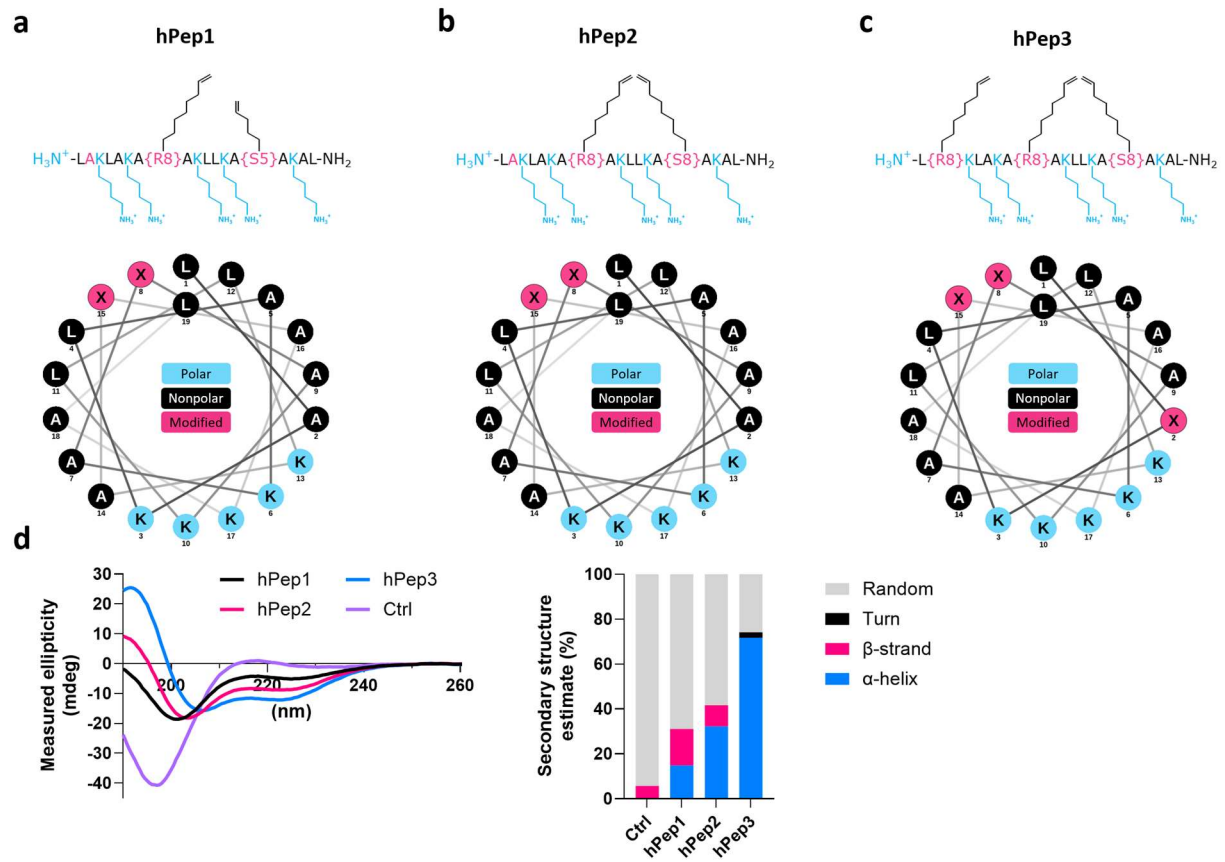
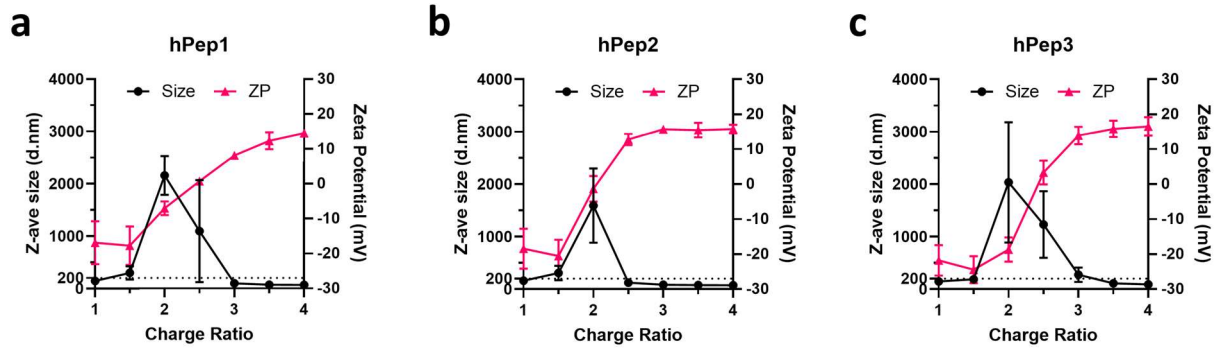


