Electronic Supplementary Material (ESI) for Biomaterials Science.


**Supplementary Data**

# Development of non-viral vectors for neuronal-targeted delivery of CRISPR-Cas9 RNA-proteins as a therapeutic strategy for neurological disorders

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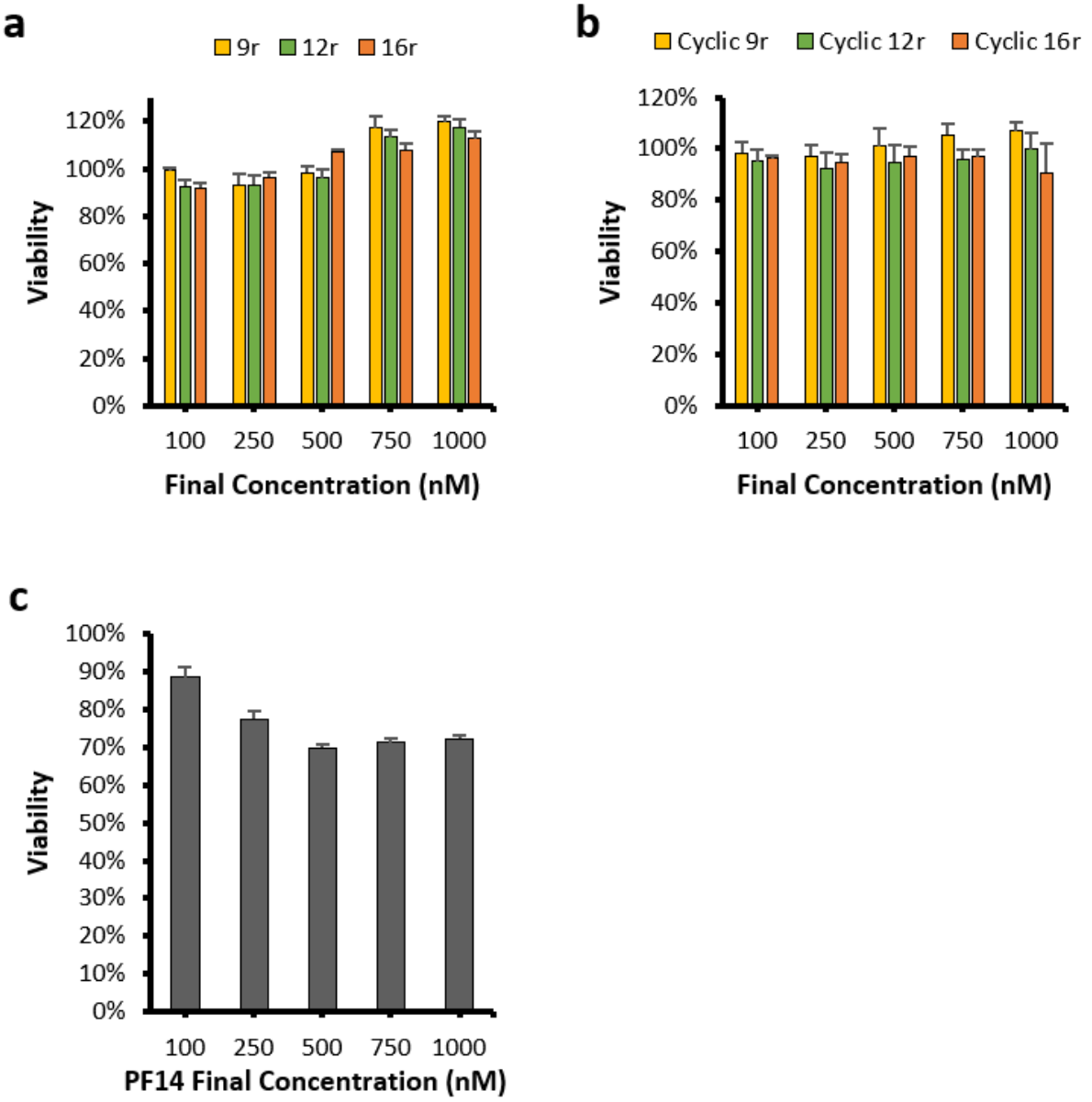
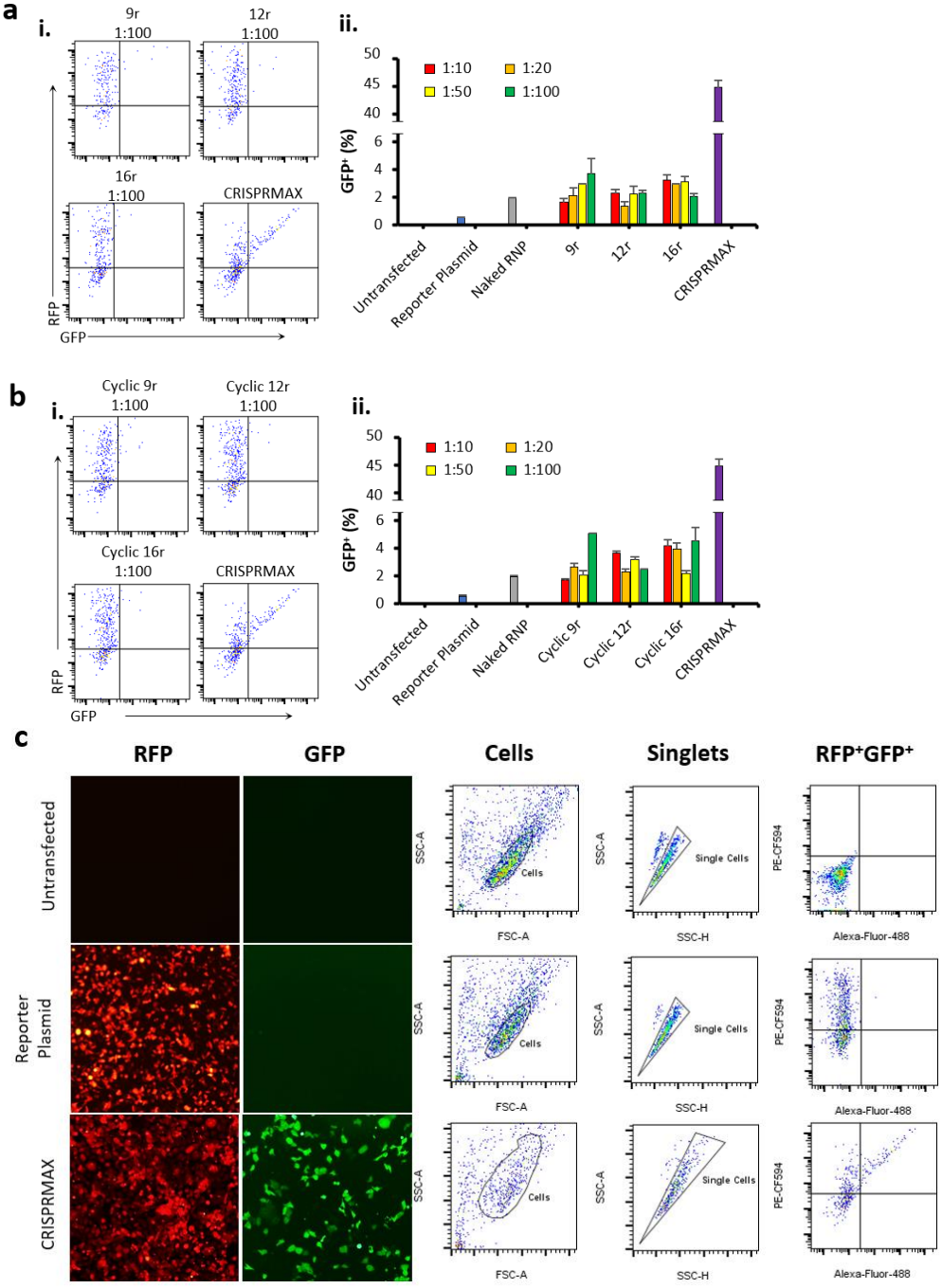
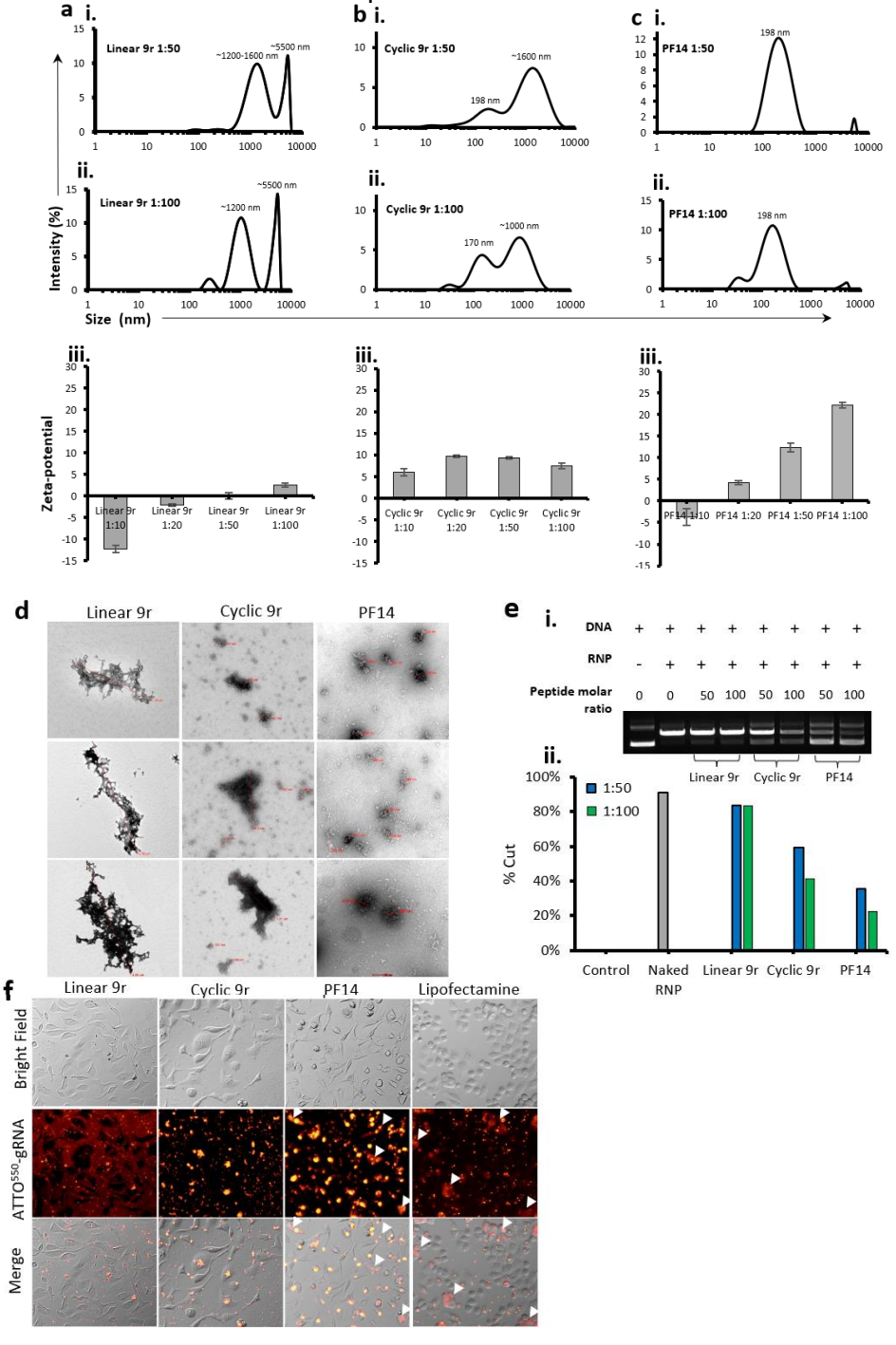
**Supplementary Figure S1. Cytotoxicity tests for cell-penetrating peptides using MTT assay.** HEK293 cells were treated with different amounts of linear (a) or cyclic (b) poly-arginine peptides, or PepFect14 peptide (c). After 24 hours, viability was measured using MTT assay. Values are expressed as a percentage of the untreated control. Error bars are standard error of $n = 3$.



