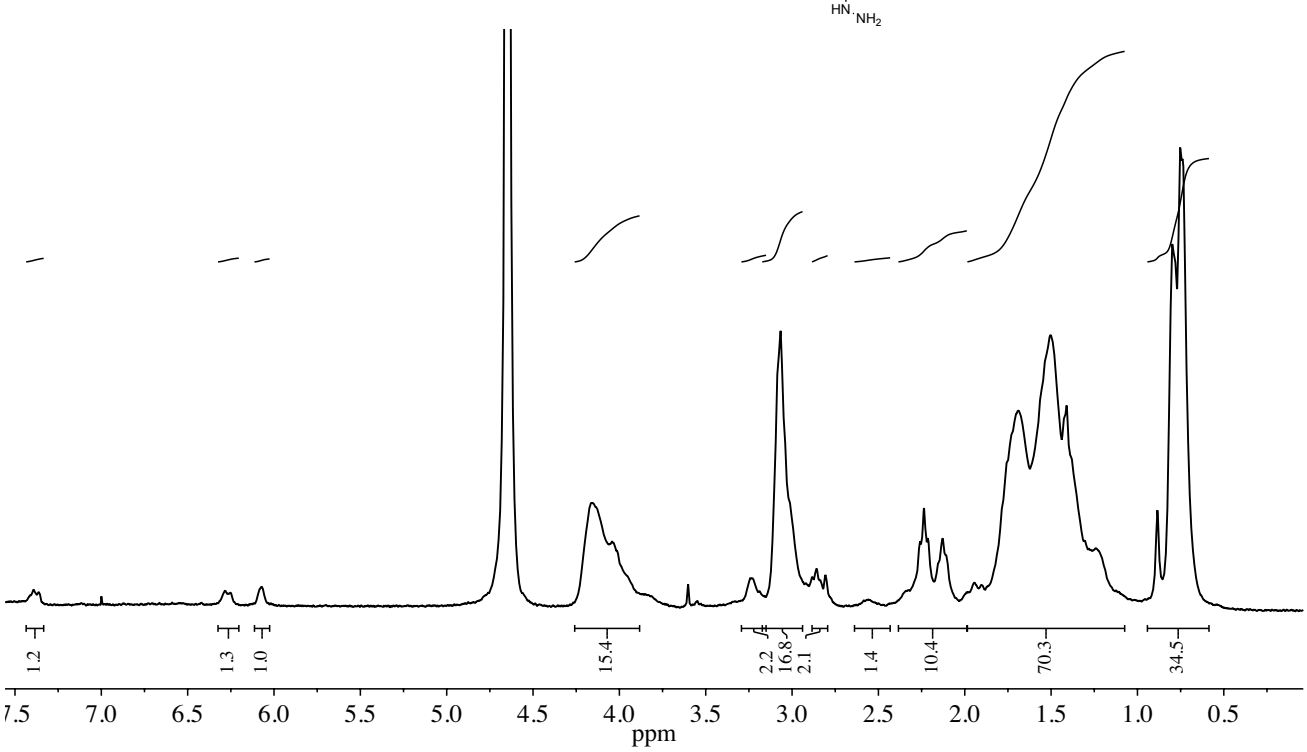
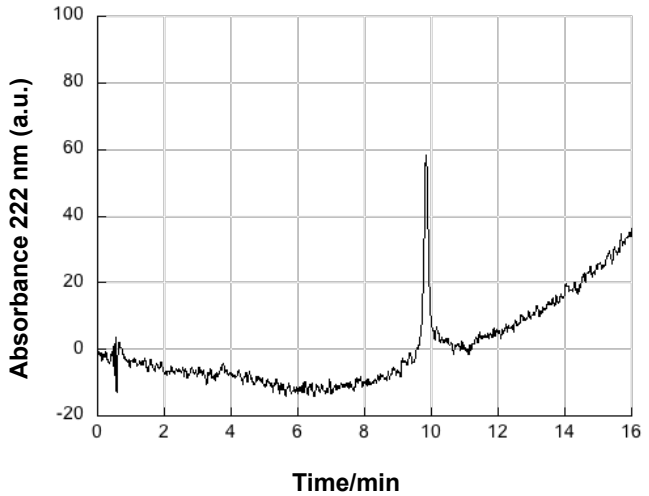


Figure S10. ^{1}H-NMR spectrum in D_2O of **TmP.** RP-HPLC [Agilent SB-C18 Column, H_2O (0.1 % TFA)/CH_3CN (0.1% TFA) 95:5 → 5:95, (0 → 15 min)]. R_t = 9.6 min and ESI-MS for **TmP.**







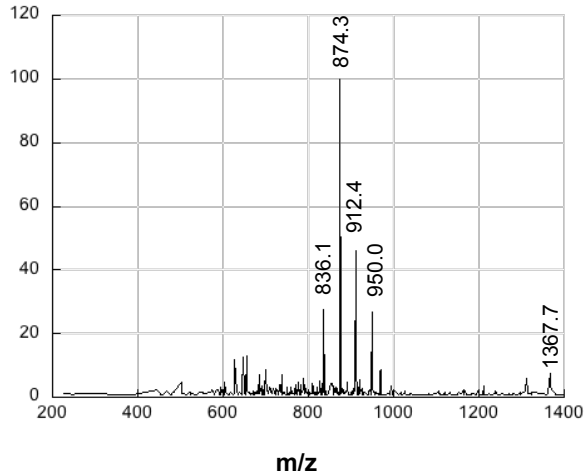


Figure S11. ^{1}H-NMR spectrum in D_2O of **DexP.** RP-HPLC [Agilent SB-C18 Column, H_2O (0.1 % TFA)/CH_3CN (0.1% TFA) 95:5 → 5:95, (0 → 15 min)]. R_t = 9.8 min and ESI-MS for **DexP.**

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