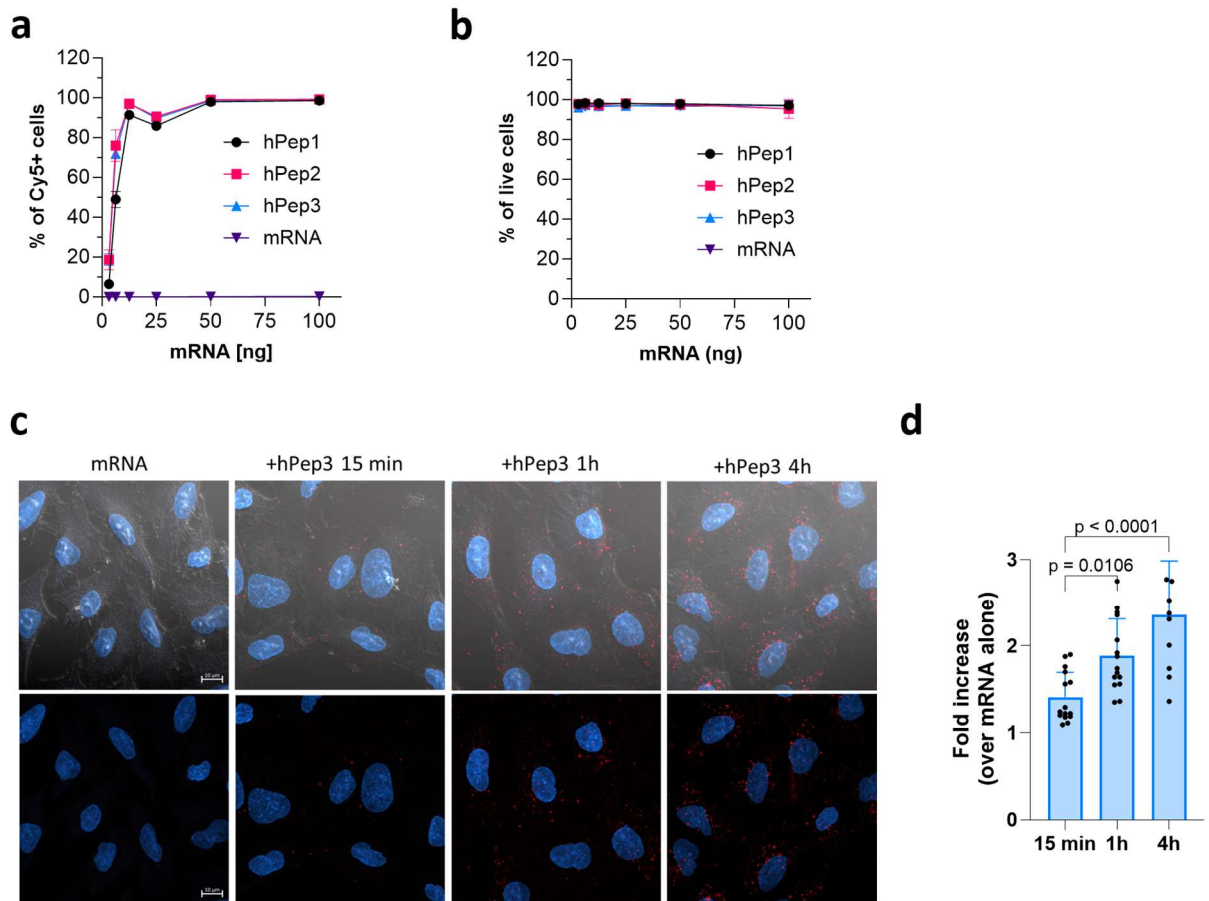
# Supplementary Data



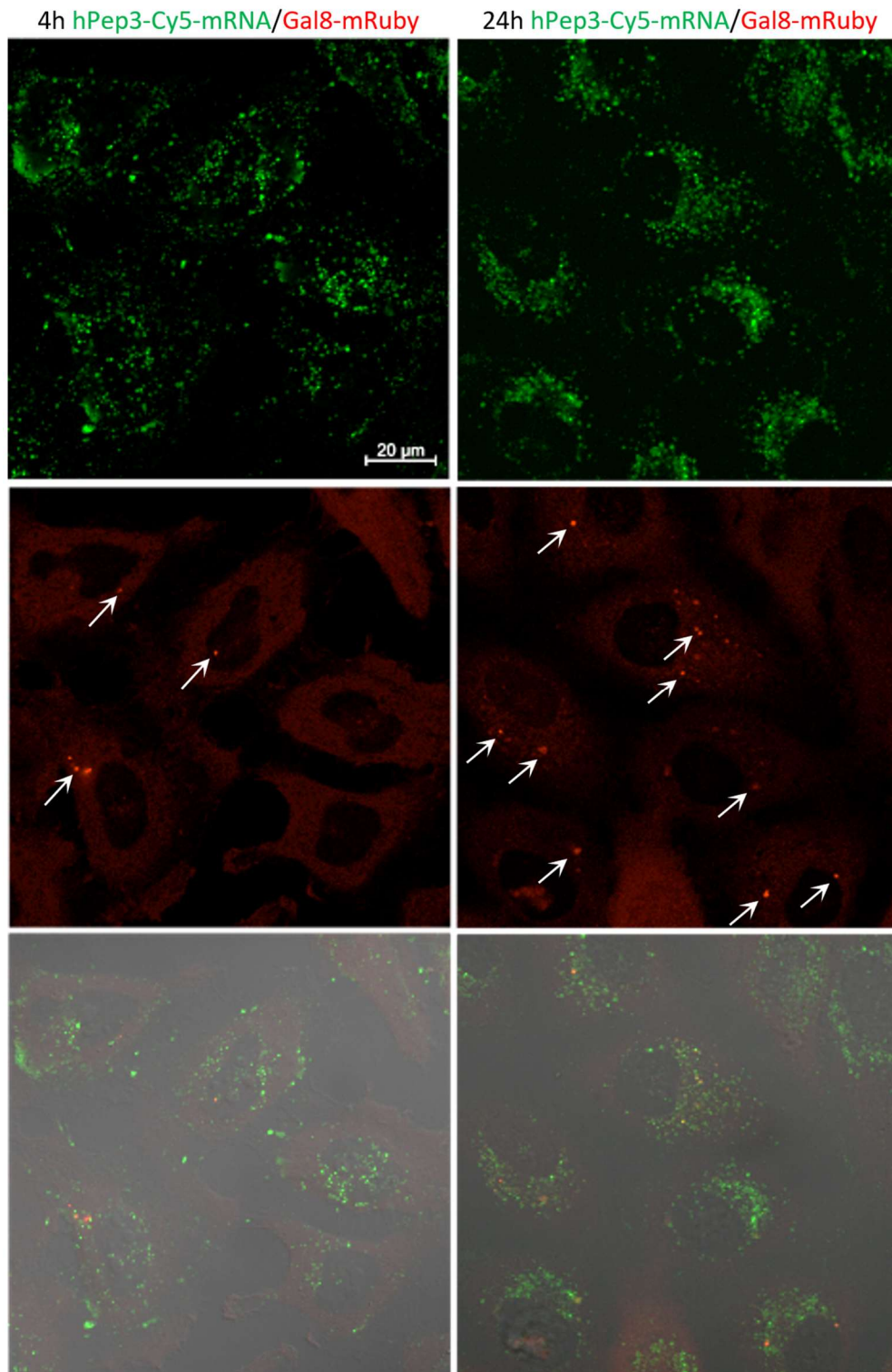
**Supplementary Figure 1. Structural design of hPep peptides.** a-c, Chemical structure and helical wheel projections of hPep peptides. The Ctrl peptide is hPep3 analog where all three octenyl-alanines are substituted by natural alanines. Helical wheel projections were created with <http://lbqp.unb.br/NetWheels/>. d, CD spectra of hPep peptides.