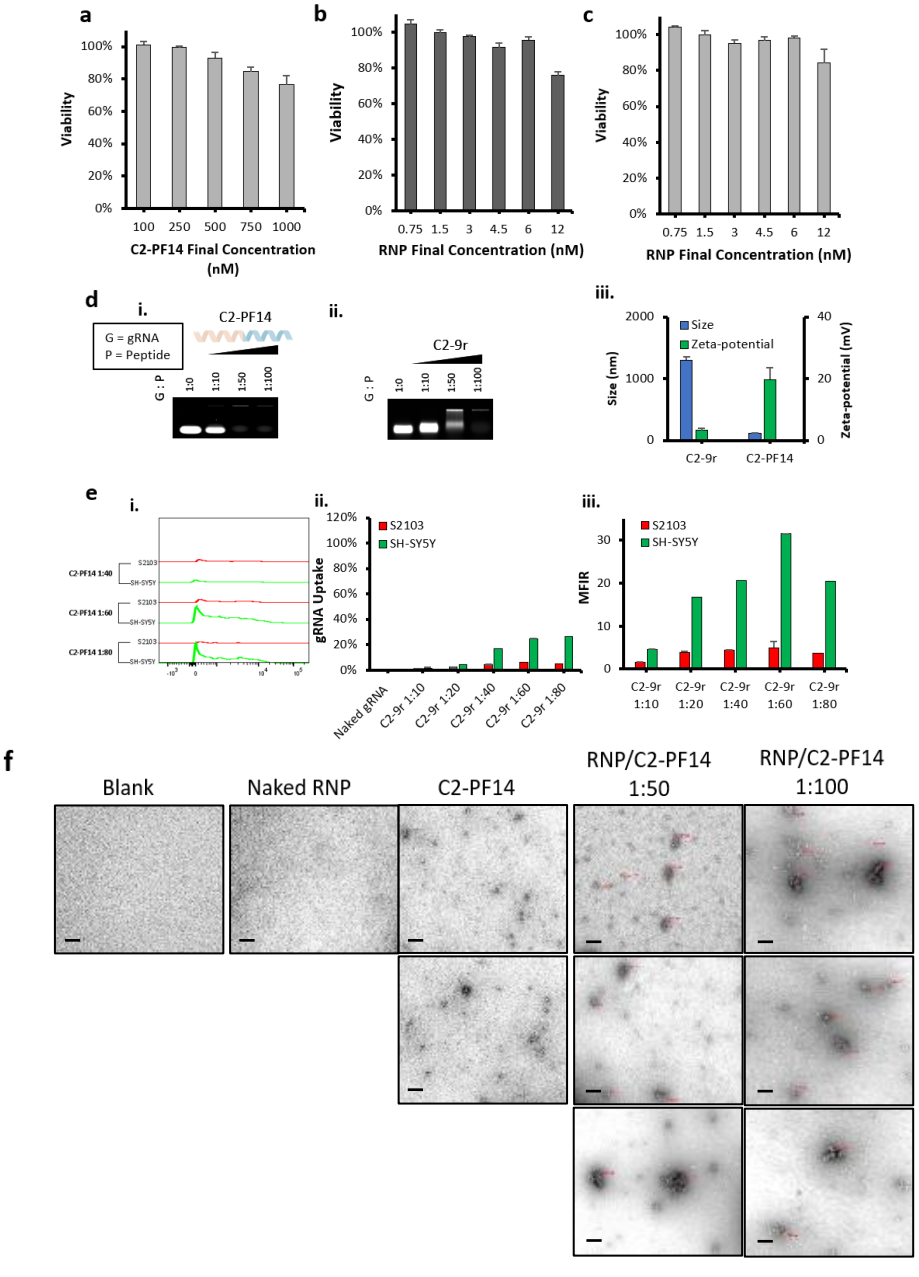
**Supplementary Figure S2. Cellular Reporter Assay for poly-arginine peptides.** To measure nuclease activity, HEK293 cells expressing the pMRS_Cas9 reporter plasmid were treated with RNP/linear (a) or RNP/cyclic (b) poly-arginine peptide complexes. Cells were treated in a 96-well plate with complexes containing 12 nM RNP using either Lipofectamine CRISPRMAX or peptides at the indicated molar ratios and incubated for 72 hours. RFP and GFP fluorescence were quantified by flowcytometry (i) and the percentage of nuclease active cells ($RFP^+GFP^+$)/(RFP+) was calculated (ii). The untreated control represents cells not transfected with the reporter plasmid; reporter plasmid was transfected with the reporter plasmid and not treated with any complexes; naked RNP were treated with RNP without peptides. Error bars are standard error ($n$ = 3). (c) Representative fluorescent microscopy images and sample gating strategy of $RFP^+GFP^+$ population in untransfected cells, cells transfected only with pMRS_Cas9 reporter plasmid, and cells transfected with RNP using Lipofectamine CRISPRMAX.



