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Supporting Information

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Folate Receptor-Mediated Delivery of Cas9 RNP for Enhanced Immune Checkpoint Disruption in Cancer Cells

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Supplementary Data

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1. Materials

1.1 Chemicals and solvents

Table S1. Chemicals and solvents in alphabetical order

Chemicals and solvents (abbreviations)	Manufacturer
1,2-Ethanedithiol (EDT)	Sigma-Aldrich, Munich, Germany
1,4-Dithiothreitol (DTT)	Sigma-Aldrich, Munich, Germany
1-Hydroxybenzotriazole (HOBt)	Sigma-Aldrich, Munich, Germany
2,5-Dihydroxybenzoic acid (DHB)	Sigma-Aldrich, Munich, Germany
2-Chlorotrityl chloride resin, 200–400 mesh, 1% DVB crosslinking	Iris Biotech, Marktredwitz, Germany
2-Hydroxy-5-methoxybenzoic acid	Sigma-Aldrich, Munich, Germany
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich, Munich, Germany
4′,6-Diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Munich, Germany
Acetonitrile (ACN)	VWR, Darmstadt, Germany
Agarose	Sigma-Aldrich, Munich, Germany
Aminoallyl-UTP-Cy3	Jena Bioscience, Jena, Germany
ATTO647N NHS-ester	ATTO-TEC, Siegen, Germany
(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (Pybop®)	Multisyntech, Witten, Germany
Chloramphenicol	Sigma-Aldrich, Munich, Germany
Chloroform-d	Sigma-Aldrich, Munich, Germany
Deuterium Oxide	Sigma-Aldrich, Munich, Germany
Dibenzocyclooctyne acid (DBCO)	Sigma-Aldrich, Munich, Germany
Dichloromethane (DCM)	Bernd Kraft, Duisburg, Germany
Dimethyl sulfoxide (DMSO)	Acros Organics, Geel, Belgium
Di-*tert*-butyl dicarbonate (Boc_2O)	Sigma-Aldrich, Munich, Germany
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich, Munich, Germany
Fatty acids	Sigma-Aldrich, Munich, Germany
Fetal bovine serum (FBS)	Life Technologies, Carlsbad, USA
N-α-Fmoc-*L*-glutamic acid α-2-phenylisopropyl ester (Fmoc-Glu-O-2-PhiPr)	VWR, Darmstadt, Germany
Fmoc-N-amido-dPEG24-acid	Quanta Biodesign, Powell, Ohio, USA
Fmoc-Stp(Boc_3)-OH building block	In-house synthesis[1]
Fmoc-α-amino acids	Iris Biotech, Marktredwitz, Germany
GelRed™	VWR, Darmstadt, Germany
Glucose	Sigma-Aldrich, Munich, Germany
Hydrazine monohydrate	Sigma-Aldrich, Munich, Germany
Hydrochloric acid solution (1 M) (1 M HCl)	Bernd Kraft, Duisburg, Germany
Imidazole	Sigma-Aldrich, Munich, Germany
Isopropyl β-*D*-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, Munich, Germany
Kanamycin	Sigma-Aldrich, Munich, Germany
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Munich, Germany

Methanol (MeOH)	Iris Biotech, Marktredwitz, Germany
Methyl *tert*-butyl ether (MTBE)	Brenntag GmbH, Essen, Germany
N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES)	Biomol GmbH, Hamburg, Germany
N,*N*-Diisopropylethylamine (DIPEA)	Iris Biotech, Marktredwitz, Germany
N,*N*-Dimethylformamide (DMF)	Iris Biotech, Marktredwitz, Germany
N^{10}-(Trifluoroacetyl)pteroic acid	Clauson-Kass A/S, Farum, Denmark
n-Hexane	Grüssing GmbH, Filsum, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, Munich, Germany
Penicillin	Biochrom, Berlin, Germany
Phenylmethylsulfonylfluorid (PMSF)	Carl Roth, Karlsruhe, Germany
Piperidine	Iris Biotech, Marktredwitz, Germany
Potassium chloride (KCl)	Sigma-Aldrich, Munich, Germany
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich, Munich, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, Munich, Germany
Sodium hydroxide (NaOH)	Sigma-Aldrich, Munich, Germany
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich, Munich, Germany
Streptomycin	Biochrom, Berlin, Germany
Sucrose	Sigma-Aldrich, Munich, Germany
Trifluoro acetic acid (TFA)	Iris Biotech, Marktredwitz, Germany
Triisopropylsilane (TIS)	Sigma-Aldrich, Munich, Germany
Triton X-100	Sigma-Aldrich, Munich, Germany
Trizma® base	Sigma-Aldrich, Munich, Germany

1.2 *In vitro* transcription templates and sgRNA sequences

The sgRNAs used for *in vitro* knockout experiments were synthesized by T7 *in vitro* transcription. The sgRNAs used for *in vivo* knockout experiments were chemically modified (2' O-methyl modification on the first 3 and last 3 RNA bases) and purchased from Integrated DNA Technologies (IDT, USA). The sequences of ssDNA oligonucleotides for assembly of dsDNA template, sgRNAs after *in vitro* transcription, and chemically modified sgRNAs are shown below.

Table S2. ssDNA oligonucleotides for assembly of dsDNA templates (5' → 3')

F-sgGFP	GCGGCCTCTAATACGACTCACTATAGGACCAGGATGGGCACCACCCGT TTTAGAGCTAGAAATAGCA
F-sgCtrl	GCGGCCTCTAATACGACTCACTATAGGGTAACCGTGCGGTCGTACGTT TTAGAGCTAGAAATAGCA
F-sgFRα	TTCTAATACGACTCACTATAGAGGGTTTAACAAGTGCGCAGGTTTTAG AGCTAGA
F-sgPD-L1_1	GCGGCCTCTAATACGACTCACTATAGGTCCAGCTCCCGTTCTACAGTTT TAGAGCTAGAAATAGCA
F-sgPD-L1_2	GCGGCCTCTAATACGACTCACTATAGTATGGCAGCAACGTCACGAGTT TTAGAGCTAGAAATAGCA
F-sgPD-L1_3	GCGGCCTCTAATACGACTCACTATAGCTTGCGTTAGTGGTGTACTGTTT TAGAGCTAGAAATAGCA
F-sgPD-L1_4	GCGGCCTCTAATACGACTCACTATAGACTTGTACGTGGTGGAGTAGTT TTAGAGCTAGAAATAGCA
F-sgPVR_1	GCGGCCTCTAATACGACTCACTATAGCTACAATTCGACAGGCGTCTGT TTTAGAGCTAGAAATAGCA
F-sgPVR_2	GCGGCCTCTAATACGACTCACTATAGCAGCCATGACAGCGGGGAGGTT TTAGAGCTAGAAATAGCA
F-sgPVR_3	GCGGCCTCTAATACGACTCACTATAGCTTCTAATCTCCACCGTAGGTTT TAGAGCTAGAAATAGCA
R-sgRNA	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTA GCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Table S3. sgRNAs after *in vitro* transcription (5' → 3')

sgGFP	GACCAGGAUGGGCACCACCCGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgCtrl	GGGUAACCGUGCGGUCGUACGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgFRα	GGGUUUAACAAGUGCGCAGUGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgPD-L1_1	GGUCCAGCUCCCGUUCUACAGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgPD-L1_2	GUAUGGCAGCAACGUCACGAGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU

sgPD-L1_3	GCUUGCGUUAGUGGUGUACUGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgPD-L1_4	GACUUGUACGUGGUGGAGUAGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgPVR_1	GCUACAAUUCGACAGGCGUCUGUUUUAGAGCUAGAAAUAGCAAGUU AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUC GGUGCUUUUUUU
sgPVR_2	GCAGCCAUGACAGCGGGGAGGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU
sgPVR_3	GCUUCUAAUCUCCACCGUAGGUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGCUUUUUUU

Table S4. Chemically modified sgRNAs obtained from commercial source (5' → 3')

sgCtrl	mG*mG*mG*rUrArArCrCrGrUrGrCrGrGrUrCrGrUrArCrGrUrUrUrUrArGrArG rCrUrArGrArArArUrArGrCrArArGrUrUrArArArArArUrArArGrGrCrUrArGrUrCr CrGrUrUrArUrCrArArCrUrUrGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrG rGrUrGrCmU*mU*mU*rU
sgPD-L1_4	mG*mA*mC*rUrUrGrUrArCrGrUrGrGrUrGrGrArGrUrArGrUrUrUrUrArGrArGr GrCrUrArGrArArArUrArGrCrArArGrUrUrArArArArArUrArArGrGrCrUrArGrUr CrCrGrUrUrArUrCrArArCrUrUrGrArArArArArGrUrGrGrCrArCrCrGrArGrUrC rGrGrUrGrCmU*mU*mU*rU
sgPVR_3	mG*mC*mU*rUrCrUrArArUrCrUrCrCrArCrCrGrUrArGrGrUrUrUrUrArGrArG rCrUrArGrArArArUrArGrCrArArGrUrUrArArArArArUrArArGrGrCrUrArGrUrCr CrGrUrUrArUrCrArArCrUrUrGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrG rGrUrGrCmU*mU*mU*rU

1.3 PCR primers

Table S5. Sequences of PCR primers (5' → 3')

PD-L1-F	TGGTTCCTTTTAAACAAGACTGGG
PD-L1-R	CGCACCACCGTAGCTGATTA
PVR-F	AGAGCCCTGGTATCGGATCT
PVR-R	ACCCACATCCCACTGCTAAG
FRα-F	GACCATGGAGCAGGAACC
FRα-R	CAGCTCCAGTTCTATTCGG

1.4 Buffers and solutions

Deionized water was purified in-house using a Millipore system (Simplicity Plus, Millipore Corp.) and was used for all experiments.

Kaiser test solutions: 80 % (w/v) phenol in EtOH; 5 % (w/v) ninhydrine in EtOH; 20 μM KCN in pyridine (2 mL of 1 mM KCN (aq) in 98 mL of pyridine).[1]

HEPES buffered glucose (HBG): 20 mM HEPES, 5 % w/v glucose, pH 7.4.

Phosphate buffered saline (PBS): 136.89 mM sodium chloride, 2.68 mM potassium chloride, 8.10 mM sodium phosphate dibasic heptahydrate, 1.47 mM potassium dihydrogen phosphate, pH 7.4.

Tris/Borate/EDTA (TBE) 10 × buffer: 1.49 M tris base, 0.89 M boric acid, 20 mM EDTA, pH 8.0.

FACS buffer: PBS buffer containing 10 % FBS, pH 7.4.

2. Methods

2.1 Synthesis of hydroxystearic acid (OHSteA)

The synthesis route is shown in Figure S1. Firstly, oleic acid (2 g, 7.08 mmol) was dissolved in TFA/CH_2Cl_2 (2:1, 30 mL) and stirred for 3 days at room temperature. Afterwards, the solvent was removed by a nitrogen flow to obtain hydroxystearic acid TFA ester (2.67 g, 6.73 mmol, yield: 95.1 %). Secondly, the hydroxystearic acid TFA ester (2.67 g, 6.73 mmol) was dissolved in 1 M NaOH methanol/water (4:1, 30 mL) mixture and stirred overnight. 1 M HCl solution was added to the reaction to adjust pH to 1-2 and then the mixture was centrifuged. The supernatant was collected and evaporated to remove the solvent. The obtained light brown powder was dissolved in water and extracted 3 times with DCM. The DCM phase was combined and evaporated to generate the white powder hydroxystearic acid (1.58 g, 5.26 mmol, yield: 78.2%).

2.2 Synthesis of DBCO agents

FolA-PEG_{24}-DBCO and PEG_{24}-DBCO were synthesized using Fmoc-Lys(Dde)-OH and Fmoc-N-amido-dPEG_{24}-acid preloaded resin, respectively.[2] For FolA-PEG_{24}-DBCO, Fmoc-Glu-O-2-PhiPr and N^{10}-(trifluoroacetyl)pteroic acid were coupled in sequence to generate folic acid. Then, a Dde deprotection step was carried out as described above. To deprotect the trifluoroacetyl group of pteroic acid, the resin was incubated 4 times with 25 % aqueous ammonia solution/DMF (1:1, 5 mL g^{-1} resin) for 30 min. Afterwards, Fmoc-dPEG_{24}-OH was introduced followed by the coupling of DBCO acid. The resin was washed 3 times with DMF and DCM sequentially and dried in vacuo. DBCO agents were cleaved off the resin by incubation with pre-cooled cleavage cocktail DCM/TFA/TIS (92.5:5:2.5, 10 mL g^{-1} resin) for 60 min. The solution was immediately precipitated in pre-cooled MTBE/n-hexane (2:8, 45 mL). Afterwards, the precipitate was dissolved in 5 mL of 0.05 M NaOH solution, and the pH was adjusted to 7. The agents were purified by dialysis using a dialysis membrane (RC, MWCO: 1000 Da) against deionized water for 48 h. Deionized water was changed every 8 hours. The solution was snap frozen and lyophilized to obtain the final products.

2.3 Analytical methods

Proton ^{1}H NMR spectroscopy

All ^{1}H NMR spectra were recorded on an Advance III HD Bruker BioSpin (400 MHz) equipped with a Cryo Probe™ Prodigy probe head without TMS. The chemical shifts were calibrated to

the residual solvent signal and were reported in parts per million (ppm). The spectra were processed in MestReNova software (MestReLab Research, SL). Integration was performed manually.

MALDI mass spectrometry

Super-DHB consisting of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (9:1, m/m) in H_2O/ACN/TFA (69.93:30:0.07) was prepared as matrix solution. 1 µL of the solution was spotted on an MTP AnchorChip (Bruker Daltonics, Bremen, Germany). After drying of the matrix, 1 µL of sample solution (1 mg/mL in water) was added to the dried matrix. All mass spectra were recorded on an Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) in the positive ion mode.

2.4 ATTO647N-Cas9 preparation

ATTO647N-Cas9 was prepared as described previously.[3] In brief, Cas9 protein was diluted in HEPES (20 mM, pH 7.4) to a concentration of 1 mg/mL, and the pH was adjusted to 8.0 using 0.2 M sodium bicarbonate solution. Afterwards, 1.5-fold molar excess of ATTO647N NHS-ester dissolved in DMSO (10 mM) was added to the Cas9 solution. The resulting solution was stirred at RT for 1 h. ATTO647N-labelled Cas9 was purified with an Äkta purifier system (GE Healthcare Bio-Sciences AB, Sweden) using storage buffer (20 mM HEPES, 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) as the solvent. The fractions containing ATTO647N-Cas9 were combined, and the concentration of ATTO647N-Cas9 was measured with a Nanodrop photometer (Thermo Scientific, USA) using an extinction coefficient of $\varepsilon/1.000 = 120\ M^{-1}\ cm^{-1}$. The ATTO647N-Cas9 solution was aliquoted and stored at −80 °C.

2.5 *In vitro* transcription of sgRNAs

In vitro transcribed sgRNAs were prepared as described previously.[3] In brief, two single-stranded DNA (ssDNA) oligonucleotides were annealed, extended using T4 DNA polymerase (NEB, Germany), and then purified with a QIAquick PCR Purification Kit (QIAGEN, Germany). The obtained DNA template was transcribed using the HiScribe T7 High Yield RNA Synthesis Kit (NEB, Germany) followed by the purification of the generated sgRNA with a RNeasy Micro Kit (QIAGEN, Germany). The purified sgRNA was heated to 80 °C for 2 min, immediately snap frozen, and stored at −80 °C. Sequences of the ssDNA oligonucleotides and sgRNAs can be found in Table S2 and S3, respectively.

2.6 Transmission electron microscopy (TEM)

Unmodified, FolA-PEG_{24}-DBCO or PEG_{24}-DBCO modified Cas9 RNP nanocarriers (modification ratio 0.75 eq) were formulated in distilled water. Hydrophilized carbon coated copper grids (Ted Pella, Redding, CA, USA, 300 mesh, 3.0 mm OD) were placed on top of 10 µL sample droplets for 20 s. Subsequently, the sample solution was removed using a filter paper, stained with 1.0 % uranyl formate in water, and allowed to dry for 20 min. All samples were analyzed using a JEM-1100 electron microscope (JEOL, Tokyo, Japan) with 80 kV acceleration voltage.

2.7 Ribogreen assay

Encapsulation efficiency of Cas9/sgRNA RNP complexes was determined using a Quant-iT™ RiboGreen® RNA assay kit (Invitrogen, CA, USA). 50 µL of RNP nanocarriers containing 250 ng sgRNA was mixed with 50 µL of TE buffer in the presence or absence of 2 % (v/v) Triton X-100 (Sigma Aldrich, Gillingham, UK). A standard curve of the sgRNA was prepared in TE buffer as well. Afterwards, the samples were added to a 96-well plate followed by incubation for 10 min at 37 °C. Subsequently, 100 µL of Ribogreen solution (diluted 1:100 in TE buffer) was added to each well. The plate was incubated for another 5 min. Ribogreen fluorescence intensity of each well was detected using a microplate reader (Spectrafluor Plus, Tecan, Männedorf, Switzerland) with excitation and emission wavelength of 485 and 528 nm, respectively. The sgRNA concentration of RNP nanocarriers with or without Triton X-100 lysis was calculated based on the sgRNA standard curve. After subtraction of the blank wells, the encapsulation efficiency (in percentage) was calculated as (1 − (nanocarriers without Triton / nanocarriers with Triton)) × 100 %. All studies were performed in triplicates.