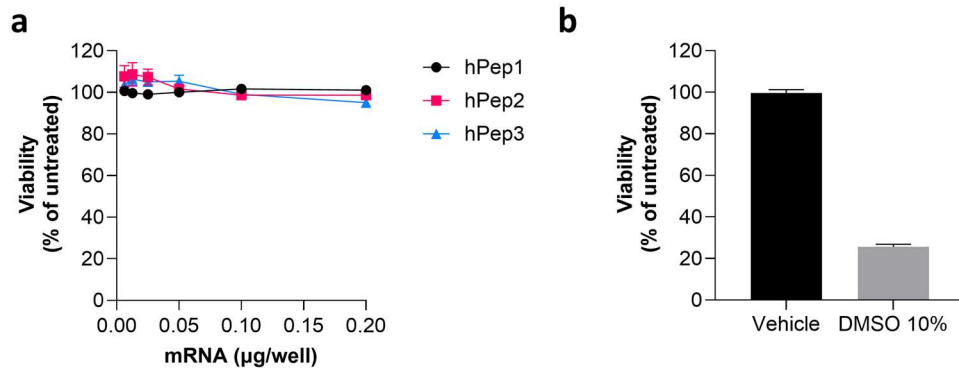
**Supplementary Figure 2. Physicochemical characterization of hPep/mRNA NP properties.** a-c, Size and zeta potential graphs of hPep/mRNA complexes formulated over a range of peptide/mRNA charge ratios with hPep1, hPep2, and hPep3, respectively. Data is presented as mean  $\pm$  SD (n=3).



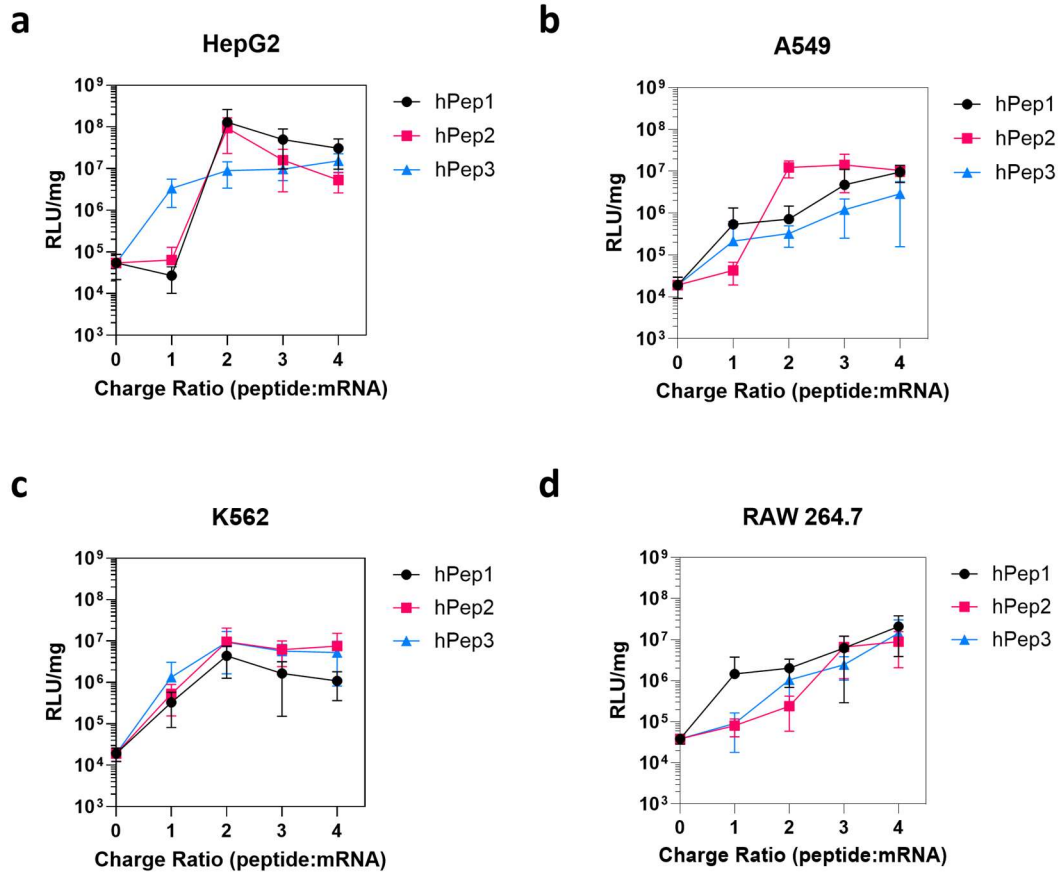
**Supplementary Figure 3. Uptake of hPep/Cy5-mRNA NPs.** A549 cells were treated for 4 h with hPep/Cy5-mRNA NPs formulated at CR4 at different doses. **a**, Percentage of Cy5 positive cells, and **b**, percentage of live cells were quantified by FACS. Data is presented as mean  $\pm$  SD ( $n=3$ ). **c**, Confocal microscopy of fixed A549 cells at different time-points after treatment with hPep3/Cy5-mRNA NPs. **d**, Statistical analysis of the uptake of hPep/Cy5-mRNA NPs (mean  $\pm$  SD). One-way ANOVA with Dunnett's multiple comparison.