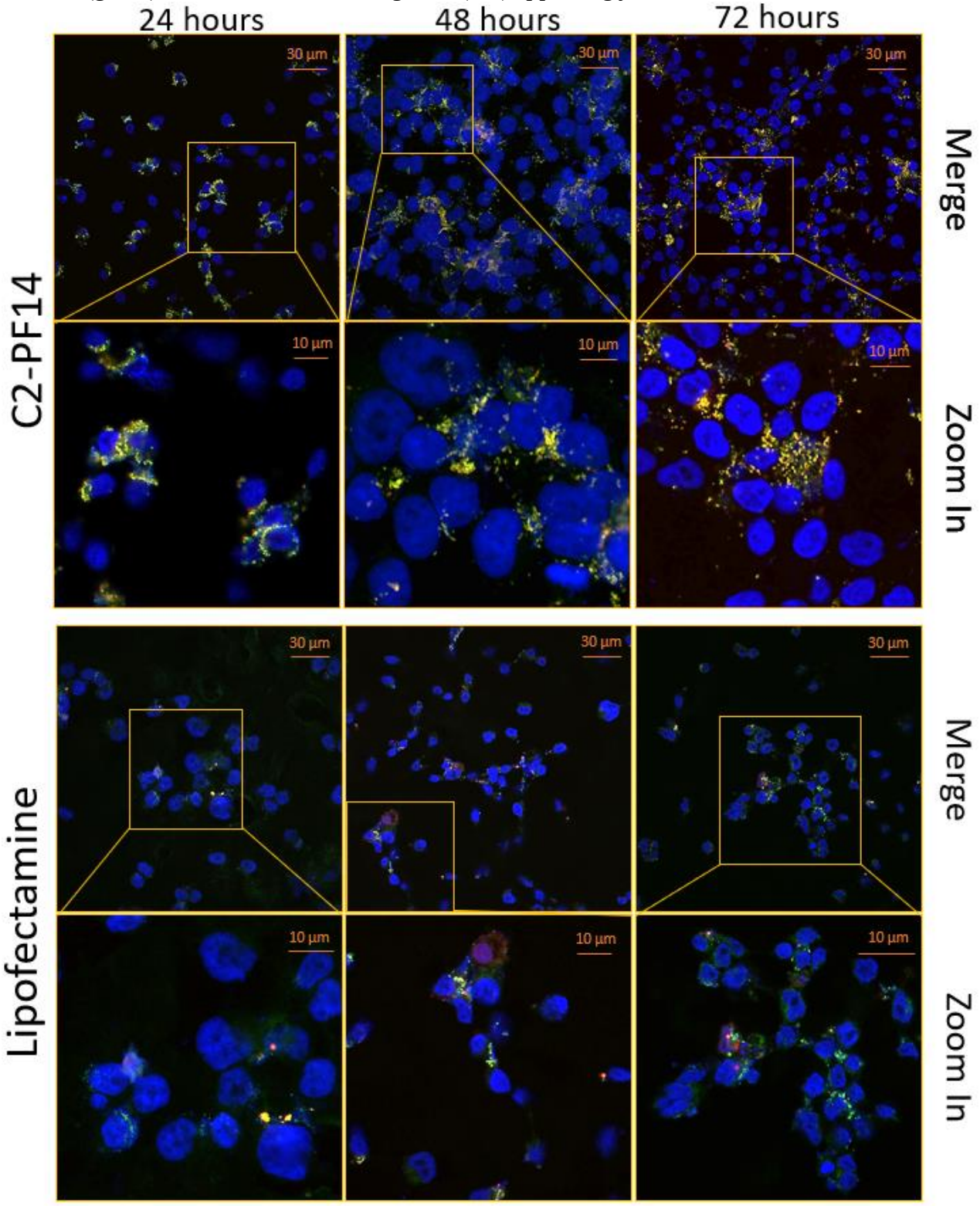
**Supplementary Figure S3. Comparing Linear 9r, Cyclic 9r and PepFect14.** (a-c) Particle size (i and ii) and zeta-potential (iii) analyses for RNP/linear 9r (a), RNP/cyclic 9r (b), and RNP/PepFect14 complexes at the indicated peptide molar ratios. Error bars are standard error of $n = 3$. (d) Representative transmission electron microscopy images of RNP complexed with linear 9r, cyclic 9r, or PepFect14 at molar ratio 1:100 (Scale bar = 200 nm). (e) *in-vitro* cleavage assay: pMRS_Cas9 reporter plasmid containing Cas9 target sequence was incubated with RNA-proteins (RNP) complexed with linear 9r, cyclic 9r or PepFect14 peptides at the indicated molar ratios at 37 $^{o}$C for 2 hours. DNA was then resolved in a 0.8% agarose gel stained with ethidium bromide at 90 V for 35 minutes and visualized using an ultraviolet transilluminator. (f) Live cell imaging: HEK293 cells were incubated with RNPs, carrying a fluorescently labelled gRNA (ATTO$^{550}$), complexed with peptides at molar ratio 1:100 or Lipofectamine CRISPRMAX and observed at 24 hours post-transfection Arrows indicate diffused complexes.