2.8 Agarose gel shift assay

A 2 % (w/v) agarose gel containing GelRed® (Biotium, Hayward, CA, USA) was prepared using 1 × TBE buffer. 20 µL of Cas9 RNP nanocarriers at a concentration of 75 nM RNP in HBG buffer was mixed with 4 µL of 6 × loading buffer followed by sample loading in the gel pockets. The electrophoresis was carried out at 100 V for 75 min.

2.9 Long-term stability

Cas9 RNP nanocarriers with or without ligand modification (0.75 eq) were prepared. Afterwards, the nanocarriers were aliquoted and directly stored at different temperatures (4 °C,

-20 °C, or -80 °C), or first lyophilized and then stored at -80 °C. At determined time points, 100 μL of RNP nanocarriers was taken or reconstituted for size and zeta potential measurements.

2.10 Cell viability assay (MTT) after eGFP reporter gene knockout

MTT assay was performed to determine the cell viability after treatments of CT26 eGFP/luc, HeLa eGFP/tub, or FRα-knockout HeLa eGFP/tub cells with Cas9 RNP nanocarriers. After 48 h total incubation, 10 μL of MTT (5 mg/mL) was added to each well. The cells were incubated for 2 h in the incubator. Afterwards, the medium was discarded, and the plates were frozen at –80 °C overnight. 100 μL of DMSO was then added to each well to dissolve the formed formazan product. The plates were incubated for 30 min at 37 °C under shaking. The absorbance of each well was measured at 590 nm with background correction at 630 nm using a microplate reader (Tecan Spark 10M, Tecan, Männedorf, Switzerland). The relative cell viability (in percentage) was calculated relative to control wells treated with HBG buffer as ([A] test/[A] control) × 100 %. All experiments were conducted in triplicate.

2.11 Endocytosis pathway study

Endocytosis pathways of Cas9 RNP nanocarriers with or without ligand modification were studied by using different endocytosis inhibitors. One day prior to the treatments, CT26 and HeLa WT cells were seeded into 24-well plates at a density of 25000 cells/well. On the next day, the medium was replaced with 500 μL of serum-free medium containing different endocytosis inhibitors (10 μM chlorpromazine, 54 μM nystatin, or 1 mM amiloride). The cells were incubated at 37 °C for 2 h. Afterwards, the medium was replaced with 375 μL of fresh medium. 125 μL of RNP nanocarriers containing 20 % of Cy3-labeled sgRNA were added to each well resulting in a final concentration of 75 nM Cas9 RNP. The cells were then incubated for 4 h. For 4 °C group, the cells were firstly placed on ice for 2 h and then treated with Cas9 RNP nanocarriers in ice-cold medium at the same RNP dose as above. Subsequently, the medium was discarded and 4000 IU/mL heparin in PBS were added. The cells were incubated on ice for 30 min. Afterwards, the cells were collected and prepared for flow cytometry analysis. The relative cellular uptake (in percentage) was calculated relative to control cells without the treatment of inhibitors as ([MFI] test/[MFI] control) × 100 %. MFI: median fluorescence intensity. All experiments were performed in triplicate.

2.12 Determination of PD-L1 gene knockout by flow cytometry

One day prior to the treatments, CT26 WT cells were seeded into 96-well plates at a density of 5000 cells/well. On the next day, the medium in each well was replaced with 75 μL of fresh medium. For the comparison of sgRNAs targeting PD-L1, RNP nanocarriers containing different sgPD-L1 sequences were prepared with or without ligand modification (0.75 eq). The sequences of sgPD-L1 are shown in Table S3. 25 μL of RNP nanocarriers was added into each well resulting in a final concentration of 75 nM RNP complexes. For the dose titration experiments, RNP nanocarriers containing sgPD-L1_4 were formulated and then diluted to prepare a series of samples with different concentrations of RNP complexes. 25 μL of the dilutions was added to each well resulting in final concentrations of RNP ranging from 1 nM to 100 nM. After 4 h incubation, the medium in each well was replaced with 100 μL of fresh medium and the cells were further incubated for 44 h. Afterwards, the cells were trypsinized and transferred to the 24-well plates. The cells were incubated for another 3 days. On the day before the flow cytometry measurements, 100 ng/mL interferon-γ (IFN-γ, BioLegend, CA, USA) was added to stimulate PD-L1 expression. The cells were harvested and resuspended in 100 μL of FACS buffer. 2 μL of APC anti-mouse PD-L1 antibody (BioLegend, CA, USA) was added to each sample. The samples were incubated for 60 min on ice in the dark followed by washing with PBS. The samples were resuspended in 600 μL of FACS buffer and analyzed by flow cytometry as described above. The APC fluorescence was detected with 633 nm excitation and 660 nm emission. All experiments were performed in triplicate.

2.13 Cell viability assay (MTT) after PD-L1 gene knockout

MTT assay was performed to determine the cell viability after treatments of CT26 WT cells with Cas9/sgPD-L1 RNP nanocarriers. Treatments were performed and MTT assay was carried out as described above. The relative cell viability (in percentage) was calculated relative to control wells treated with HBG buffer as ([A] test/[A] control) × 100 %. All experiments were performed in triplicate.

2.14 Determination of PD-L1 gene knockout by CLSM

One day prior to the CLSM experiments, CT26 WT cells treated with Cas9/sgPD-L1_4 RNP nanocarriers (75 nM RNP) were seeded in 8-well Ibidi μ-slides (Ibidi GmbH, Germany) at a density of 30000 cells/well. 100 ng/mL IFN-γ was added to stimulate PD-L1 expression. On the next day, the medium was replaced with 300 μL of fresh medium. 2 μL of APC anti-mouse PD-L1 antibody was added to each well. The slides were incubated for 60 min on ice in the

dark. Afterwards, the cells were prepared for CLSM as described above. APC emission was recorded at 660 nm.

2.15 Sanger sequencing and TIDE analysis of PD-L1 gene edits

Limiting dilution was performed to generate single cell clones from Cas9/sgPD-L1_4 RNP treated CT26 WT cells. Genomic DNA of the clones was extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The sgPD-L1_4 target regions of PD-L1 gene were amplified using OneTaq DNA polymerase (NEB, Frankfurt am Main, Germany) with the PD-L1-F and PD-L1-R primers (see Table S5). The PCR products were gel purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Purified products at concentrations of 5 - 10 ng/µL were sequenced by Eurofins GATC Biotech (Germany) with the primer PD-L1-F. For the TIDE analysis, genomic DNA of the cells was extracted 5 days after the treatment. The samples were processed as described above. The raw sequence chromatograms were analyzed by TIDE analysis (https://tide.deskgen.com/). The Indel size range was set to 25 bp, and the default values for the other parameters were used.

2.16 Cell viability assay (MTT) after PVR gene knockout

One day prior to the treatments, CT26 WT cells were seeded into 96-well plates at a density of 5000 cells/well. On the next day, the medium in each well was replaced with 75 µL of fresh medium. RNP nanocarriers containing different sgPVR sequences and sgCtrl were prepared with or without ligand modification (0.75 eq). The sequences of sgPVR and sgCtrl are shown in Table S3. 25 µL of RNP nanocarriers was added into each well resulting in a final concentration of 75 nM RNP complexes. After 4 h, the medium in each well was replaced with 100 µL of fresh medium and the cells were incubated for 44 h. Afterwards, MTT assay was performed as described above. The relative cell viability (in percentage) was calculated relative to control wells treated with HBG buffer as ([A] test/[A] control) × 100 %. All experiments were performed in triplicate.

2.17 Apoptosis assay after PVR gene knockout

Assessment of cell apoptosis after PVR gene knockout was performed using an Annexin V-FITC Apoptosis Detection Kit (BioVision, USA). CT26 WT cells were seeded into 24-well plates at a density of 50000 cells/well 24 h prior to the treatments. On the next day, the medium in each well was replaced with 375 µL of fresh medium. 125 µL of RNP nanocarriers containing sgPVR_3 or sgCtrl with or without ligand modification (0.75 eq) was added to each well

resulting in a final concentration of 75 nM RNP complexes. After 4 h, the medium in each well was replaced with 500 μL of fresh medium and the cells were incubated for 20 h. Afterwards, the cells were harvested and resuspended in 500 μL of 1 × Annexin V-FITC binding buffer, followed by the addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI, 50 μg/mL). The samples were incubated in the dark for 5 min at RT and then analyzed by flow cytometry. The FITC fluorescence was detected with 488 nm excitation and 520 nm emission. The PI fluorescence was assayed with 488 nm excitation and 640 nm emission. All experiments were performed in triplicate.

2.18 Apoptosis assay after treatments with apoptosis inducers

Assessment of cell apoptosis after treatments with different apoptosis inducers was carried out using the same detection kit as described above. CT26 WT cells were seeded into 24-well plates at a density of 50000 cells/well one day prior to the treatments. On the next day, the medium in each well was replaced with 500 μL of fresh medium containing different apoptosis inducing agents (10 μM doxorubicin, 10 μM etoposide, or 35 μM cycloheximide). The cells were incubated for 24 h. Afterwards, the cells were collected and prepared for flow cytometry analysis as described above.

2.19 Determination of PVR expression after treatments with apoptosis inducers

PVR expression of CT26 WT cells after treatments with different apoptosis inducers was detected by flow cytometry. The treatments were performed as described in section 2.18. Afterwards, the cells were harvested and then stained with PE anti-mouse PVR antibody (BioLegend, San Diego, CA, USA) for flow cytometry analysis. The PE fluorescence was detected with 488 nm excitation and 615 nm emission.

2.20 Cellwatcher study

CT26 WT cells were seeded into 48-well plates at a density of 20000 cells/well 24 h prior to the treatments. On the next day, the medium in each well was replaced with 150 μL of fresh medium. The dynamics of cell growth was real-time monitored up to 114 h using a Cellwatcher device (PHIO, Germany). At 1 h after recording, 50 μL of RNP nanocarriers containing sgPVR_3 or sgCtrl with or without ligand modification (0.75 eq) was added to each well resulting in a final concentration of 75 nM RNP complexes. The cell growth curves were generated based on the automated detection of cells and analysis algorithms.

2.21 Determination of PVR gene knockout by flow cytometry

One day prior to the treatments, CT26 WT cells were seeded into 24-well plates at a density of 50000 cells/well. On the next day, the medium in each well was replaced with 375 µL of fresh medium. 125 µL of RNP nanocarriers (75 nM RNP) containing sgPVR_3 or sgCtrl with or without ligand modification (0.75 eq) was added to each well. After 4 h, the medium in each well was replaced with 500 µL of fresh medium. On day 3 or day 4 after the treatments, the samples were prepared and analyzed using PE anti-mouse PVR antibody (BioLegend, San Diego, CA, USA). The PE fluorescence was detected with 488 nm excitation and 615 nm emission. All experiments were performed in triplicate.

2.22 Determination of PVR gene knockout by CLSM

CT26 WT cells were seeded in 8-well Ibidi µ-slides (Ibidi GmbH, Germany) at a density of 50000 cells/well 24 h prior to the treatments. On the next day, the medium was replaced with 225 µL of fresh medium. 75 µL of RNP nanocarriers (25 nM RNP) containing sgPVR_3 was added to each well. After 4 h, the medium in each well was replaced with 300 µL of fresh medium. The cells were further incubated for 3 days. Afterwards, the medium was replaced with 300 µL of fresh medium. 2 µL of PE anti-mouse PVR antibody (BioLegend, San Diego, CA, USA) was added to each well. The slides were incubated for 60 min on ice in the dark. Subsequently, the cells were washed, fixed, stained and imaged as described above. PE fluorescence was detected with 488 nm excitation and emission was recorded at 615 nm.

2.23 Colony formation assay

CT26 WT cells were seeded in 24-well plates at a density of 25000 cells/well 24 h prior to the treatments. On the next day, the medium was replaced with 375 µL of fresh medium. 125 µL of RNP nanocarriers (75 nM) containing sgPVR_3 or sgCtrl with or without ligand modification (0.75 eq) was added to each well. After 4 h, the cells were typsinized and counted using a cell counter (Z2 Coulter, Beckman Coulter, CA, USA). Afterwards, 250 treated cells were immediately re-plated in the 12-well plates and incubated in the incubator. The medium in each well was replaced with fresh medium every 3 days. After 3 weeks, the colonies were fixed with 4 % PFA, stained with crystal violet (0.5 % w/v) and counted under a microscope. At least 50 cells were counted as a colony. The relative colony formation ratio (in percentage) was calculated relative to control wells treated with HBG as (colony number of test / colony number of control) × 100 %. All experiments were performed in triplicate.

2.24 Sanger sequencing and TIDE analysis of PVR gene edits

Genomic DNA of CT26 WT cells was extracted 3 days after the treatments with Cas9/sgPVR_3 RNP nanocarriers using a QIAamp DNA Mini Kit. The sgPVR_3 target regions of PVR gene were amplified using PVR-F/PVR-R primers (see Table S5), gel purified, sequenced and analyzed by TIDE analysis as described above.

3. Supporting Figures

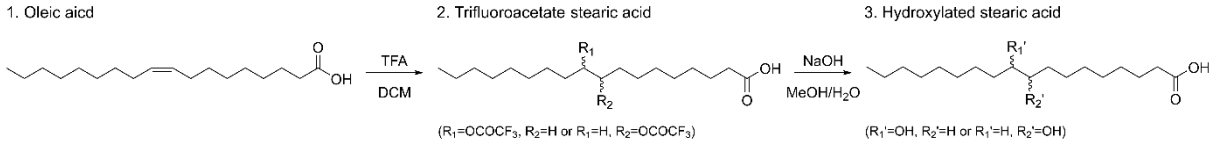


Figure S1. Synthetic route to 9- or 10-hydroxystearic acid.

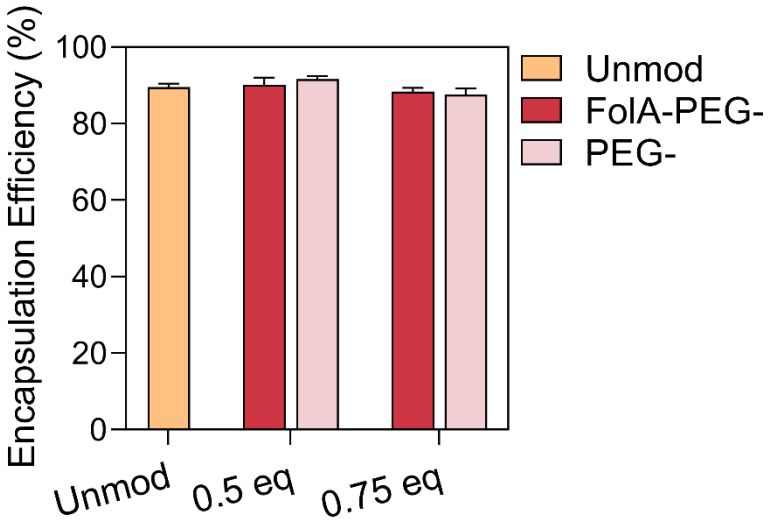


Figure S2. Cas9/sgRNA RNP encapsulation efficiency of nanocarriers at FolA-PEG- or PEG- to oligomer ratios of 0, 0.5, and 0.75 eq. Data were obtained by Ribogreen assay and are presented as mean ± SD (n=3).

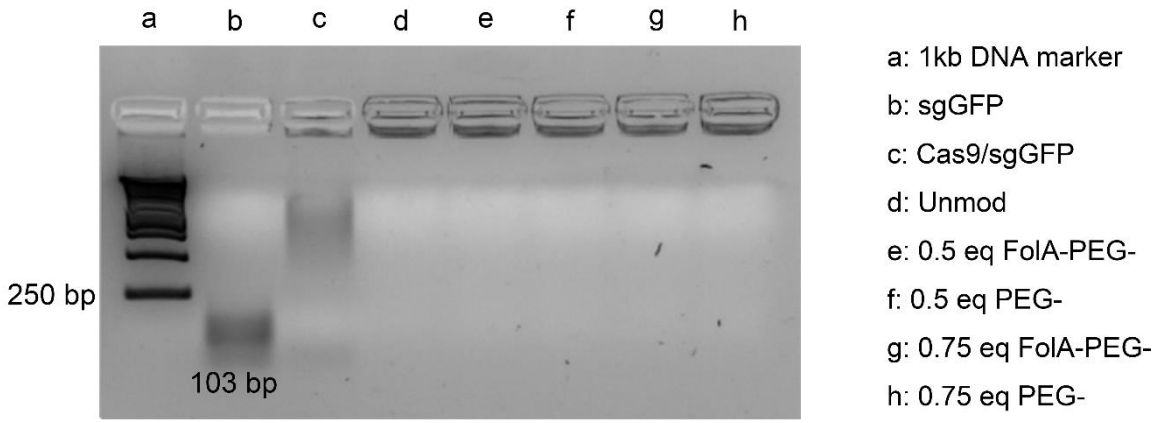


Figure S3. Agarose gel shift assay of Cas9 RNP nanocarriers. Different formulations of Cas9 RNP nanocarriers with or without 0.5 eq or 0.75 eq of ligand modification were prepared using 1.25 μg Cas9 protein and 0.25 μg sgGFP. Free sgGFP, and free Cas9/sgGFP RNP were used as controls.