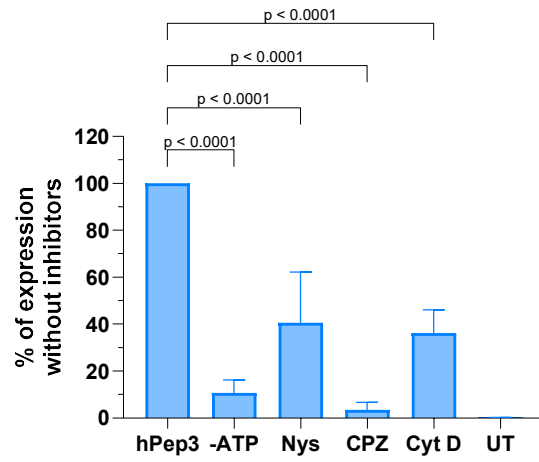
**Supplementary Figure 4. Endosomal release of hPep3/mRNA NPs.** Confocal microscopy of Gal8-mRuby expressing HeLa cells treated with hPep3/Cy5-mRNA NPs at CR4 at the indicated time-points. Arrows indicate endosomal membrane disruption sites.



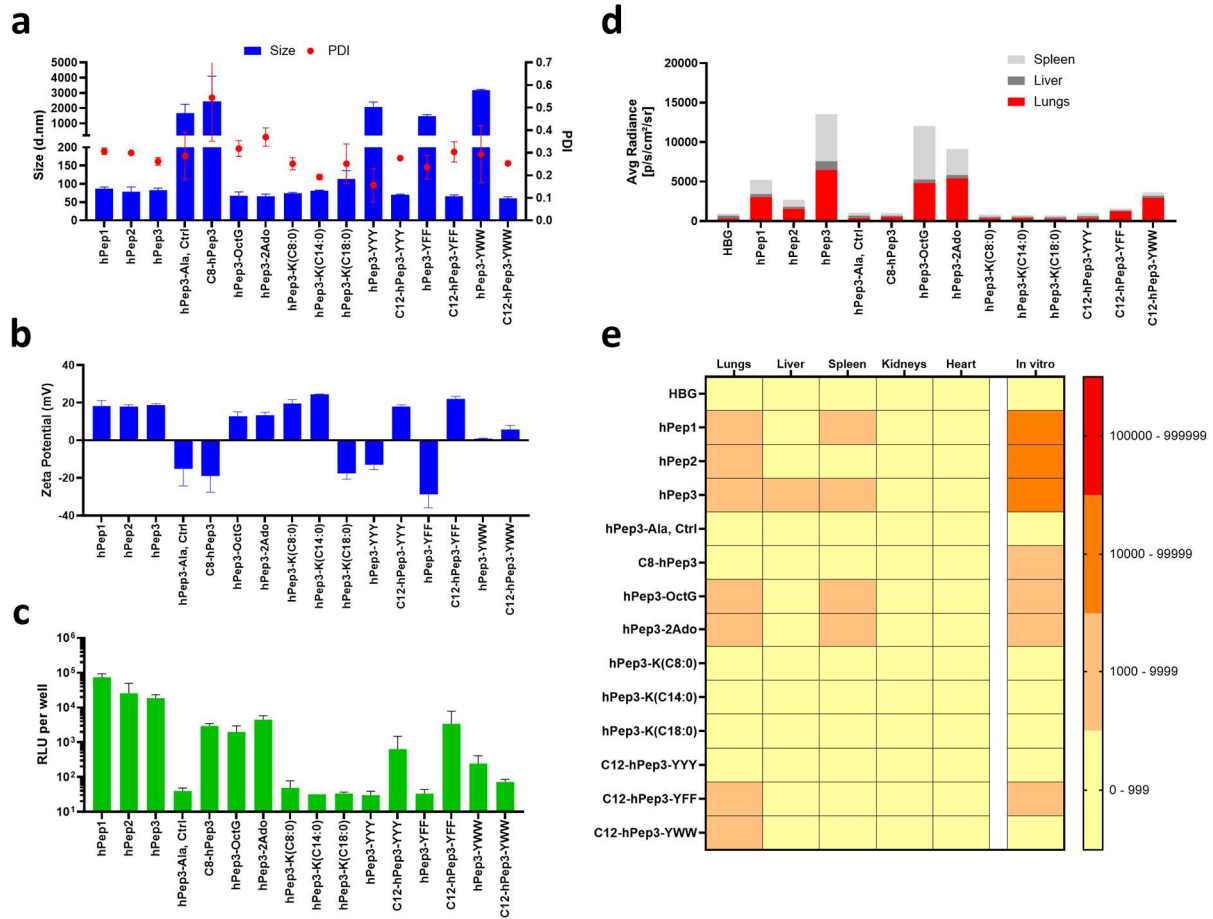
**Supplementary Figure 5. Dose-dependent evaluation of hPep/mRNA NPs on cell viability in A549 cells using WST-1 assay.** **a**, Viability of cells after 24-hour treatment with PNPs formulated at CR4, **b**, negative control formulation buffer (vehicle) and positive control (DMSO 10%). Data is presented as mean  $\pm$  SD (n=3).