**Supplementary Figure S4. Neuronal cell targeting.** (a) C2-PF14 peptide MTT assay: SH-SY5Y cells were treated with different amounts of C2-PF14 peptide. After 24 hours, viability was measured. Values are expressed as a percentage of the untreated control. (b) and (c) RNP/C2-PF14 MTT Assay: SH-SY5Y cells were treated with RNP/C2-PF14 complexes (b) and RNP/C2-PF14 complexes formulated in PVA-PEG solution (c) at molar ratio 1:50 (RNP:C2PF14), at different final concentrations of RNP as indicated. After 24 hours, viability was measured. Values are expressed as a percentage of the untreated control. Error bars are standard error of $n = 3$. (d) Gel-shift assay: ATTO$^{550}$ fluorescently labelled gRNA was incubated with C2-PF14 (i) or C2-9r (ii) at the indicated molar ratios and subjected to gel electrophoresis. iii. Particle size and zeta potential analyses of RNP/C2-9r and RNP/C2-PF14 complexes. (e) Neuronal SH-SY5Y and non-neuronal S2103 cells were incubated with fluorescently labelled gRNA-ATTO$^{550}$/C2-9r complexes. **ii.** Quantitative representation of the percentage of uptake (cells carrying a signal from fluorescently labelled gRNA-ATTO$^{550}$). **iii.** Quantitative representation of the mean fluorescence intensity ratio (MFIR) of the degree of uptake in cells. Error bars are standard error of $n = 3$. (f) Representative transmission electron microscopy images of blank (buffer), naked RNP, C2-PF14, and RNP/C2-PF14 nanocomplexes formed at the indicated molar ratios. (Scale bar = 200 nm).