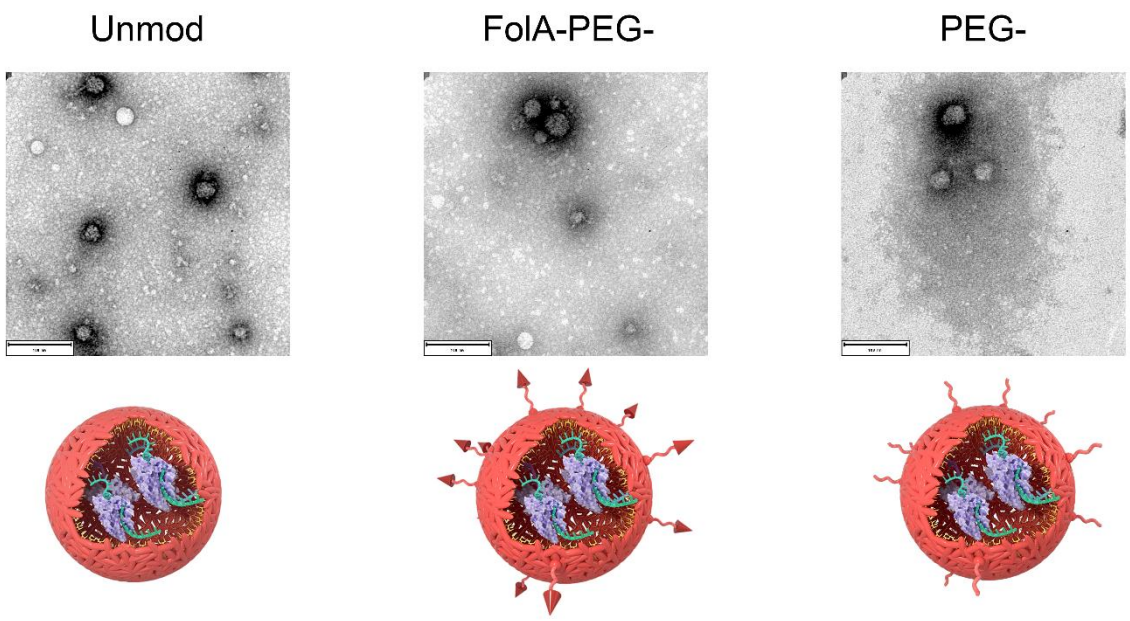


Figure S4. Transmission electron microscopy (TEM) images of 3 different formulations of Cas9 RNP nanocarriers. Scale bar: 100 nm.

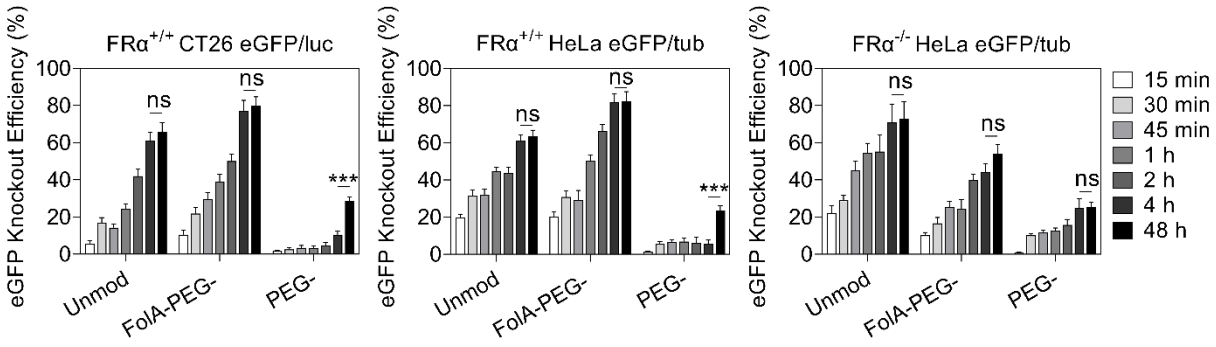


Figure S5. eGFP knockout efficiency of Cas9 RNP nanocarriers incubated for different times in CT26 eGFP/luc cells (0.75 eq modification), HeLa eGFP/tub or FRα-knockout HeLa eGFP/tub cells (0.5 eq modification). Cas9/sgGFP RNP was used at 75 nM. Evaluation was performed 5 days after the treatments.

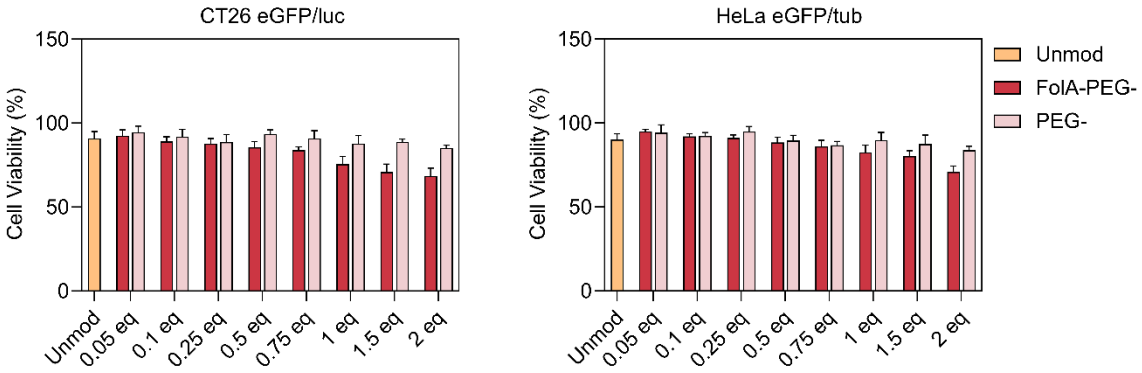


Figure S6. Viability of CT26 eGFP/luc and HeLa eGFP/tub cells after treatments with Cas9/sgGFP RNP nanocarriers with or without ligand modification at different equivalents. CT26 eGFP/luc and HeLa eGFP/tub cells were treated with the Cas9 RNP nanocarriers at the RNP dose of 75 nM for 4 h. Metabolic activity of the cells was determined using a MTT assay 48 h after transfection. Data are presented as % cell viability with respect to the control cells ± SD (n=3).

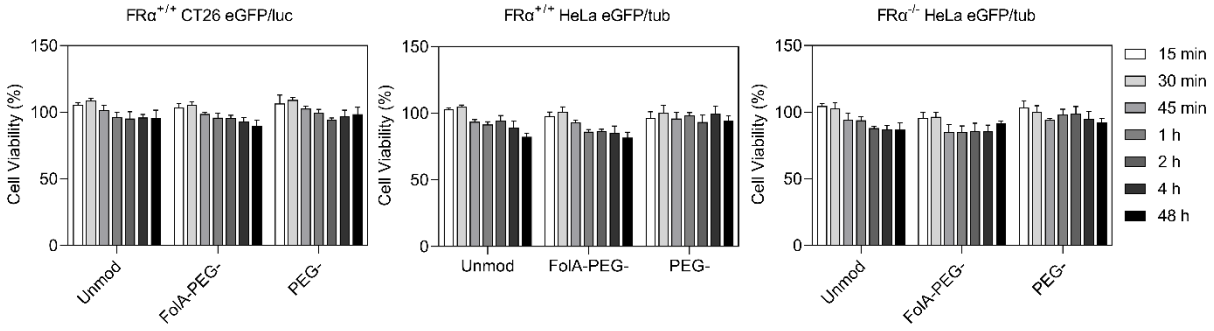


Figure S7. Viability of CT26 eGFP/luc, HeLa eGFP/tub, and FRα-knockout HeLa eGFP/tub cells after treatments with Cas9/sgGFP RNP nanocarriers with or without ligand modification (0.75 eq for CT26 cells and 0.5 eq for HeLa cells) for different times. CT26 eGFP/luc, HeLa eGFP/tub, and FRα-knockout HeLa eGFP/tub cells were treated with the Cas9 RNP nanocarriers at a RNP dose of 75 nM for 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, or 48 h. Metabolic activity of the cells was determined using a MTT assay 48 h after transfection. Data are presented as % cell viability with respect to the control cells ± SD (n=3).

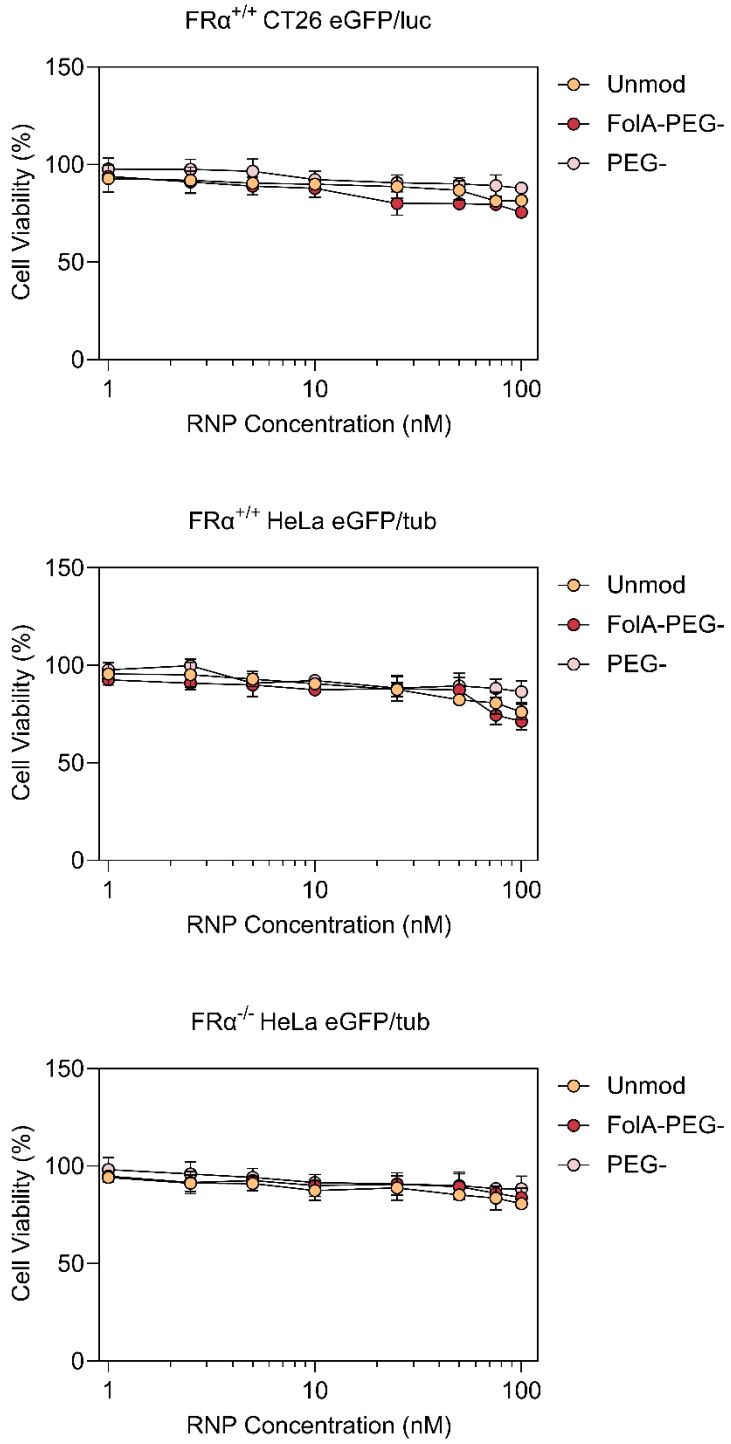


Figure S8. Viability of CT26 eGFP/luc, HeLa eGFP/tub, and FRα-knockout HeLa eGFP/tub cells after treatments with Cas9/sgGFP RNP nanocarriers with or without ligand modification (0.75 eq for CT26 cells and 0.5 eq for HeLa cells) at different RNP doses. CT26 eGFP/luc, HeLa eGFP/tub, and FRα-knockout HeLa eGFP/tub cells were treated with the Cas9 RNP nanocarriers at RNP doses ranging from 1 nM to 100 nM for 4 h. Metabolic activity of the cells was determined using a MTT assay 48 h after transfection. Data are presented as % cell viability with respect to the control cells ± SD (n=3).

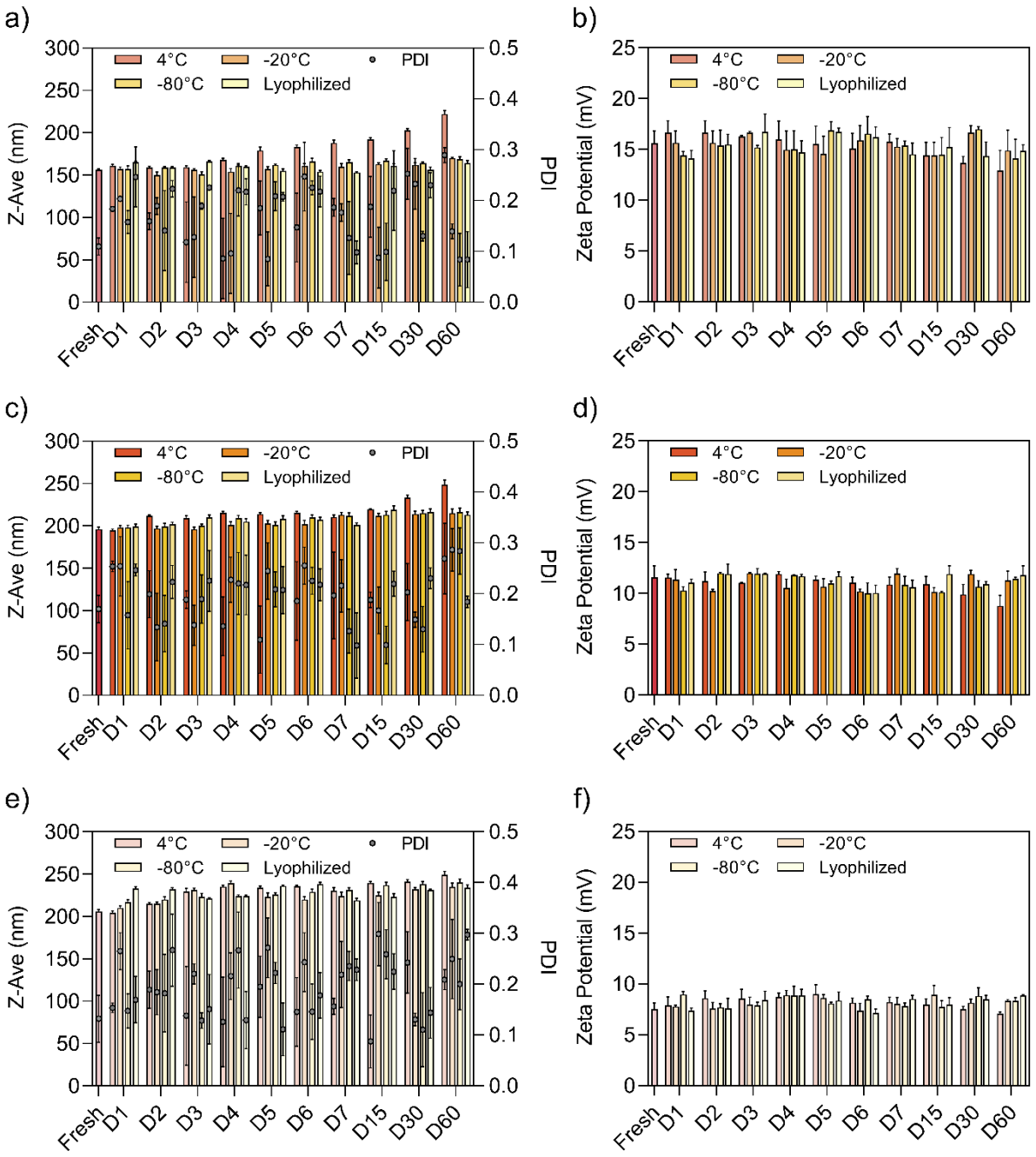


Figure S9. Long-term storage stability of nanoparticles determined by dynamic light scattering (DLS). a) Hydrodynamic particle size (Z-average) and polydispersity index (PDI) of a) unmodified RNP nanocarriers, c) FolA-PEG RNP nanocarriers, and e) PEG RNP nanocarriers. Zeta potential of b) unmodified RNP nanocarriers, d) FolA-PEG RNP nanocarriers, and f) PEG RNP nanocarriers. Three technical replicates were measured.