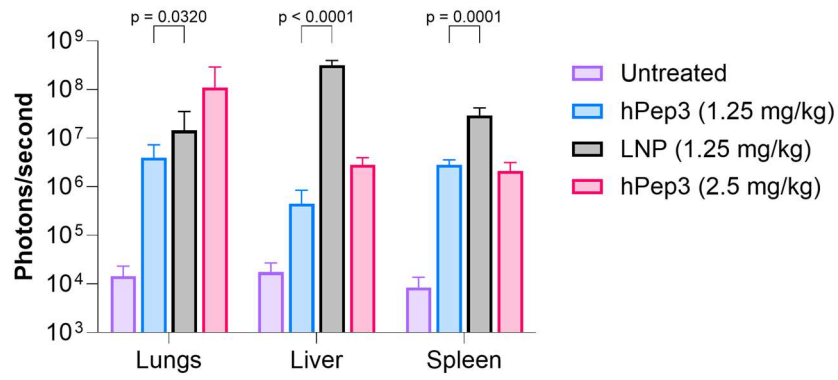
**Supplementary Figure 6. CR-dependent transfection efficacy of hPep/mRNA NPs in different cell lines. a-d,** Cell lines were treated for 24 h with hPep3/Fluc NPs formulated at different CRs and corresponding luciferase expressions are presented. Data is presented as mean  $\pm$  SD (n=3).



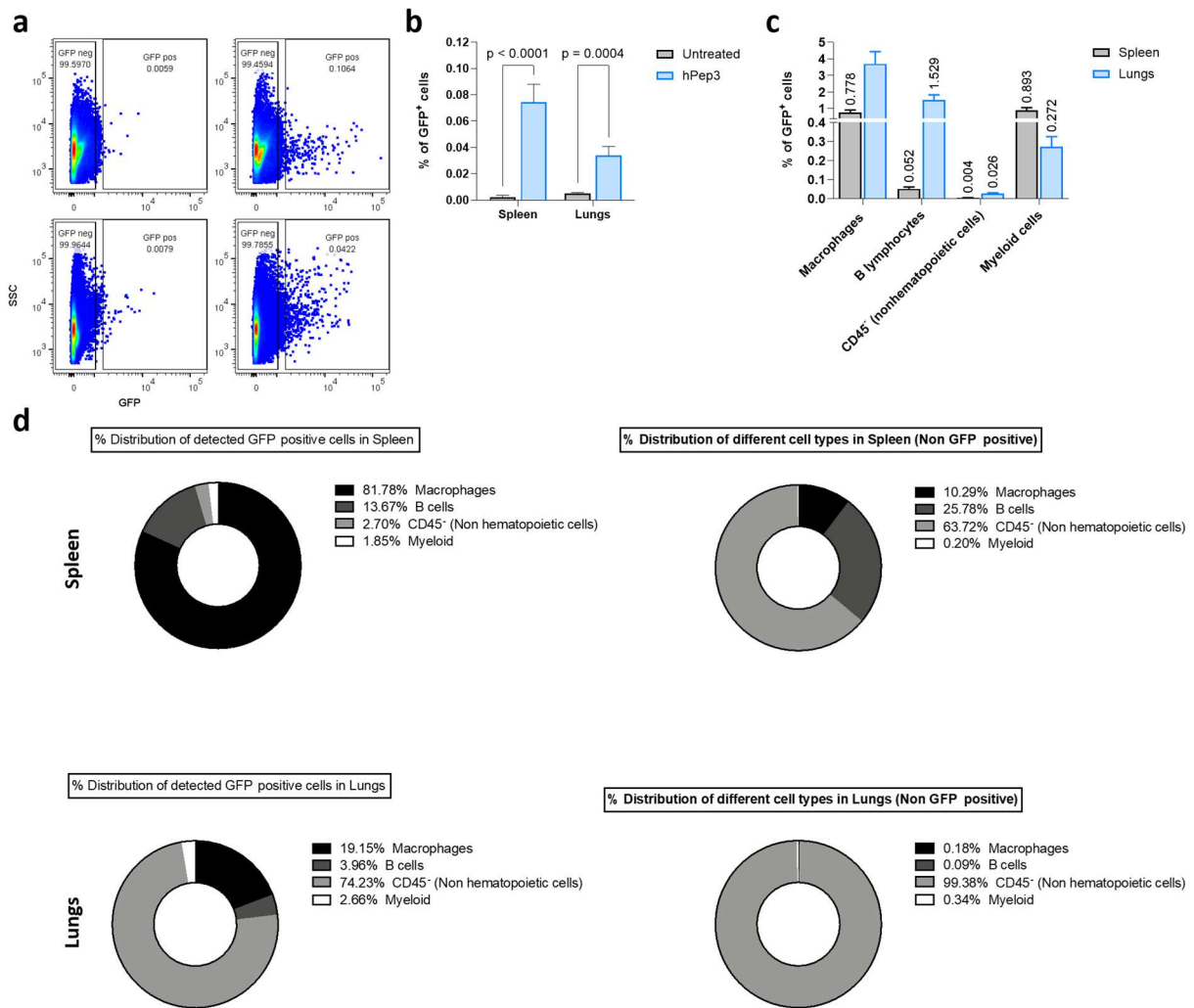
**Supplementary Figure 7. Effect of pharmacological endocytosis inhibition on the luciferase encoding mRNA delivery efficacy with hPep3/Fluc NPs in A549 cells.** Data is presented as mean  $\pm$  SD, n=3. One-way ANOVA with Dunnett's multiple comparison