**Supplementary Figure S5. C2-PF14 delivers RNP complexes to neuronal SH-SY5Y cells and maintains Cas9/gRNA co-localization.** SH-SY5Y cells were treated with Cas9/ATTO$^{550}$-gRNA RNP complexes (3 nM RNP) using C2-PF14 (a) or Lipofectamine RNAiMAX (b). Cells were stained with Cas9 specific antibody (Alexa-fluor 488) and fixed using 4% paraformaldehyde at 24, 48, and 72 hours and imaged using a confocal microscope. Images were taken at representative fields with blue DAPI-stained nuclei, and co-localization of Cas9 (green) and ATTO$^{550}$-labelled gRNA (red) appearing yellow.



**Tables:**

**Table 1: Peptides used for complexing and delivering RNA-proteins (RNP):**

| Peptide | Sequence |
|---|---|
| 6r | rrrrrr |
| 9r | rrrrrrrr |
| 12r | rrrrrrrrrrr |
| 16r | rrrrrrrrrrrrrrr |
| 20r | rrrrrrrrrrrrrrrrrrr |
| Cyclic 9r | CrrrrrrrrC |
| Cyclic 12r | CrrrrrrrrrrrC |
| Cyclic 16r | CrrrrrrrrrrrrrrrC |
| PepFect14 | Stearyl-AGYLLGKLL-Orn-Orn-LAAAAL-Orn-Orn-LL |
| RI-C2-9r | rrrrrrrr-Sar-Sar-Sar-Sar-arkGrsntfidc |
| RI-C2-PF14 | Stearyl-AGYLLGKLL-Orn-Orn-LAAAAL-Orn-Orn-LL-Sar-Sar-Sar-Sar-arkGrsntfidc |

**Table 2: Primers used for cloning, sequencing and amplification of gRNAs:**

| Primer | Sequence | Experiment |
|---|---|---|
| pRG2S_Forward | 5’-CTGCCCGGCGCCTACAAGAC-3’ | Cloning Cas9 target sequence |
| pRG2S_Reverse | 5’-CGTAGCCTTCGGGCATGGCG-3’ | |
| SNCA_Forward | 5’-CACTCATGGCTTTACATTCCTGATCGT-3’ | Amplifying SNCA target sequence for mutation analysis |
| SNCA_Reverse | 5’-TCAGGTAGCCGTTCCCCACAGTAA-3’ | |
| M13_Forward (-20) | 5’-GTAAAACGACGGCCAG-3’ | Sanger's sequencing of the edited gene targets |
| U6-gRNA_Forward | 5’-GCGGCCGCGAGGGCCTATTT-3’ | Amplifying U6 gRNA expression fragments |
| U6-gRNA_Reverse | 5’-GGATCCTAGTACTCGAGAAAAAAAGCACCG-3’ | |

**Table 3: Guide RNAs (gRNA) used for targeting reporter and endogenous genes:**

| gRNA | Sequence | Experiment |
|---|---|---|
| Cas9_Target_gRNA2 | 5’-CTGCTGTGACTGCTTGTAGA-3’ | Reporter assay for the detection of CRISPR-Cas9 activity |

| | | |
|---|---|---|
| U6-pRG2S-Cas9-gRNA2 | GCGGCCGCGAGGGCCTATTTCCCA TGATTCCTTCATATTTGCATATACG ATACAAGGCTGTTAGAGAGATAAT TAGAATTAATTTGACTGTAAACAC AAAGATATTAGTACAAAATACGTG ACGTAGAAAGTAATAATTTCTTGG GTAGTTTGCAGTTTTAAAATTATGT TTTAAAATGGACTATCATATGCTTA CCGTAACTTGAAAGTATTTCGATTT CTTGGGTTTATATATCTTGTGGAAA GGACCCACCTTGTTGGCTGCTGTGA CTGCTTGTAGAGTTTTAGAGCTAGA AATAGCAAGTTAAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGG CACCGAGTCGGTGCTTTTTTTCTCG AGTACTAGGATCC | Reporter assay for the detection of CRISPR-Cas9 activity |
| gSNCA3 sgRNA | 5'- AAAGAGCAAGTGACAAATGT-3' | Inducing mutation in and knocking out *SNCA* gene |