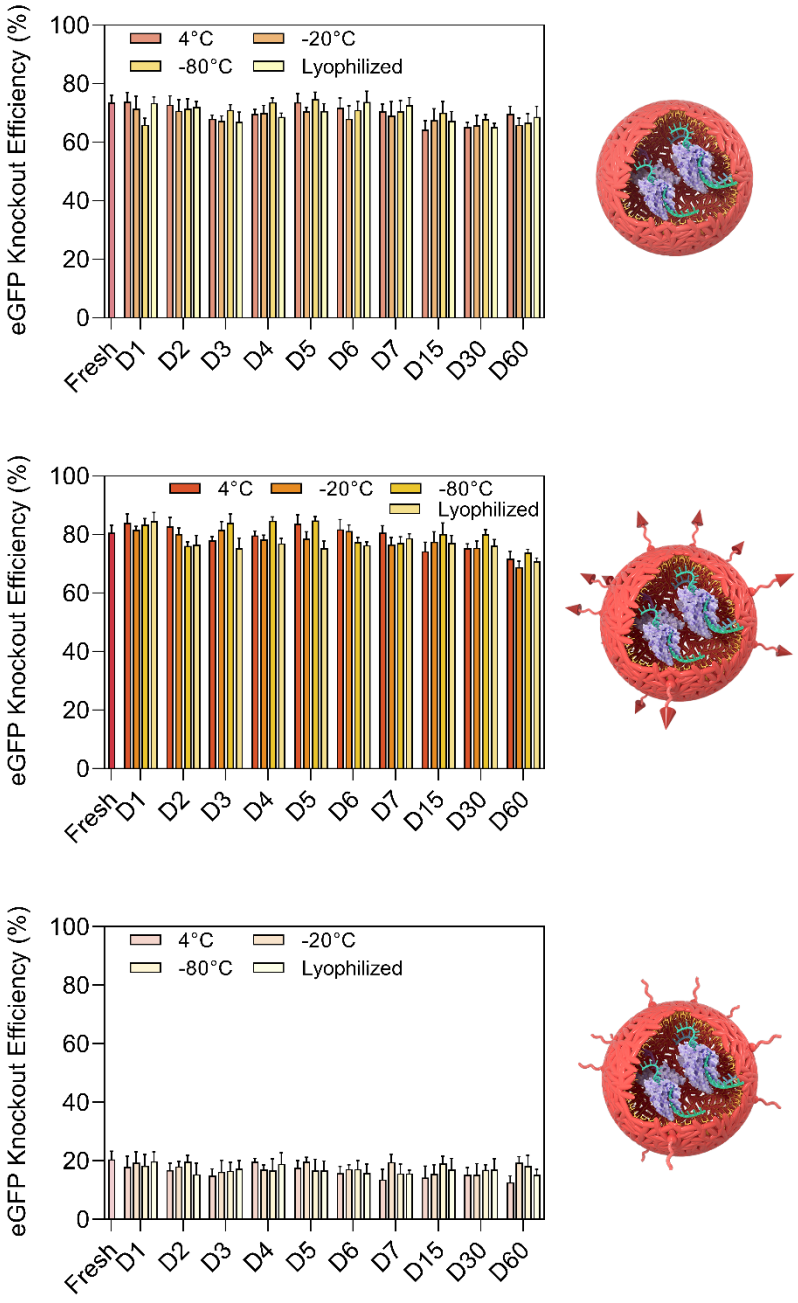


Figure S10. Effect of different long-term storage conditions on the eGFP knockout efficiency in HeLa eGFP/tub cells. Cas9/sgGFP RNP nanocarriers with or without 0.5 eq of ligand modification were prepared and stored under four conditions (4 °C, -20 °C, -80 °C or -80 °C after lyophilization) before use. HeLa eGFP/tub cells were incubated with the Cas9 RNP nanocarriers at the RNP dose of 75 nM for 4 h after different storage times. Flow cytometry was conducted 48 h after transfection. From top to bottom: unmodified nanocarriers, FolA-PEG nanocarriers, and PEG nanocarriers. Data are presented as the mean ± SD (n=3).

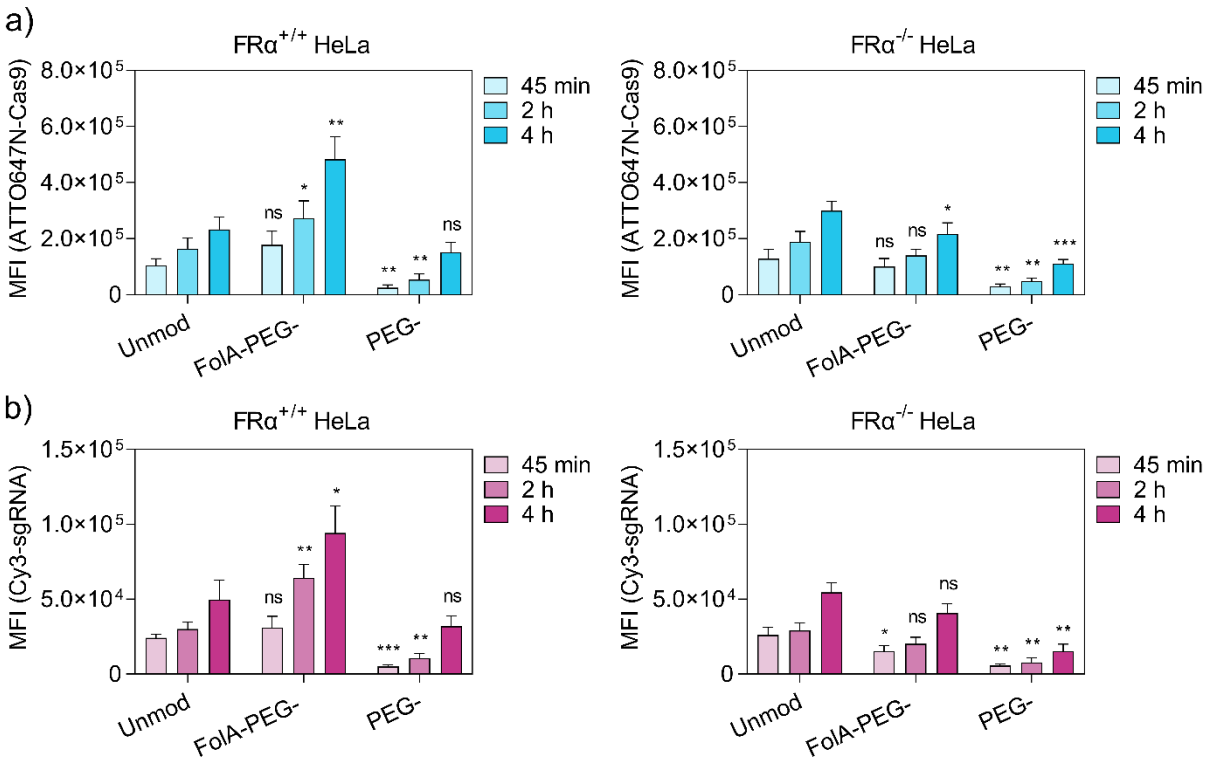


Figure S11. Cellular uptake of Cas9 RNP nanocarriers with or without ligand modification (0.5 eq) determined by flow cytometry. FRα-positive or FRα-knockout HeLa cells were incubated with the Cas9 RNP nanocarriers containing 20 % ATTO647N-labeled Cas9 protein and 20 % Cy3-labeled sgRNA at a RNP dose of 75 nM. Flow cytometry was conducted 45 min, 2 h, or 4 h after RNP treatment. a) Comparison of the measured median fluorescence intensities (MFI) of Cas9 protein. b) Comparison of the measured median fluorescence intensities (MFI) of sgRNA. The data are presented as the mean ± SD (n=3).

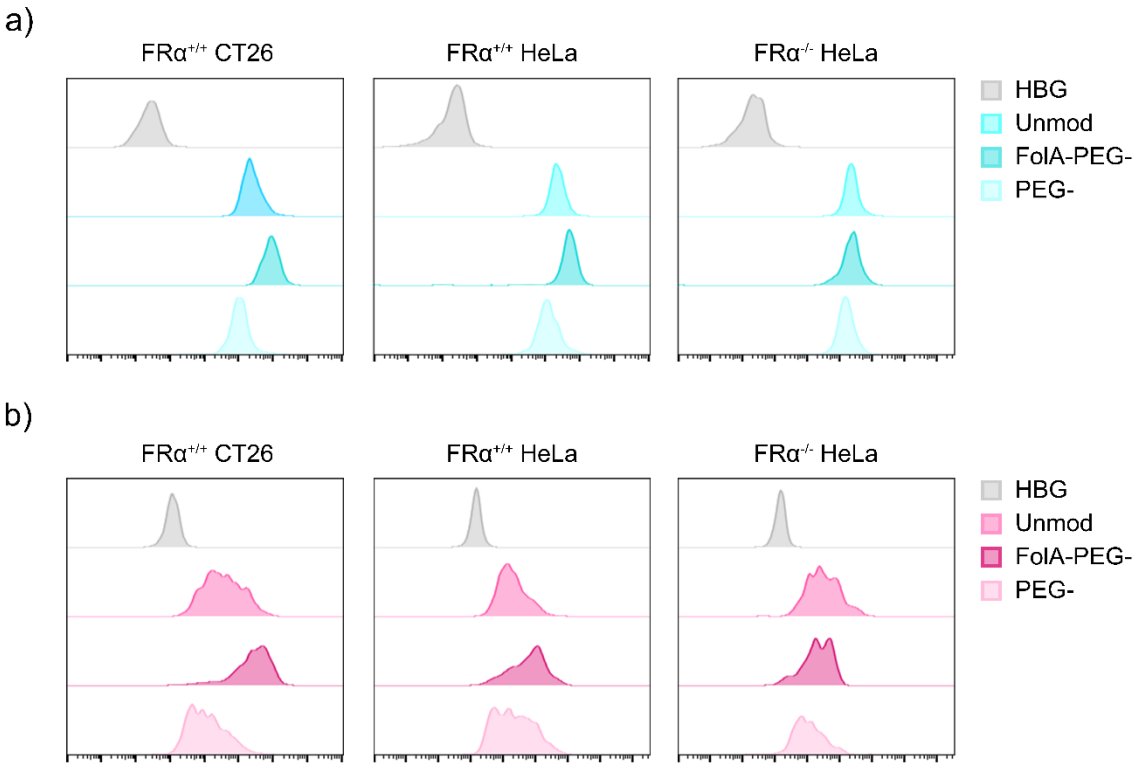


Figure S12. Cellular uptake of Cas9 RNP nanocarriers with or without ligand modification (0.75 eq for CT26 cells and 0.5 eq for HeLa cells) determined by flow cytometry. CT26, HeLa, and FRα-knockout HeLa cells were incubated with the Cas9 RNP nanocarriers containing 20 % ATTO647N-labeled Cas9 protein and 20 % Cy3-labeled sgRNA at the RNP dose of 75 nM. Flow cytometry was conducted 4 h after RNP transfection. a) Flow cytometry histograms of Cas9 protein uptake of each sample. b) Flow cytometry histograms of sgRNA uptake of each sample.

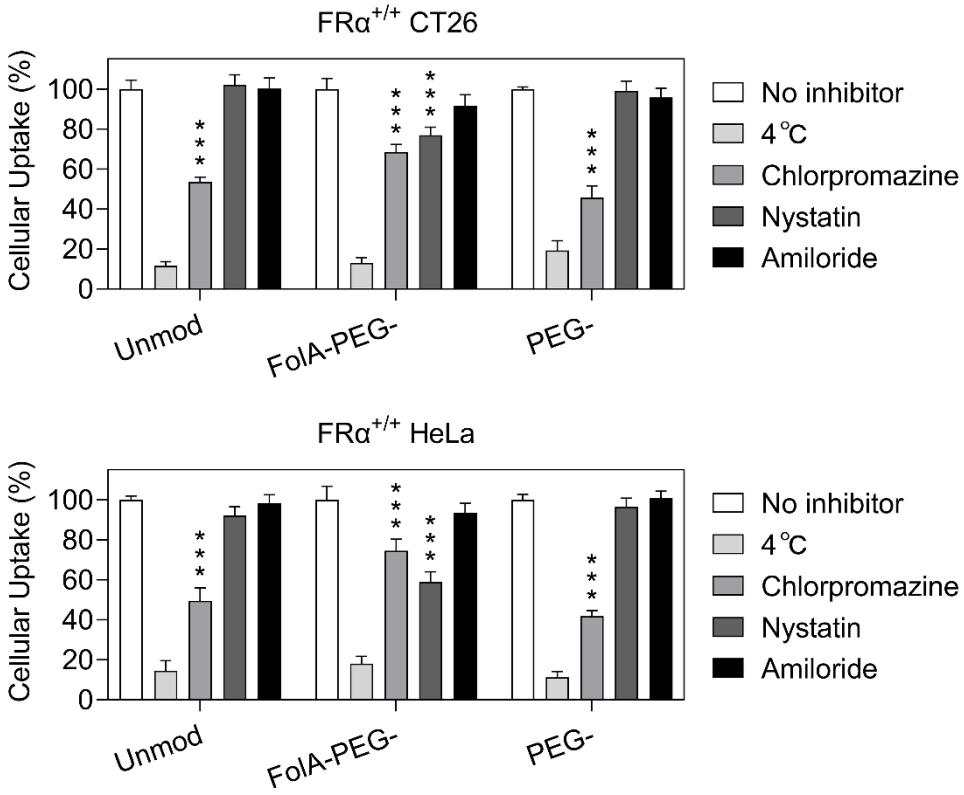


Figure S13. Endocytosis pathway study of different Cas9 RNP nanocarriers. CT26 and HeLa cells were pre-treated with different inhibitors followed by incubation with the Cas9 RNP nanocarriers containing 20 % Cy3-labeled sgRNA at the RNP dose of 75 nM. Flow cytometry was conducted 4 h after RNP transfection. 4 °C: energy-dependent endocytosis, Chlorpromazine: clathrin-mediated endocytosis, Nystatin: caveolae-mediated endocytosis, Amiloride: macropinocytosis. Data are presented as mean ± SD (n = 3), *** $p \leqslant 0.001$.

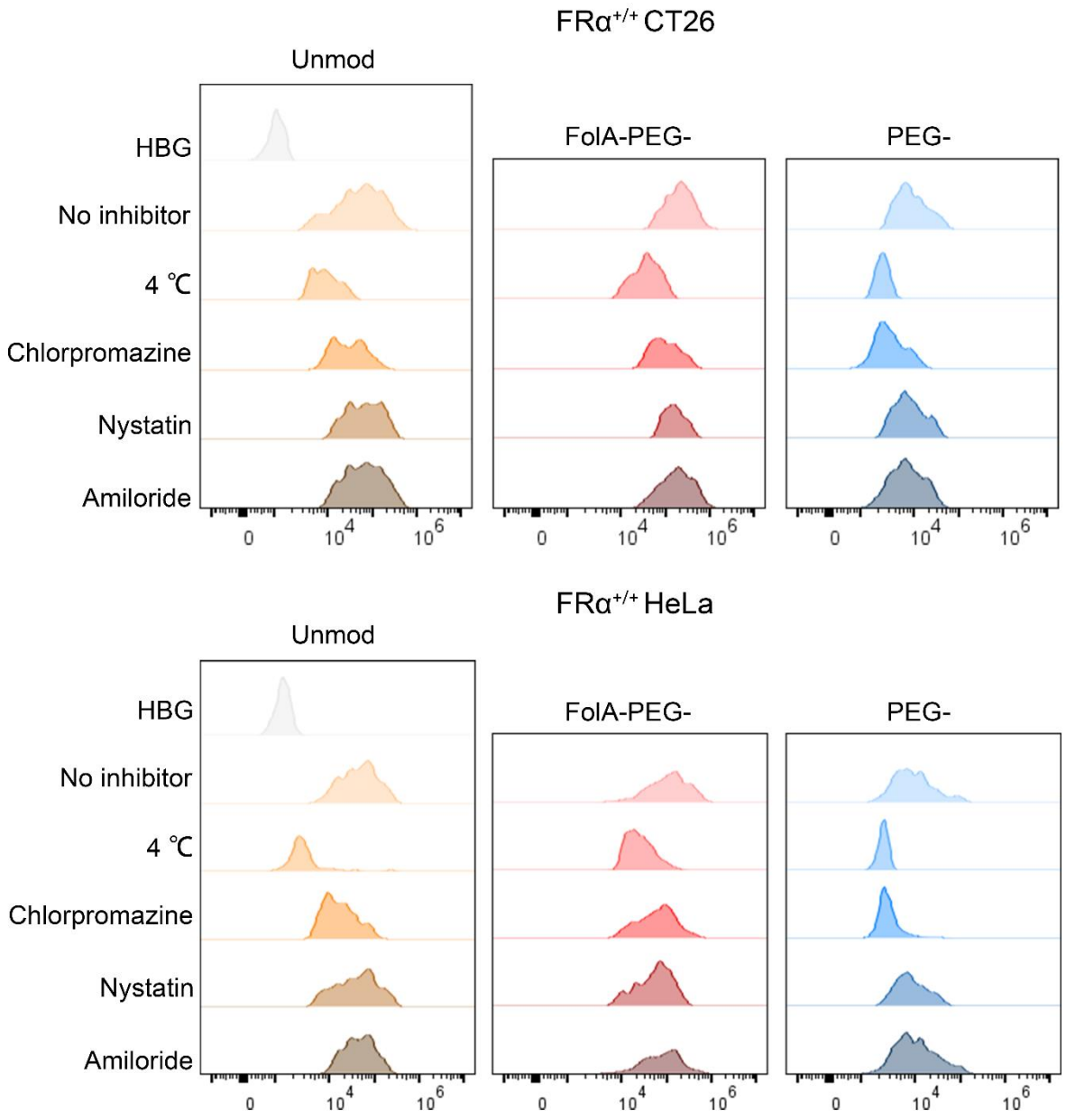


Figure S14. Histograms of flow cytometry showing the cellular uptake of Cas9 RNP nanocarriers (20 % Cy3-labeled sgRNA) in CT26 and HeLa cells pre-treated with different inhibitors.

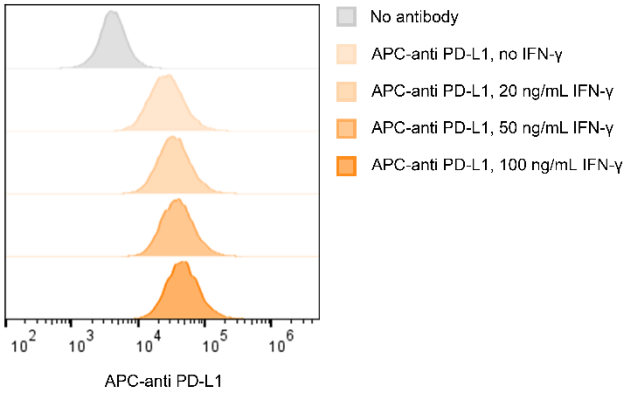


Figure S15. PD-L1 expression of CT26 WT cells stimulated by different concentrations of interferon-γ (IFN-γ). CT26 WT cells were incubated with or without 20, 50 or 100 ng/mL IFN-γ for 24 h. The flow cytometry experiment was conducted after PD-L1 was stained with APC-anti-PD-L1 antibody. The figure shows a flow cytometry histogram plot of each sample.