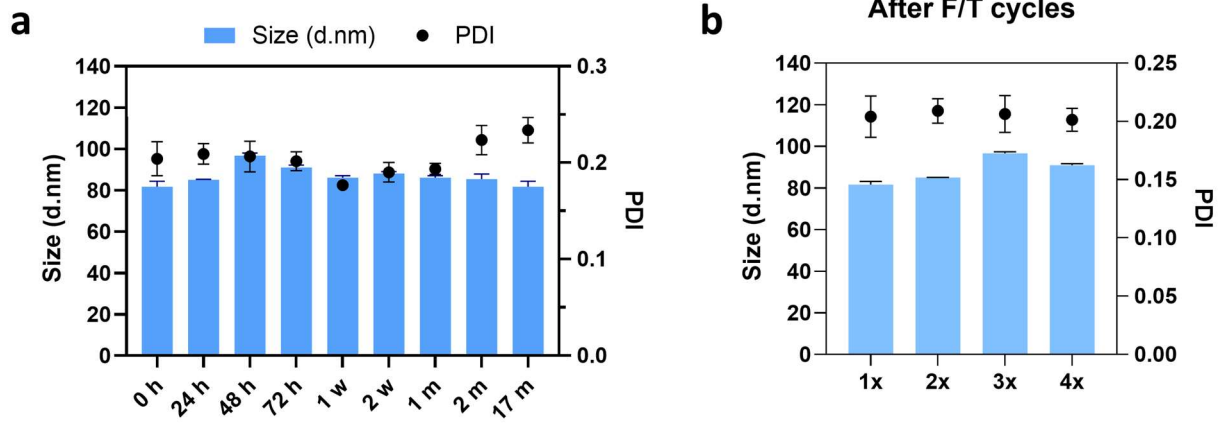
**Supplementary Figure 8. Characterization of peptide/mRNA NPs with hPep analogs and their efficacy in vitro and in vivo.** **a-b**, Size and zeta potential of hPep/mRNA formulations at CR4 as measured by DLS. **c**, A549 cells were treated for 24 h with hPep/Fluc-mRNA NPs formulated at CR4, and corresponding luciferase expressions are presented. **d**, For in vivo evaluation Balb/c mice (n=3 per group) were intravenously administered with hPep/mRNA NPs formulated at CR4 at a final dose of 1 mg/kg. After 24 h luciferase expression was quantified from dissected tissues with IVIS. **e**, Summary of in vivo and in vitro data. Data is presented as mean  $\pm$  SD (n=3).