**DNA sequences and maps of plasmids used in this study:**

**Cas9 Target Sequence: (yellow, cloned between BamHI and BstXI)**

5'-
atggccaagaagcccgtgcagctgcccggcgcctacaagaccgacatcaagctggacatcacctcccacaacgaggactacaccat
cgtggaacagtacgagcgcgccgagggccgccactccaccggcgccgaattcGCGTCCGCGCCATGGCCATCT
ACAAGCAGTCACAGCAg-3'

**pMRS_Cas9 sequence and map:**

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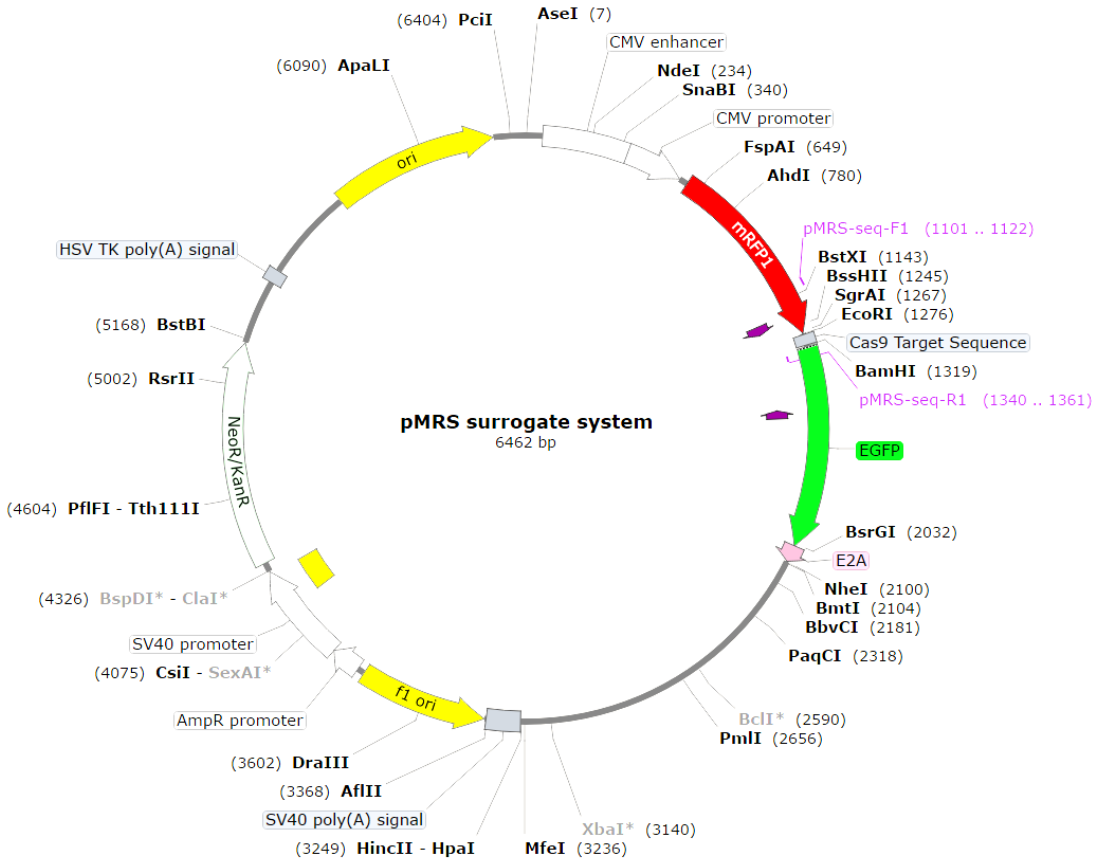


**pRG2S_Cas9 sequence and map:**

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