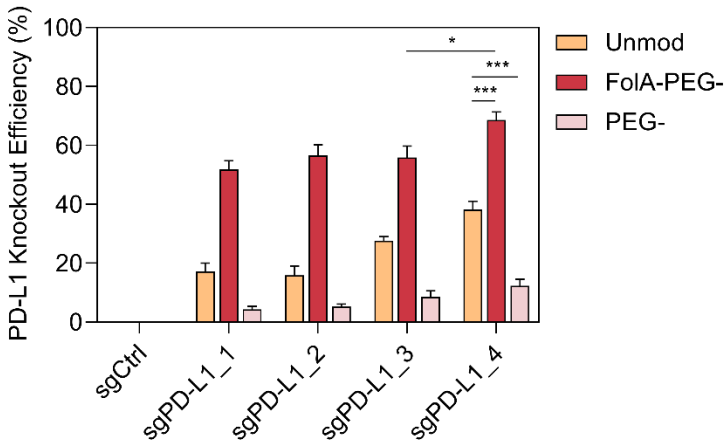


Figure S16. Screening of sgPD-L1. CT26 WT cells were incubated with Cas9 RNP nanocarriers containing 4 different sgPD-L1 sequences with or without 0.75 eq of ligand modification at the RNP dose of 75 nM for 4 h. Flow cytometry was conducted 48 h after transfection. The figure shows the PD-L1 knockout efficiency of Cas9 RNP nanocarriers containing 4 different sgPD-L1 sequences. Data are presented as the mean ± SD (n=3).

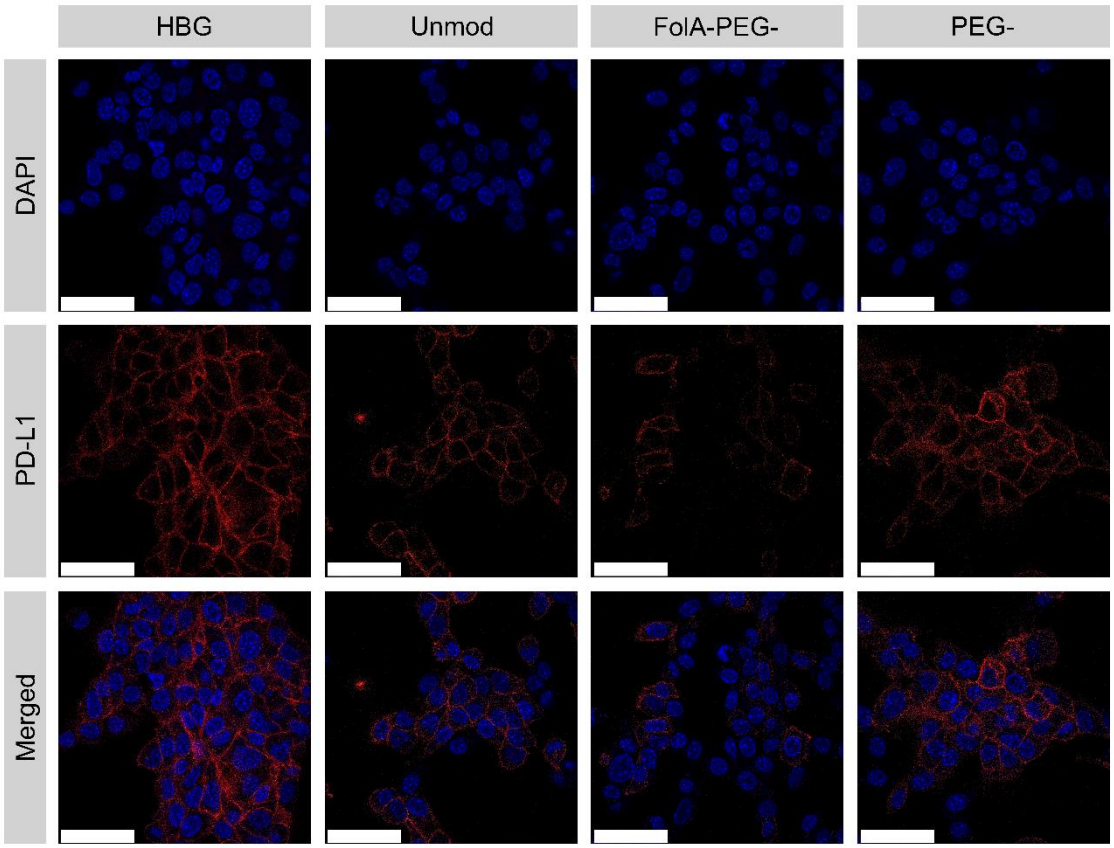


Figure S17. Confocal laser scanning microscopy (CLSM) images of CT26 WT cells after treatments with Cas9/sgPD-L1_4 RNP nanocarriers. CT26 WT cells treated with the Cas9 RNP nanocarriers containing 75 nM Cas9/sgPD-L1_4 RNP with or without 0.75 eq of ligand modification were recorded. First row: nuclear staining with DAPI; second row: fluorescence of APC-anti-PD-L1 antibody; third column: merge of two channels. A subset of the data is shown in Figure 4d of the main manuscript. Scale bar: 50 μm.

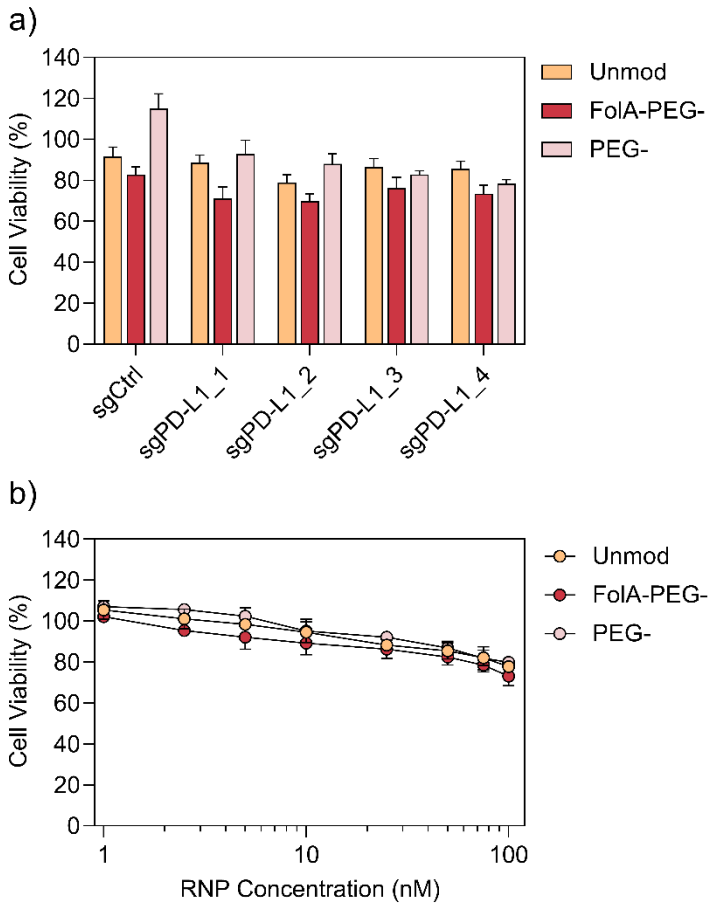


Figure S18. Viability of CT26 WT cells after treatments with Cas9/sgPD-L1 RNP nanocarriers with or without 0.75 eq of ligand modification. CT26 WT cells were incubated with the Cas9 RNP nanocarriers containing 4 different sgPD-L1 sequences at the RNP dose of 75 nM or the Cas9 RNP nanocarriers containing sgPD-L1_4 at various RNP doses ranging from 1 nM to 100 nM for 4 h. Metabolic activity of the cells was determined using a MTT assay 48 h after transfection. Data are presented as % cell viability with respect to the control cells ± SD (n=3).

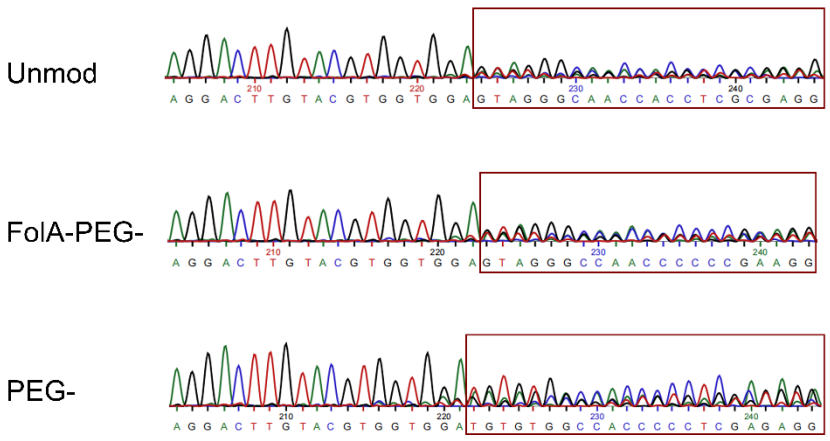


Figure S19. Sanger sequencing maps of PD-L1 gene at the target loci. Sanger sequencing maps of PD-L1 target locus of genomic DNA isolated from CT26 WT cells treated with sgPD-L1_4 loaded Cas9 RNP nanocarriers (75 nM RNP).

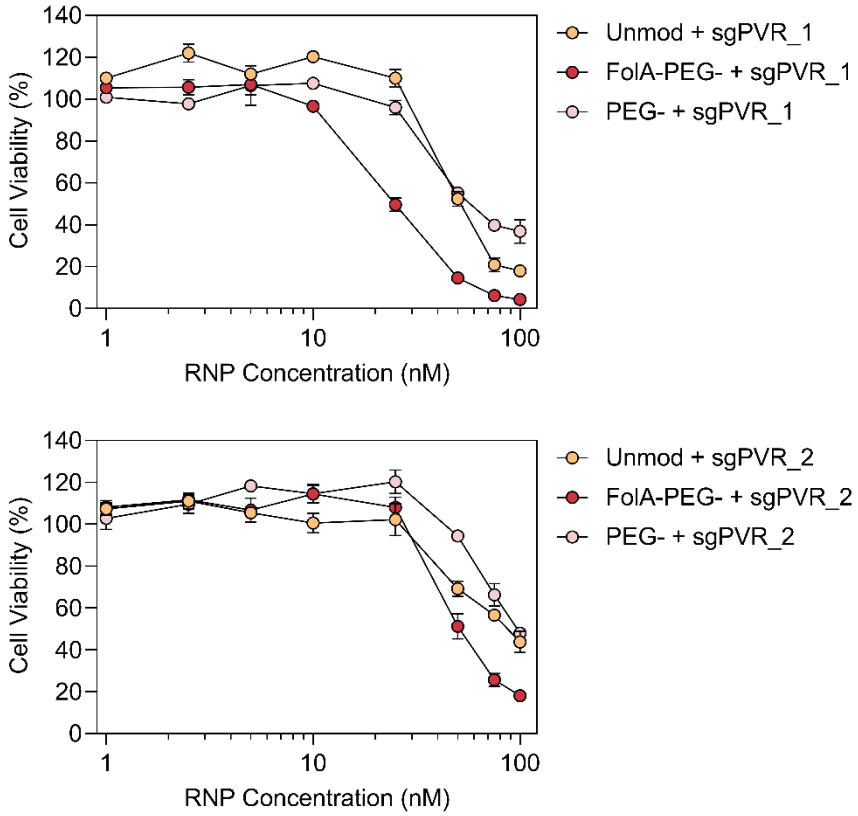


Figure S20. Viability of CT26 WT cells after treatments with Cas9/sgPVR RNP nanocarriers with or without 0.75 eq of ligand modification. CT26 WT cells were incubated with the Cas9 RNP nanocarriers containing sgPVR_1 or sgPVR_2 at various RNP doses ranging from 1 nM to 100 nM for 4 h. Metabolic activity of the cells was determined using a MTT assay 48 h after transfection. Data are presented as % cell viability with respect to the control cells ± SD (n=3).

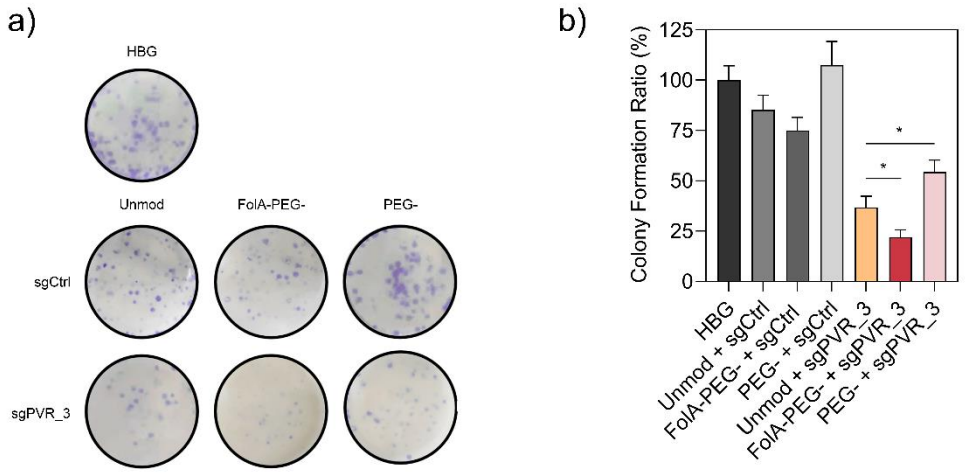


Figure S21. PVR knockout effect on CT26 WT cells evaluated by colony formation assay. CT26 WT cells were incubated with Cas9 RNP nanocarriers containing sgPVR_3 or sgCtrl with or without 0.75 eq of ligand modification at a RNP dose of 75 nM for 4 h. 250 cells of each group were subsequently re-plated and crystal violet staining was performed 3 weeks after re-plating. a) Representative colony formation images of CT26 WT cells treated with different RNP nanocarriers. b) Colony formation ratio of CT26 WT cells treated with different RNP nanocarriers. Data are presented as % colony formation ratio with respect to the control cells treated with HBG buffer ± SD (n=3).

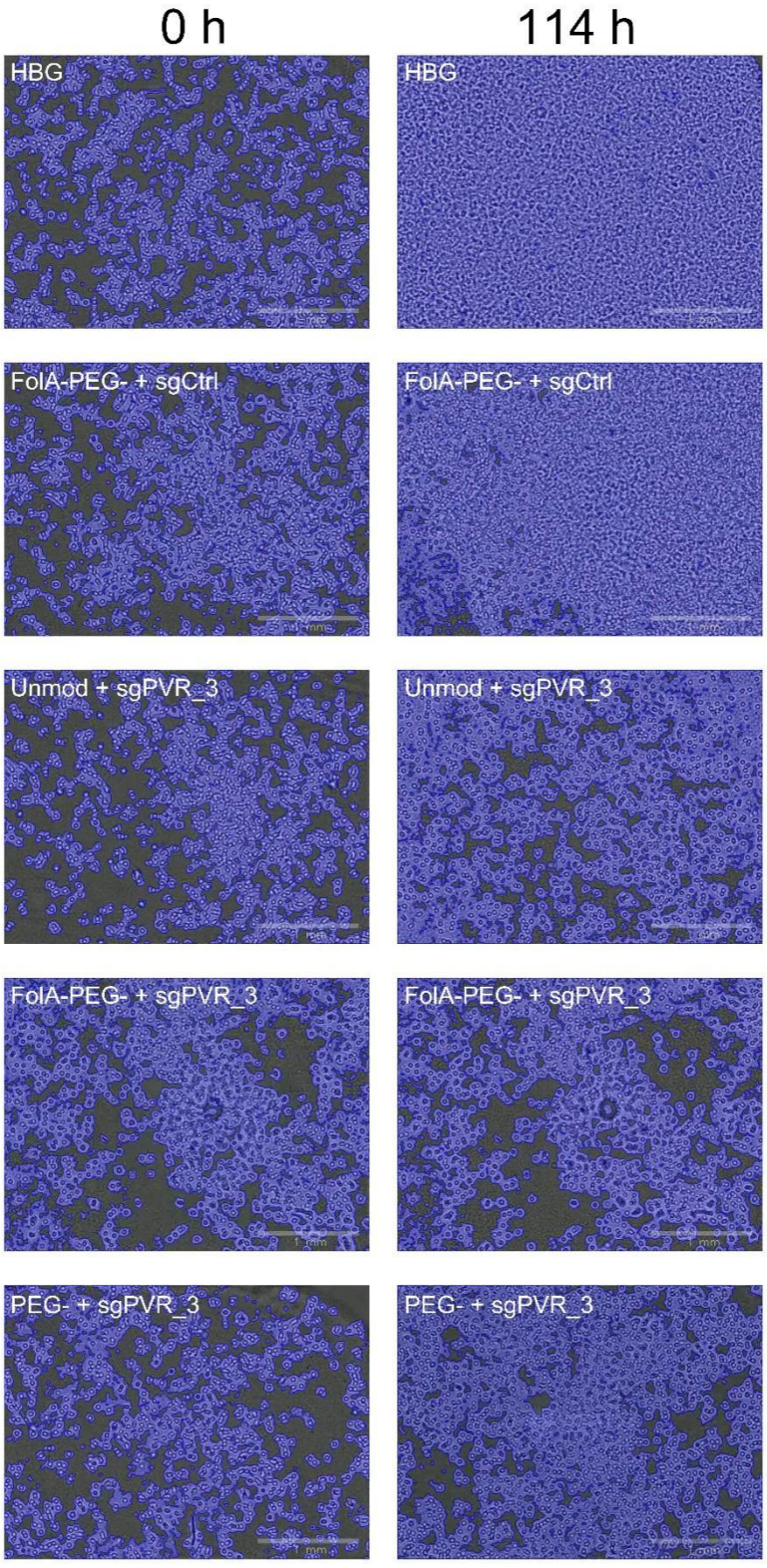


Figure S22. Cellwatcher images of CT26 WT cells treated with Cas9/sgPVR_3 RNP nanocarriers at starting and terminal time points. Cell growth kinetics were recorded from 0 h to 114 h. The RNP nanocarriers with or without 0.75 eq of ligand modification at the RNP dose of 75 nM were added to the cells 1 h after recording. Cell growth curves can be found in Figure 5d of the main manuscript.