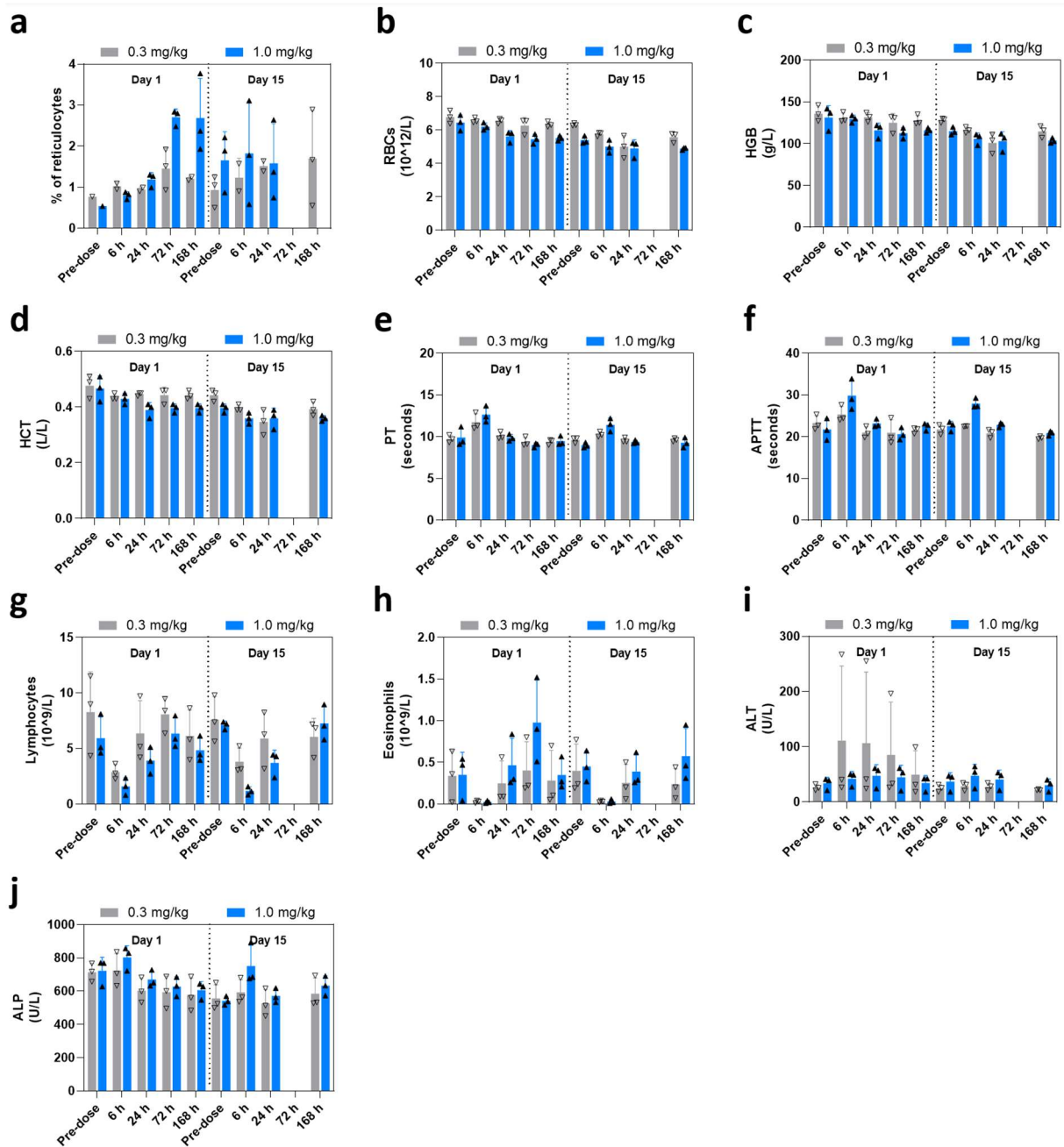
**Supplementary Figure 9. In vivo efficacy comparison between hPep3/mRNA NPs and MC3 LNPs.** Animals were treated with bulk mixed hPep3/mRNA NPs and microfluidically formulated LNPs at 1.25 mg/kg mRNA dose. After 24 h tissues were dissected and measured under IVIS. Data is presented as mean ± SD (n=3). Two-way ANOVA with Dunnett's multiple comparison.



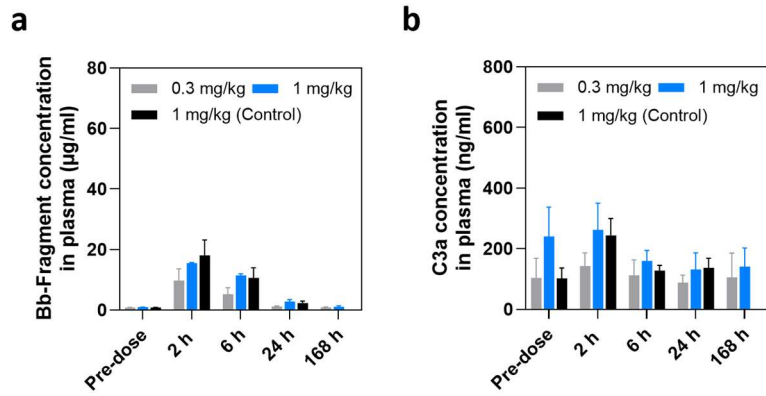
**Supplementary Figure 10. Target cell type identification of hPep3/EGFP-mRNA PNPs in vivo.** **a**, Mice were treated with hPep3/EGFP mRNA NPs i.v. (n=3 animals per group), followed by flow cytometry analysis of the tissues 24 hours post-injection. **b**, Quantification of the overall EGFP expression in different tissues by FACS. Two-way ANOVA with Šidák's multiple comparison. **c**, **d**, Cell type-specific expression of EGFP mRNA, verified 24 hours after injection of hPep3/mRNA PNPs using FACS. (n=3 animals per group). Data is presented as mean ± SD.



**Supplementary Figure 11. Short- and long-term stability of microfluidically formed hPep/mRNA formulations in cryostorage.** **a**, Formulation was carried out with NanoAssemblr™ Blaze™ instrument in combination with NxGen 500 cartridge with hEPO mRNA and stored at -80°C over time and after subjected to **b**, freeze-thaw cycles. DLS data is presented as mean ± SD.



**Supplementary Figure 12. Clinical parameters of repeated administration hPep3/hEPO mRNA PNPs in NHPs.** a, Blood reticulocytes percentage, b, red blood cell count, c, hemoglobin concentration, d, hematocrit level, e, prothrombin time, f, activated partial thromboplastin time, g, lymphocyte count, h, eosinophil count, i, alanine transaminase concentration and j, alkaline phosphatase concentration. Data is presented as mean  $\pm$  SD.



**Supplementary Figure 13. Complement activation after single administration of hPep3/hEPO mRNA and hPep3/Control mRNA formulations in NHPs is mild and transient.** a, Bb-fragment and b, C3a factor concentrations were measured from plasma at noted time-points. Data is presented as mean  $\pm$  SD (n=3 animals per group).

**Supplementary Table 1. Summary of all developed hPep analogs and their chemical properties**

Name	Structure	Charge	Mw (g/mol)	$\alpha$ -helical content %	NP formation
<b>hPep1</b>	NH <sub>2</sub> -LAKLAKA{ <b>R8</b> }AKLLKA{ <b>S5</b> }AKAL-NH <sub>2</sub>	+6	2040.4	14.9	+
<b>hPep2</b>	NH <sub>2</sub> -LAKLAKA{ <b>R8</b> }AKLLKA{ <b>S8</b> }AKAL-NH <sub>2</sub>	+6	2084.4	32.2	+
<b>hPep3</b>	NH <sub>2</sub> -L{ <b>R8</b> }KLAKA{ <b>R8</b> }AKLLKA{ <b>S8</b> }AKAL-NH <sub>2</sub>	+6	2193.9	71.7	+
hPep3-Ala, Ctrl	NH <sub>2</sub> -LAKLAKAAAKLLKAAAKAL-NH <sub>2</sub>	+6	1863.5	0.0	-
C8-hPep3	Octanoyl-L{ <b>R8</b> }KLAKA{ <b>R8</b> }AKLLKA{ <b>S8</b> }AKAL-NH <sub>2</sub>	+5	2320.4	15.3	-
hPep3-OctG	NH <sub>2</sub> -L{ <b>OctG</b> }KLAKA{ <b>OctG</b> }AKLLKA{ <b>OctG</b> }AKAL-NH <sub>2</sub>	+6	2157.4	57.7	+
hPep3-2Ado	NH <sub>2</sub> -L{ <b>2Ado</b> }KLAKA{ <b>2Ado</b> }AKLLKA{ <b>2Ado</b> }AKAL-NH <sub>2</sub>	+6	2241.4	64.0	+
hPep3-K(C8:0)	NH <sub>2</sub> -L{ <b>K(C8:0)</b> }KLAKA{ <b>K(C8:0)</b> }AKLLKA{ <b>K(C8:0)</b> }AKAL-NH <sub>2</sub>	+6	2414.3	28.6	+
hPep3-K(C14:0)	NH <sub>2</sub> -L{ <b>K(C14:0)</b> }KLAKA{ <b>K(C14:0)</b> }AKLLKA{ <b>K(C14:0)</b> }AKAL-NH <sub>2</sub>	+6	2666.8	72.8	+
hPep3-K(C18:0)	NH <sub>2</sub> -L{ <b>K(C18:0)</b> }KLAKA{ <b>K(C18:0)</b> }AKLLKA{ <b>K(C18:0)</b> }AKAL-NH <sub>2</sub>	+6	2835.1	69.4	+
hPep3-YYY	NH <sub>2</sub> -LYKLAKAYAKLLKAYAKAL-NH <sub>2</sub>	+6	2139.8	0.0	-
C12-hPep3-YYY	Lauryl-LYKLAKAYAKLLKAYAKAL-NH <sub>2</sub>	+5	2322.1	4.2	+
hPep3-YFF	NH <sub>2</sub> -LYKLAKAFKLLKAFKAL-NH <sub>2</sub>	+6	2107.8	0.0	-
C12-hPep3-YFF	Lauryl-LYKLAKAFKLLKAFKAL-NH <sub>2</sub>	+5	2290.1	11.8	+
hPep3-YWW	NH <sub>2</sub> -LYKLAKAWAKLLKAWAKAL-NH <sub>2</sub>	+6	2185.9	0.0	-
C12-hPep3-YWW	Lauryl-LYKLAKAWAKLLKAWAKAL-NH <sub>2</sub>	+5	2368.2	28.4	+

{R8} = (R)-2-(7-octenyl)alanine

{S5} = (S)-2-(4-pentenyl)alanine

{S8} = (S)-2-(7-octenyl)alanine

{OctG} = L-octylglycine

{2Ado} = (S)-2-amino-dodecanoic acid

{K(C8:0)} = N(6)-(octanoyl)lysine

{K(C14:0)} = N(6)-(myristoyl)lysine

{K(C18:0)} = N(6)-(stearoyl)lysine

**Supplementary Table 2. Formulation process parameters for NHP trial formulations.**

Parameter	Description
Instrument	NanoAssemblr™ Blaze™, Cytiva
Mixer type	NxGen 500
hPep3/mRNA w/w	8.0
hPep3 in water	2.4 mg/ml
mRNA in water	0.3 mg/ml
Flow rate ratio (FRR)	1:1
Total flow rate (TFR)	100 ml/min
Output mRNA concentration	0.15 mg/ml
Tangential flow filtration (TFF) settings	<p><b>FLuc NPs</b> 150 ml/min, transmembrane pressure (TMP) 4 psi, shear rate <math>\sim 5500 \text{ sec}^{-1}</math> through a D02-E030-05-N (115 cm<sup>2</sup>) column with L/S 16 tubing.</p> <p><b>hEPO NPs</b> 150 ml/min, TMP 4 psi, shear rate <math>\sim 5500 \text{ sec}^{-1}</math> through a S02-E030-05-N (790 cm<sup>2</sup>) column with L/S 17 tubing.</p>
mRNA target concentration after TFF	$\geq 1.4 \text{ mg/ml}$
Dilution	1:1 with 2X HEPES buffered sucrose (SH)
Sterile filtration	0.22 $\mu\text{m}$ PES, Minisart®, 25 mm filter
mRNA and encapsulation efficiency quantification	RiboGreen assay with heparin treatment
mRNA dilution to target concentration	0.6 mg/ml, diluted with sterile 1X SH buffer (10 mM HEPES, 10% w/v sucrose, pH7.4)
Aliquotation and storage	5 ml aliquots stored at -80 °C
NP characterization	DLS, Cryo-EM, endotoxin testing, peptide concentration with BCA assay

**Supplementary Table 3. Final NHP trial formulation parameters**

	<b>hEPO</b>	<b>FLuc</b>
Peptide/mRNA w/w (final)	5,85	3,85
Starting mRNA amount	190 mg	40 mg
mRNA amount in formulation	148 mg	35 mg
Yield (mRNA)	78%	87%