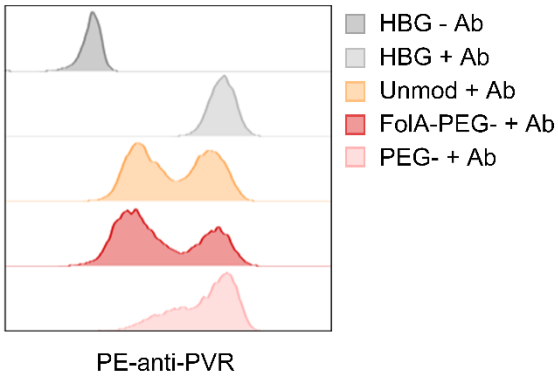


Figure S23. PVR knockout evaluation of CT26 WT cells treated with Cas9/sgPVR_3 RNP nanocarriers by flow cytometry. CT26 WT cells were incubated with the Cas9 RNP nanocarriers with or without 0.75 eq of ligand modification at the RNP dose of 75 nM for 4 h. Flow cytometry was conducted after PVR was stained with PE-anti-PVR antibody 3 days after transfection. The figure shows a flow cytometry histogram plot of each sample.

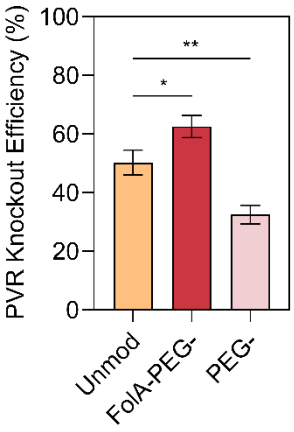


Figure S24. PVR knockout efficiency of Cas9/sgPVR_3 RNP nanocarriers in CT26 WT cells. CT26 WT cells were incubated with the Cas9 RNP nanocarriers with or without 0.75 eq of ligand modification at the RNP dose of 75 nM for 4 h. PVR knockout efficiency of each group was determined by flow cytometry 4 days after treatment. Data are presented as the mean ± SD (n=3).

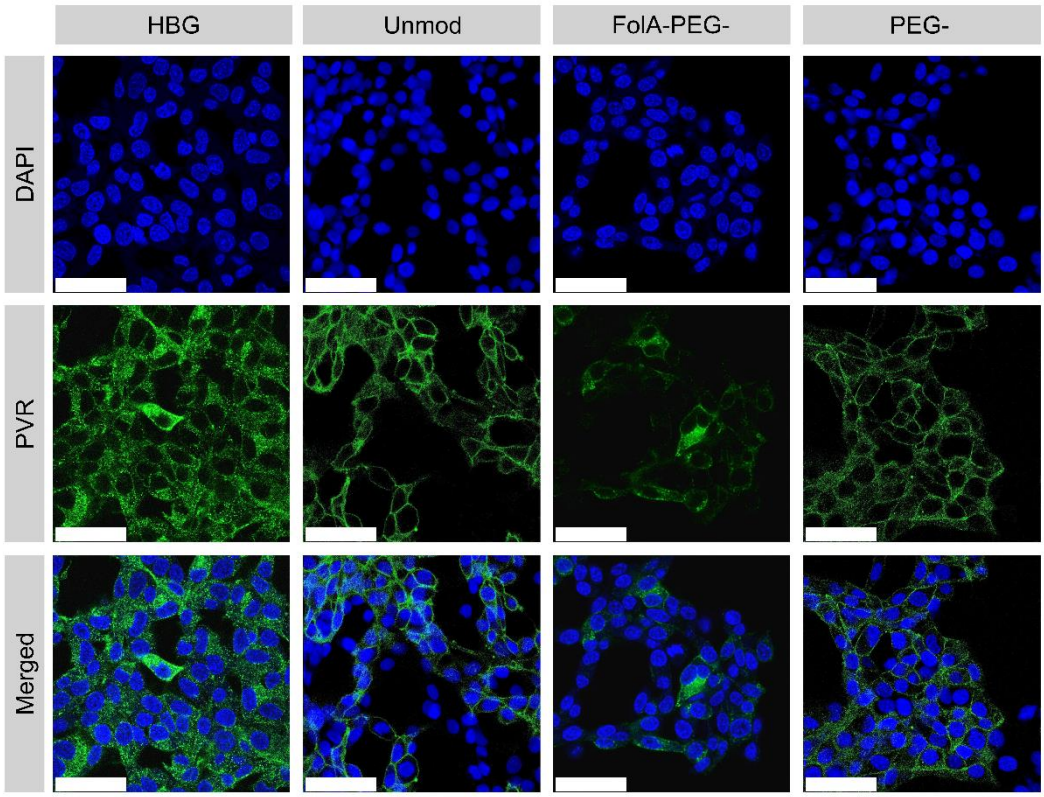


Figure S25. Confocal laser scanning microscopy (CLSM) images of CT26 WT cells after treatments with different Cas9/sgPVR_3 RNP nanocarriers. CT26 WT cells were treated with Cas9 RNP nanocarriers containing 25 nM Cas9/sgPVR_3 RNP with or without 0.75 eq of ligand modification and were imaged by CLSM after 4 h. First row: nuclear staining with DAPI; second row: staining with PE-anti-PVR antibody; third column: merge of both channels. A subset of the data is shown in Figure 5f of the main manuscript. Scale bar: 50 µm.

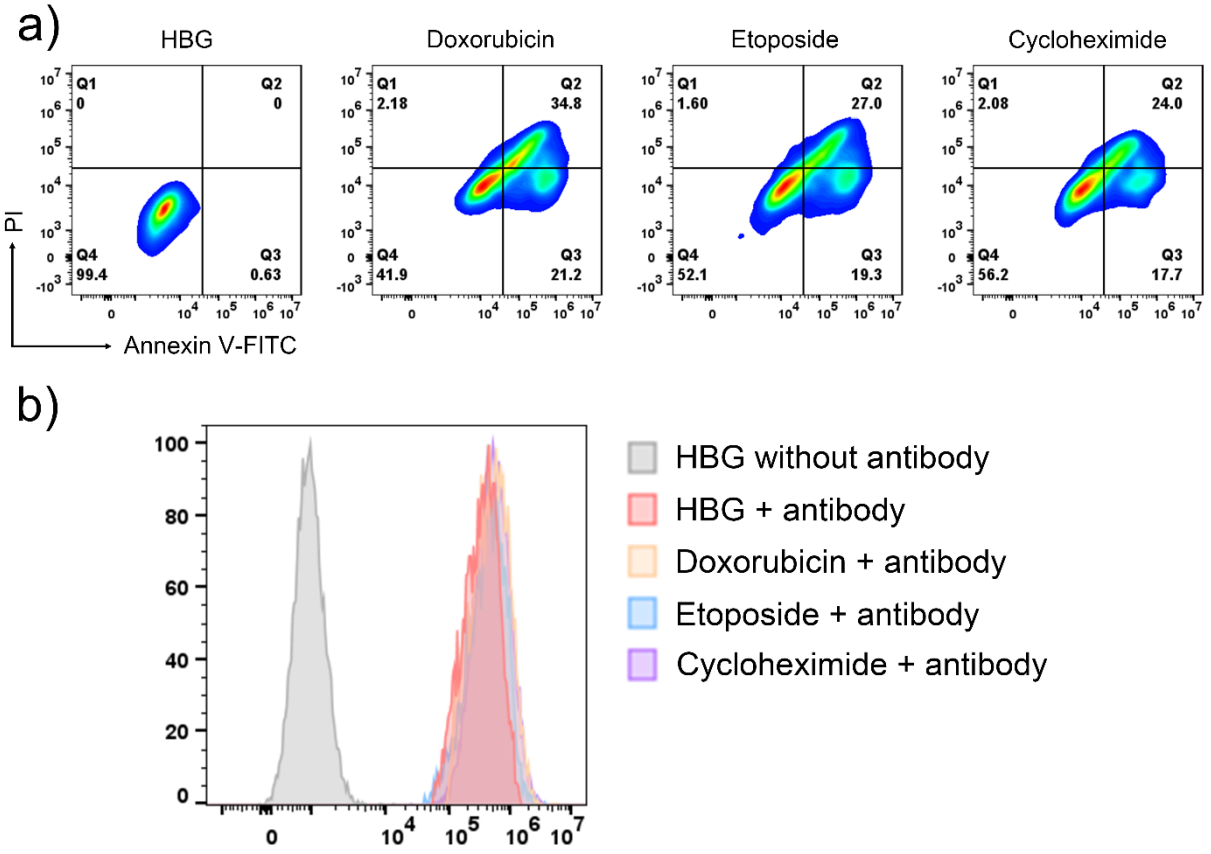


Figure S26. PVR expression of CT26 WT cells treated with different apoptosis inducers. a) Apoptosis induction by doxorubicin (10 µM), etoposide (10 µM), or cycloheximide (35 µM) as determined by flow cytometry with Annexin V-FITC/PI staining. Q1: necrotic cells; Q2: late apoptotic cells; Q3: early apoptotic cells; Q4: live cells. b) Histograms of flow cytometry showing the PVR expression of CT26 WT cells after treatments with different apoptosis inducers. PVR was stained with PE-labeled anti-PVR antibody.

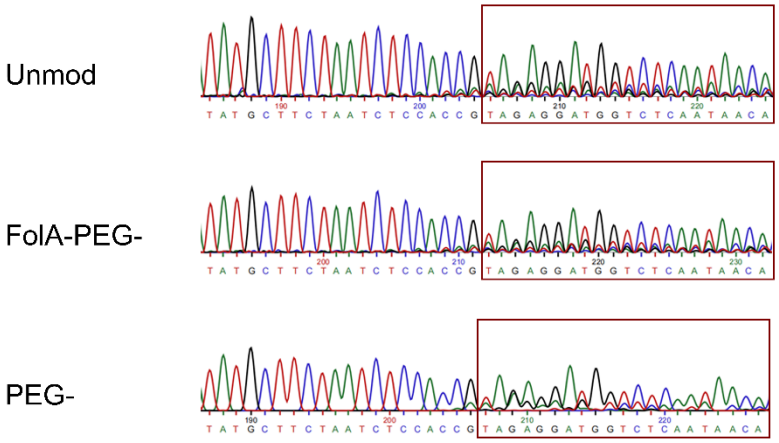


Figure S27. Sanger sequencing maps of PVR gene at the target loci. Sanger sequencing maps of PVR target locus of genomic DNA isolated from CT26 WT cells treated with sgPVR_3 loaded Cas9 RNP nanocarriers (75 nM RNP).

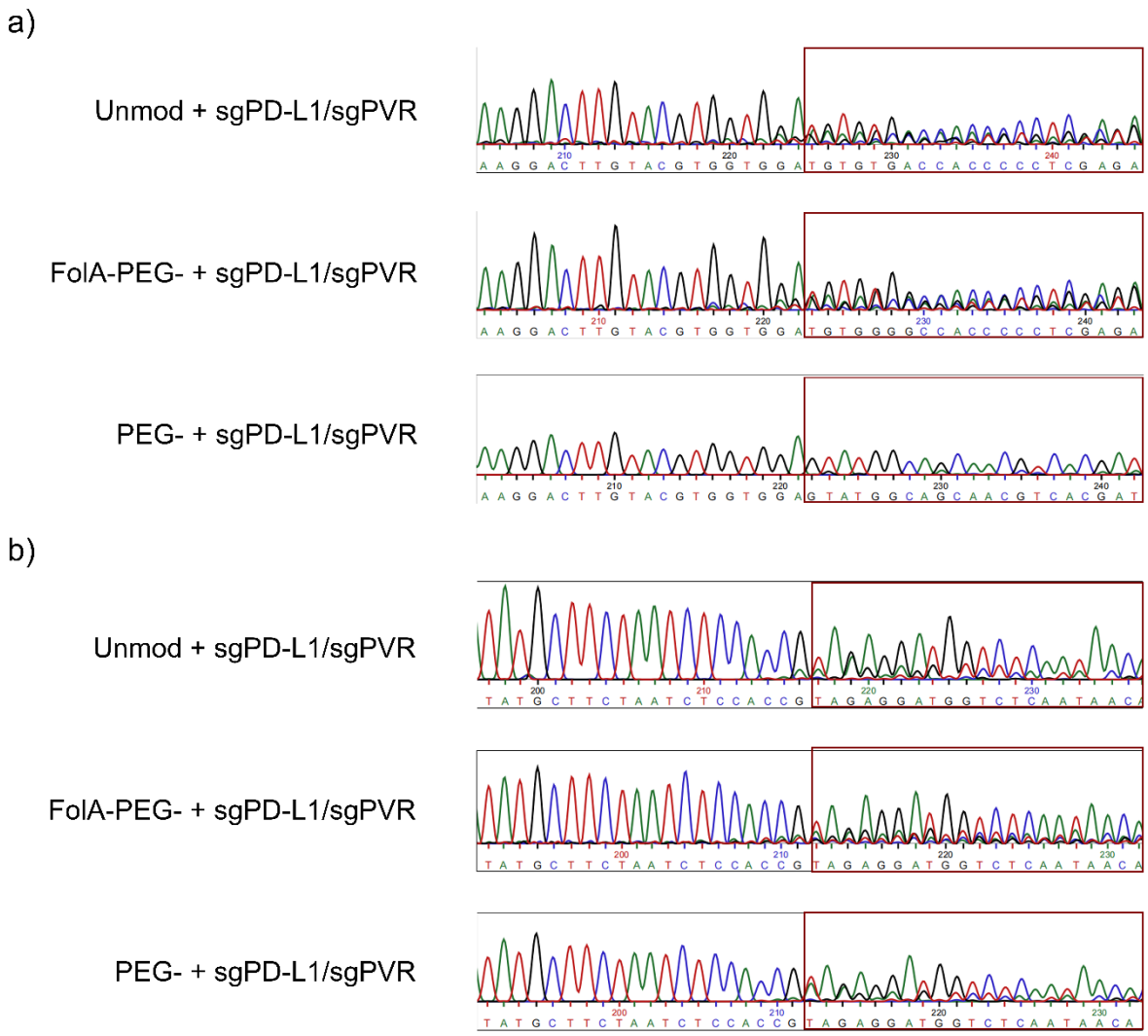


Figure S28. Sanger sequencing maps of PD-L1 and PVR genes at the target loci. Sanger sequencing maps of a) PD-L1 target locus and b) PVR target locus of genomic DNA isolated from CT26 WT cells treated with sgPD-L1_4/sgPVR_3 dual loaded Cas9 RNP nanocarriers.

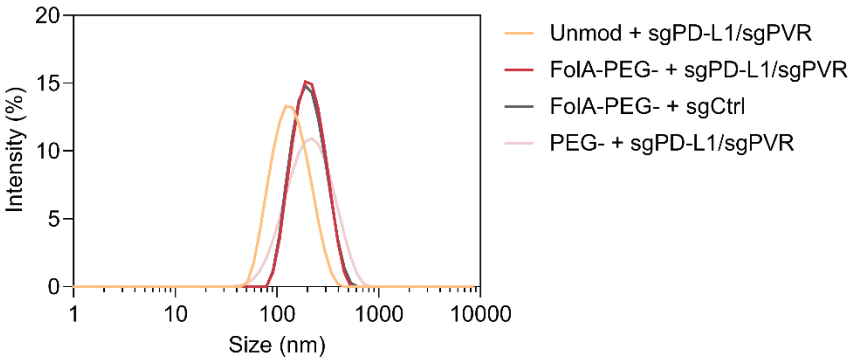


Figure S29. Particle size of Cas9 RNP nanocarriers for *in vivo* studies determined by DLS. sgPD-L1/sgPVR dual loaded or sgCtrl Cas9 RNP nanocarriers containing 125 µg Cas9 protein, 12.5 µg sgPD-L1_4, and 12.5 µg sgPVR_3 or 25 µg sgCtrl were prepared in 50 µL of HBG for each mouse. The figure shows the intensity size distribution of each sample

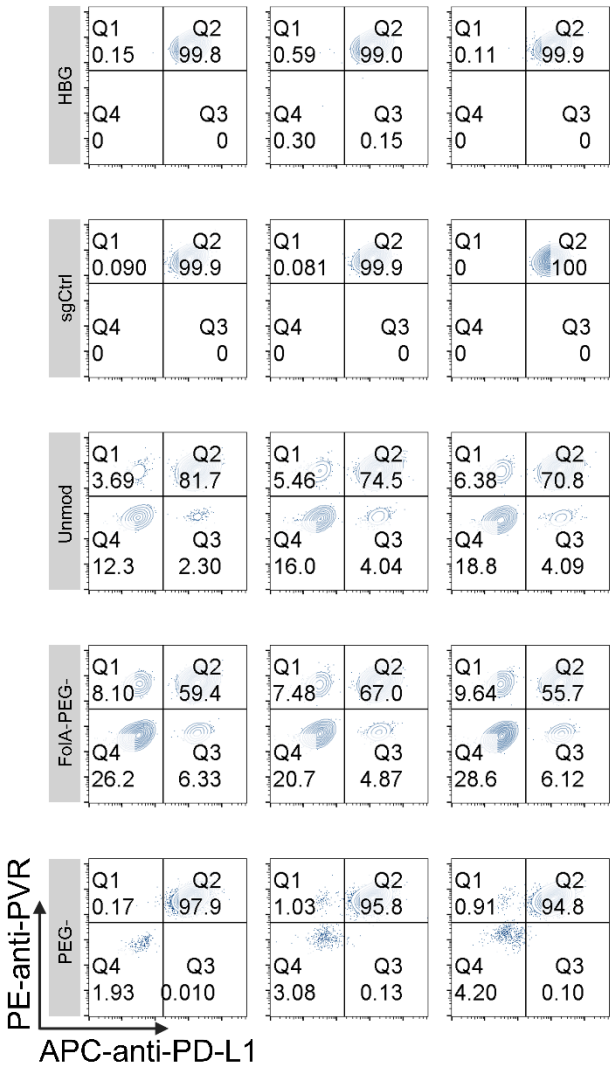


Figure S30. Evaluation of dual PD-L1/PVR knockout *in vivo* by flow cytometry. PD-L1 and PVR knockout efficiencies were determined by flow cytometry of homogenized CT26 tumors treated with HBG buffer and Cas9 RNP nanocarriers containing Cas9/sgCtrl or Cas9/sgPD-L1_4 and Cas9/sgPVR_3 (1:1) (0.75 eq modification). Data are presented as mean ± SD (n=3).

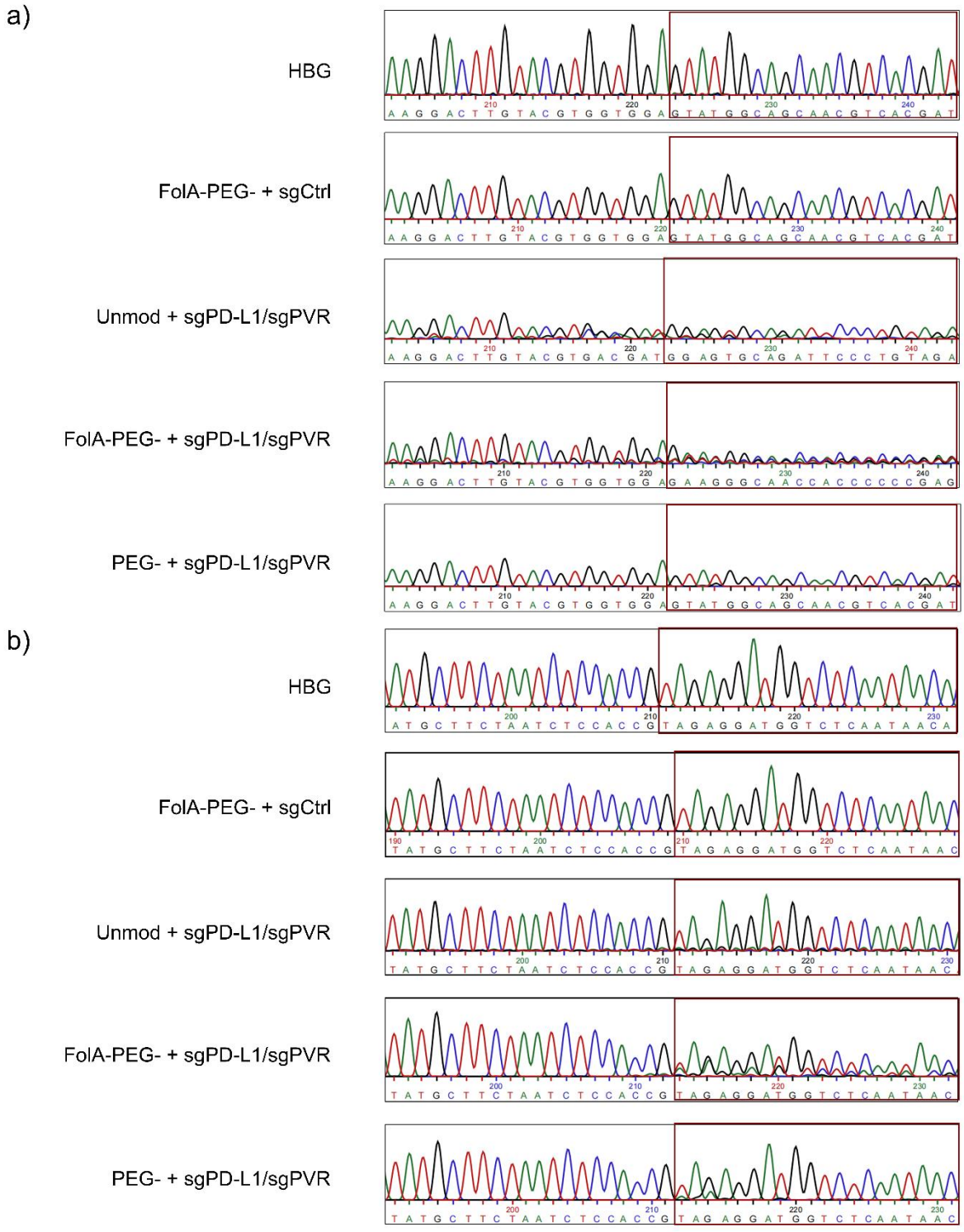


Figure S31. Sanger sequencing maps of PD-L1 and PVR at the target loci from CT26 tumor tissues. Sanger sequencing maps of a) PD-L1 target locus and b) PVR target locus of genomic DNA isolated from CT26 tumor tissues treated with HBG, sgCtrl-containing FolA-PEG nanocarriers, sgPD-L1_4/sgPVR_3 dual loaded unmodified nanocarriers, sgPD-L1_4/sgPVR_3 dual loaded FolA-PEG nanocarriers, and sgPD-L1_4/sgPVR_3 dual loaded PEG nanocarriers.

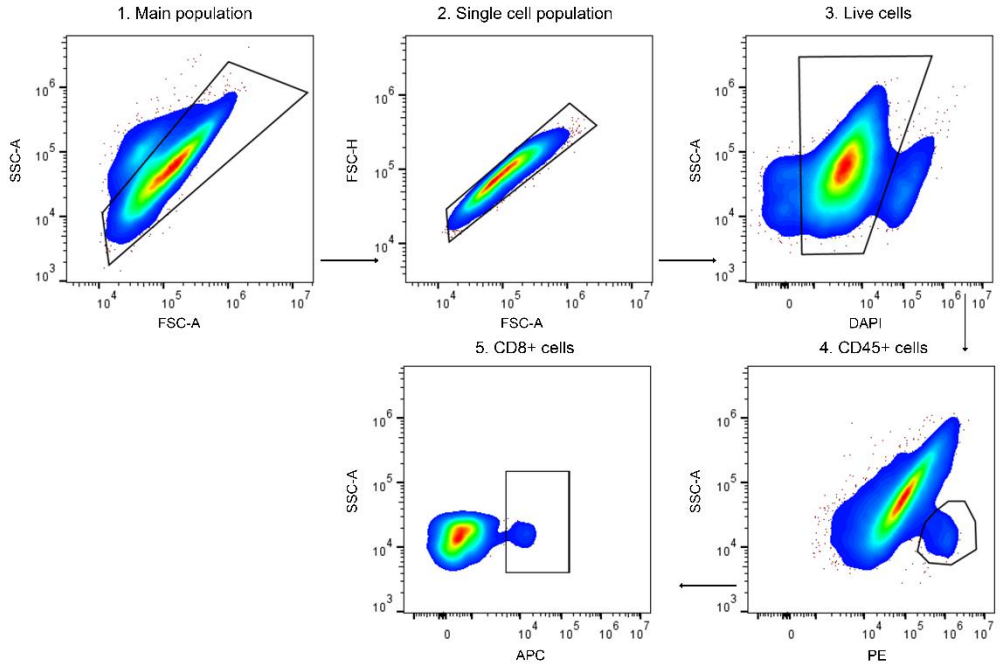


Figure S32. Gating strategy to determine CD8+ T cells in single cell tumor suspensions.

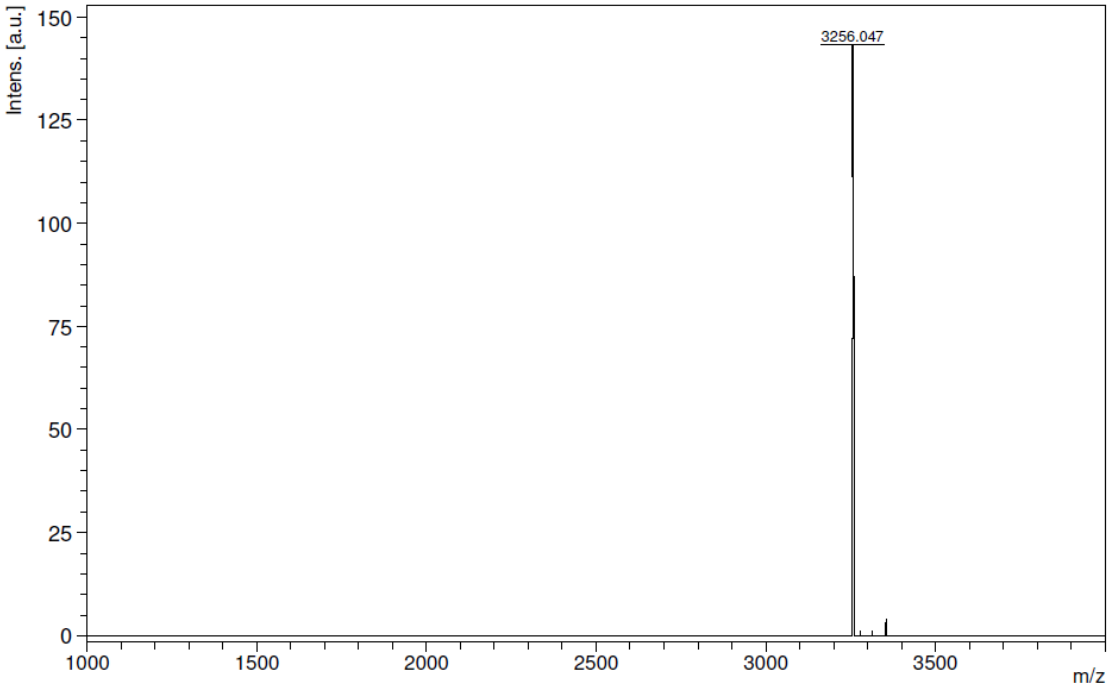
4. Analytical data

4.1 MALDI-TOF mass spectrometry

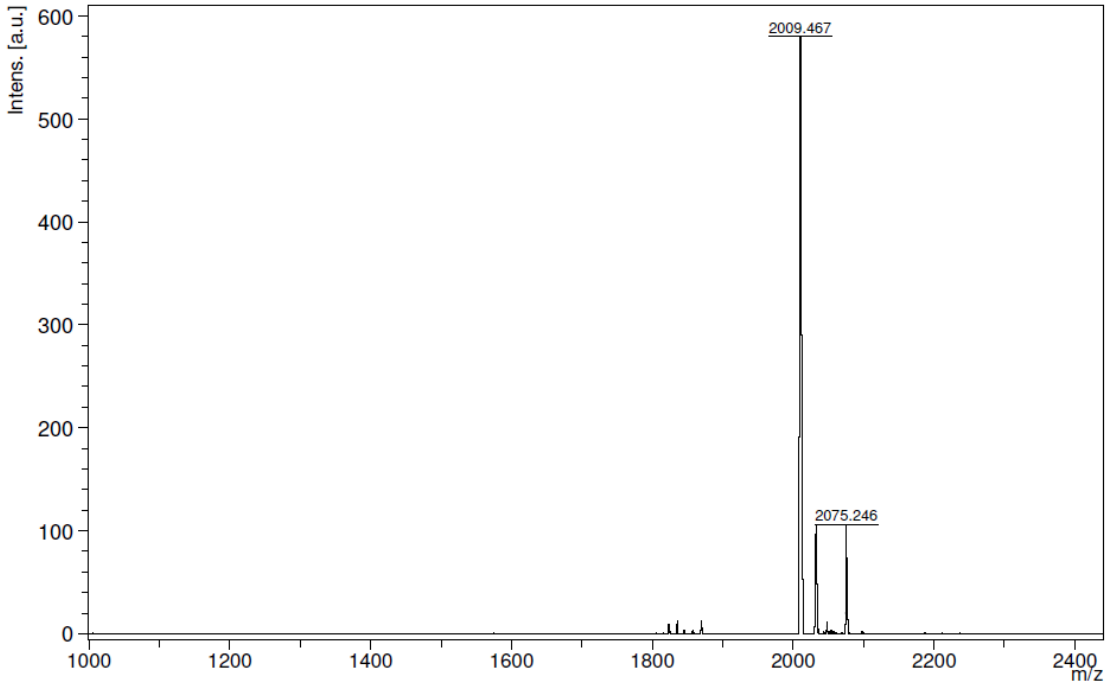
Table S6. Summary of peptides, ligands, and their conjugate mass data

ID	Description	Molecular formula	$[M+H]^+$ calc.	$[M+H]^+$ found
1445	T-OHSteA	C162H268N36O30S2	3263.0	3256.0
-	FolA-PEG_{24}-DBCO	C97H149N11O34	2012.0	2009.5
-	PEG_{24}-DBCO	C72H120N2O28	1460.8	1459.4
-	FolA-PEG_{24}-1445	C259H417N47O64S2	5275.0	5266.5
-	PEG_{24}-1445	C234H388N38O58S2	4723.8	4720.3

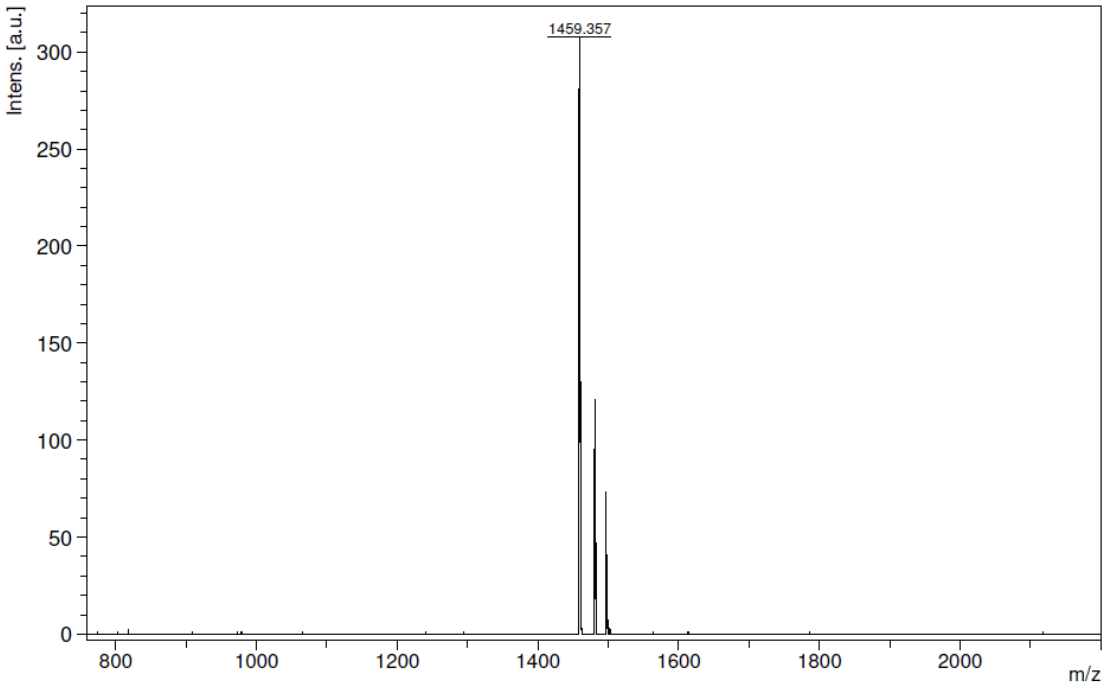
MALDI-TOF MS of 1445



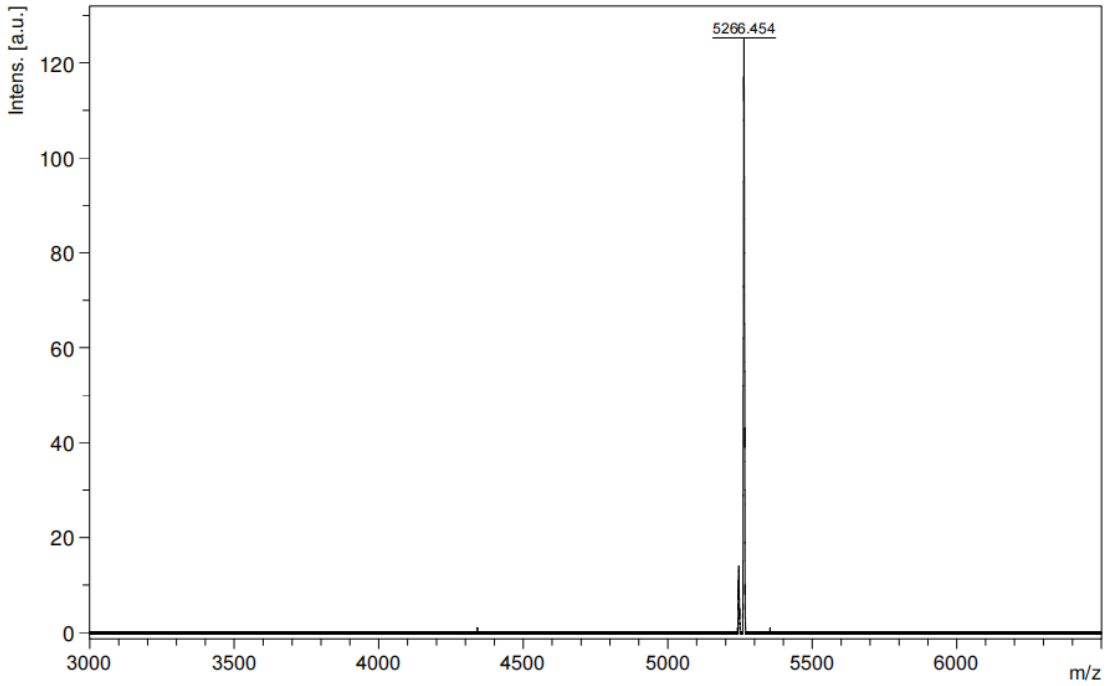
MALDI-TOF MS of FolA-PEG$_{24}$-DBCO



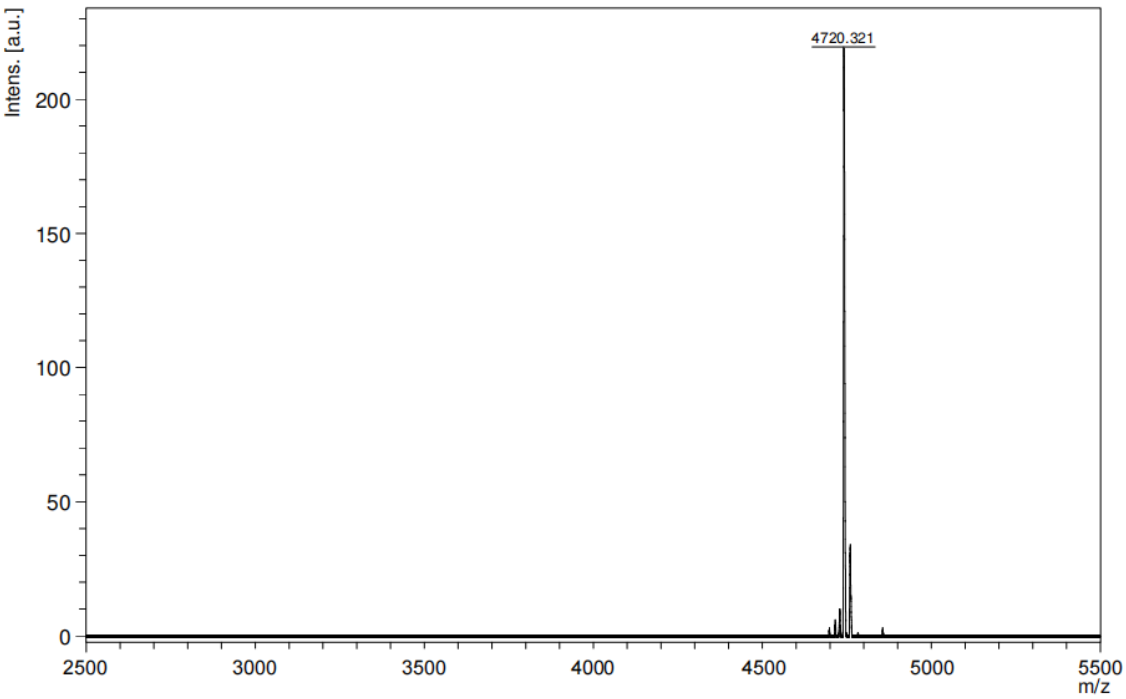
MALDI-TOF MS of PEG$_{24}$-DBCO



MALDI-TOF MS of FolA-PEG$_{24}$-1445

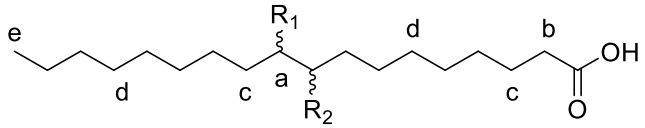


MALDI-TOF MS of PEG$_{24}$-1445

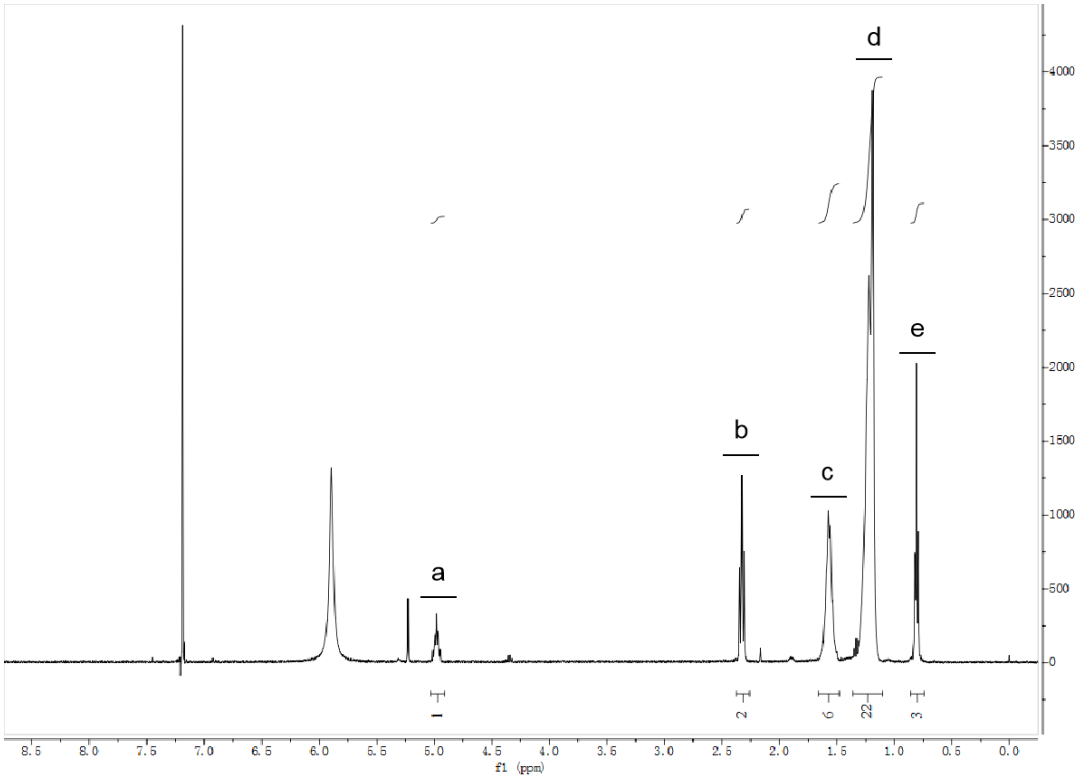


4.2 ^{1}H-NMR spectroscopy

Trifluoroacetate stearic acid

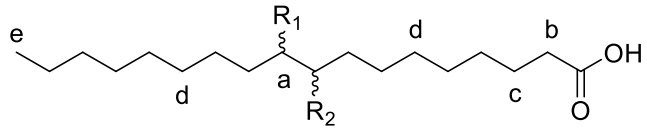


(R_1=$OCOCF_3$, R_2=H or R_1=H, R_2=$OCOCF_3$)

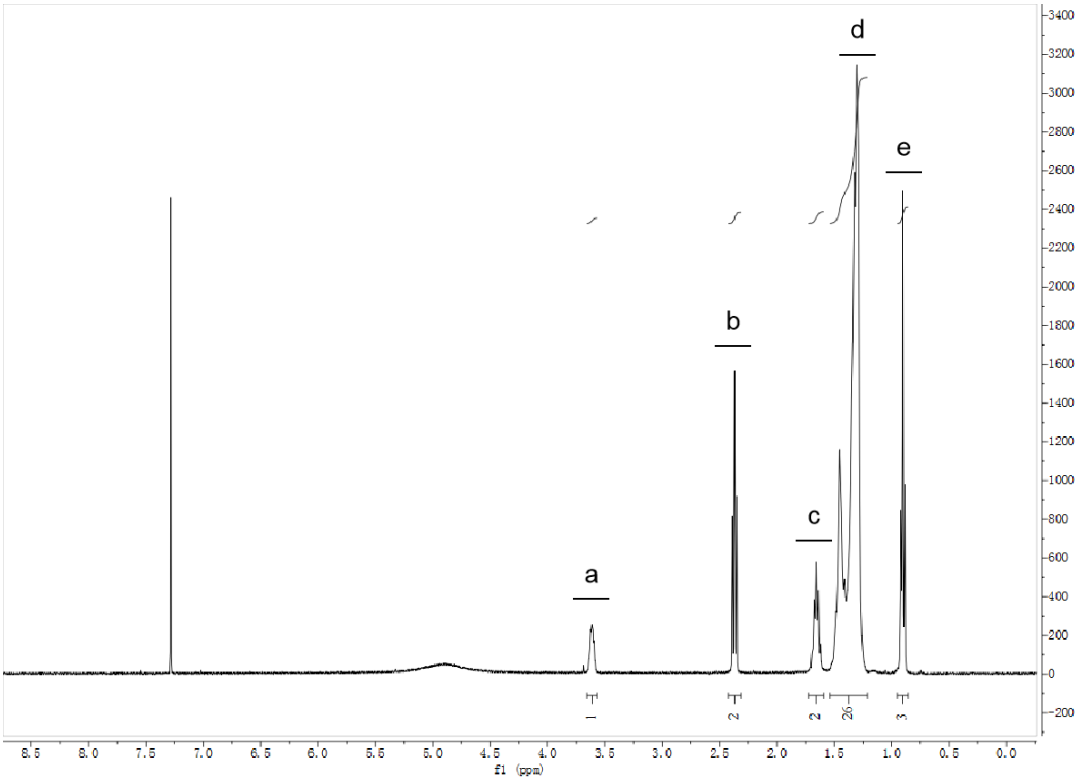


^{1}H NMR (400 MHz, Chloroform-d) δ (ppm) = 5.02-4.90 (quint, 1H, =C*H*-$OCOCF_3$), 2.33-2.26 (t, 2H, α*H* trifluoroacetate stearic acid), 1.66-1.48 (m, 6H, β*H* trifluoroacetate stearic acid, -C*H*2-CH(CH2)-$OCOCF_3$), 1.39-1.13 (m, 22H, -CH_2-), 0.83-0.74 (t, 3H, -CH_3).

Hydroxystearic acid (OHSteA)



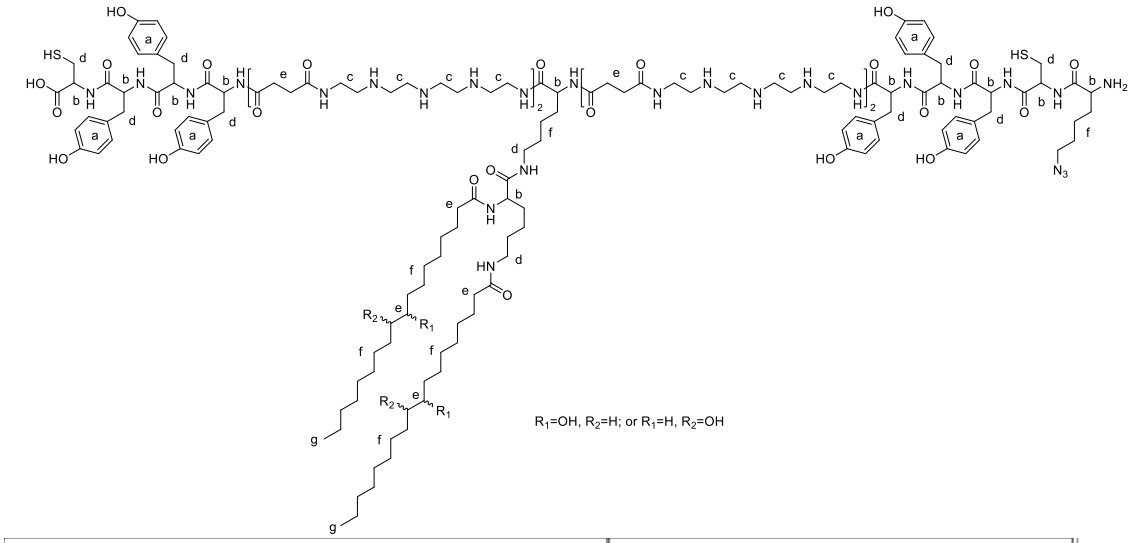
(R_1=OH, R_2=H or R_1=H, R_2=OH)

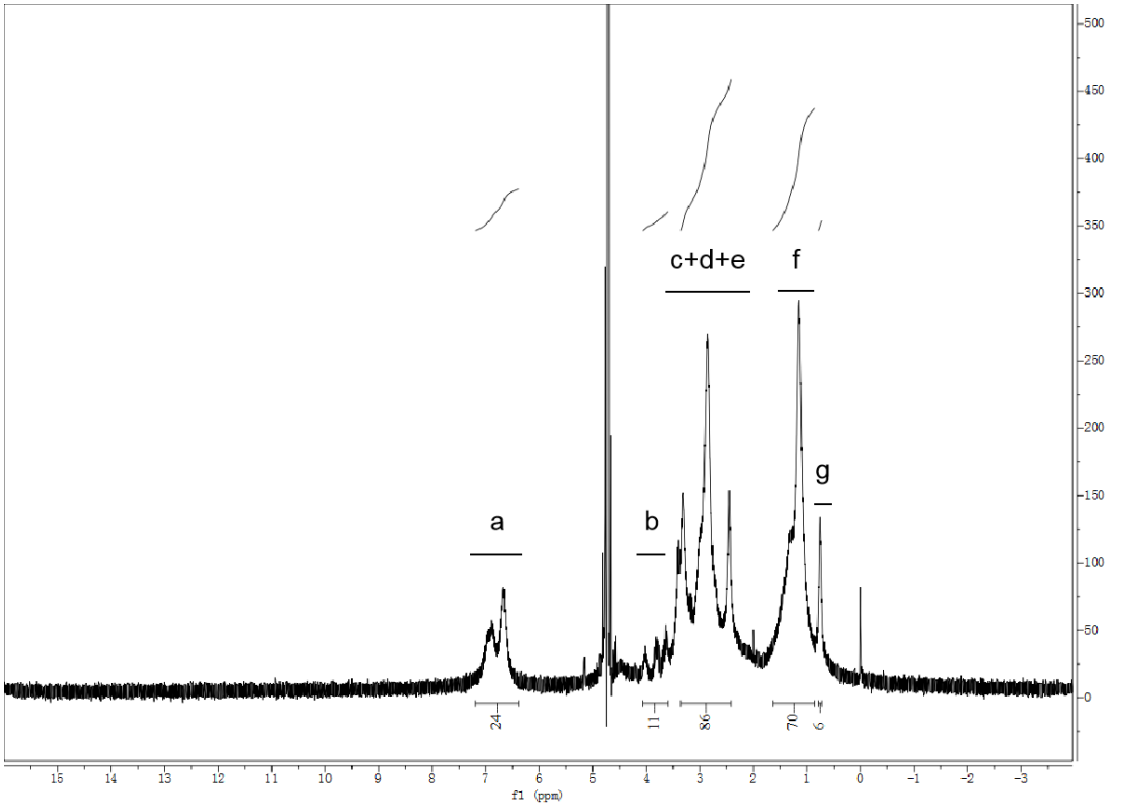


^{1}H NMR (400 MHz, Chloroform-d) δ (ppm) = 3.66-3.57 (quint, 1H, =C*H*-OH), 2.43-2.31 (t, 2H, α*H* hydroxystearic acid), 1.72-1.59 (quint, 2H, β*H* hydroxystearic acid), 1.39-1.13 (m, 26H, -CH_2-), 0.95-0.86 (t, 3H, -CH_3).

T-OHSteA (1445)

Sequence (C→N): C-Y_3-Stp_2-K(K-$OHSteA_2$)-Stp_2-Y_3-C-K(N_3)





^{1}H NMR (400 MHz, Deuterium Oxide) δ (ppm) = 7.20-6.38 (m, 24H, -C*H*- tyrosine), 4.12-3.57 (m, 11H, α*H* amino acids), 3.38-2.33 (m, 86H, -CH_2- Tp, ε*H* lysine, ε*H* tyrosine, ε*H* cysteine, -CO-CH_2-CH_2-CO- Stp, -CO-CH_2- hydroxystearic acid, =C*H*-OH hydroxystearic acid), 1.64 -0.86 (m, 70H, βγδ*H* lysine, βγδ*H* azidolysine, -CH_2- hydroxystearic acid), 0.81-0.66 (s, 6H, -CH_3 hydroxystearic acid).

5. References

[1] E. Kaiser, R. L. Colescott, C. D. Bossinger, P. Cook, *Analytical biochemistry* **1970**, 34, 595.

[2] P. M. Klein, S. Kern, D.-J. Lee, J. Schmaus, M. Höhn, J. Gorges, U. Kazmaier, E. Wagner, *Biomaterials* **2018**, 178, 630.

[3] J. Kuhn, Y. Lin, A. Krhac Levacic, N. Al Danaf, L. Peng, M. Höhn, D. C. Lamb, E. Wagner, U. Lächelt, *Bioconjugate Chemistry* **2020**, 31, 729.