Supporting Information

Efficient mRNA Delivery Using Lipid Nanoparticles Modified with Fusogenic Coiled-coil Peptides

Ye Zeng,[a] Mengjie Shen,[a] Roy Pattipeiluhu,[a] Xuequan Zhou,[a] Yun Zhang [a], Thomas Bakkum,[a] Thomas H. Sharp,[b] Aimee L. Boyle,[a] Alexander Kros,*[a]

[a] Department of Supramolecular & Biomaterials Chemistry, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands

[b] Department of Cell and Chemical Biology, Section Electron Microscopy, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

*Correspondence:
Alexander Kros, Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2333 CC Leiden, The Netherlands
Tel/Fax:
E-mail: a.kros@chem.leidenuniv.nl

Experimental section

1. Chemicals.

All Fmoc-protected amino acids were purchased from Novabiochem, TentaGel S RAM resin (S30023) was purchased from Rapp Polymere GmbH. Piperidine, trifluoroacetic acid, acetonitrile, dimethylformamide (DMF) were purchased from Biosolve; dichloromethane (DCM), and ethanol were purchased from Sigma-Aldrich. 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2K), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (PE-NBD), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-LR), were purchased from Avanti Polar Lipids, DLin-MC3-DMA was purchased from Biorbyt (Cambridge, England), and dynasore, wortmannin, nocodazole, pitstop2, genistein, methyl-β-cyclodextrin (MβCD), sodium azide (NaN_3), cholesterol was purchased from Sigma-Aldrich. Lyso-tracker deep red and lipofectamine 3000 was purchased from Thermofisher. Triton™ X-100 was purchased from Acros Organics. QuantiT™ RiboGreen® RNA reagent and rRNA standards were purchased from Life Technologies. WST-1 reagent was purchased from Sigma-Aldrich. Nucleic acid: Alexa488-nucleic acid (Alexa488-5'-AACCATACACCTACTACCTCA-3') was purchased from Integrated DNA technology; cleancap EGFP-mRNA was purchased from Trilink biotechnology.

2. Lipopeptide synthesis and purification.

Peptide E3 $(EIAALEK)_3$, K3 $(KIAALKE)_3$, E4 $(EIAALEK)_4$ and K4 $(KIAALKE)_4$ were synthesized using Fmoc chemistry and standard solid-phase peptide synthesis protocols on a 0.1 mmol scale as described previously.[1] Fmoc deprotection was performed using 20% piperidine in DMF at 90 °C for 60 s. Amide coupling was achieved using 5 eq. of protected amino acid, 5 eq. DIC as activator and 5 eq. Oxyma as activator base, heated at 95 °C for 240 seconds. Lipidated peptides (CPK3, CPE3, CPK4, CPE4) were made on resin via the coupling of 2.5 equivalents of N_3- PEG_4-COOH, with 2.5 eq of HBTU and 5 eq. of DIPEA in DMF overnight at room temperature. After washing the resin with DMF, the azide was reduced using 10 eq. of PME3 (1 M in toluene), with 4:1 dioxane:water as solvent for 2.5 hours. The resin was then washed thoroughly with 4:1 dioxane:water, MeOH and DMF. Lipidation was achieved using 2 eq. cholesteryl hemisuccinate, 2 eq. HBTU and 4 eq. DIPEA in 1:1 DMF:DCM. After the final coupling the resin was washed with DMF, MeOH, and

DCM, dried under vacuum, and the peptide was cleaved using a mixture of TFA:TIPS:EDDT:water (92.5:2.5:2.5:2.5) for 1 hour, after which the peptide was precipitated in cold diethyl ether, collected via centrifugation and lyophilized. All peptides were purified by HPLC on a Shimadzu system consisting of two KC-20AR pumps and an SPD-20A or SPD-M20A detector equipped with a Kinetix Evo C18 column. Eluents consisted of 0.1% TFA in water (A) and 0.1% TFA in MeCN (B), with all peptides eluted using a gradient of 20-90% B over 35 minutes, with a flow rate of 12 mL/min. Collected fractions were checked for purity via LC-MS, with the pure fractions being pooled and lyophilized. LC-MS spectra were recorded using a Thermo Scientific TSQ quantum access MAX mass detector connected to an Ultimate 3000 liquid chromatography system fitted with a 50x4.6 mm Phenomenex Gemini 3 µm C18 column (Fig. S10).

3. Lipid nanoparticles formulation.

Lipids and lipopeptides were combined at the desired molar ratios and concentrations from stock solutions dissolved in chloroform:methanol (1:1). Solvents were evaporated under a nitrogen flow and residual solvent was removed *in vacuo* for at least 30 minutes. The lipid film was dissolved in absolute ethanol and used for assembly (total [lipid] was 1 µmol). A solution of mRNA was made by diluting nucleic acid (Alexa488-nucleic acid or EGFP-mRNA) in 50 mM citrate buffer (pH = 4, RNase free H_2O). The solutions were loaded into two separate syringes and connected to a T-junction microfluidic mixer. The solutions were mixed in a 3:1 flow ratio of nucleic acid:lipids (1.5 mL/min for the nucleic acid solution, 0.5 mL/min for the lipids solution, N/P ratio was 16:1). After mixing, the solution was directly loaded in a 20 k MWCO dialysis cassette (Slide-A-Lyzer™, Thermo Scientific) and dialyzed against 1 x PBS overnight. After overnight dialysis, mRNA encapsulation efficiency was determined by Quant-iT™ RiboGreen™ RNA Assay Kit as described below. For confocal cellular uptake experiments, 1 mol% of PE-LR was added with the other lipids.

4. Biophysical characterization.

The size and zeta potential of LNPs were measured using a Malvern zetasizer Nano ZS. Long term stability of LNPs was assessed by measuring the hydrodynamic radius using DLS for 10 days.

The morphology of LNPs was analyzed by cryogenic transmission electron microscopy (cryo-EM). Vitrification of concentrated LNPs (lipids ~10 mM) was performed using a Leica EM GP operating at 21 °C and 95 % room humidity (RH). Sample suspensions were placed on glow discharged 100 µm lacey carbon film supported on 200 mesh copper grids (Electron Microscopy Sciences). Optimal results were achieved using a 60-second pre-blot and a 1-second blot time. After vitrification, sample grids were maintained below -170 °C, and imaging was performed on a Tecnai T12 (Thermo Fisher) with a biotwin lens and LaB6 filament operating at 120 keV equipped with an Eagle 4 K×4 K CCD camera (Thermo Fisher). Images were acquired at a nominal underfocus of -2 to -3 µm (49,000× magnification) with an electron dose of ∼2000 e/nm2. The size distribution of LNPs was based on 100 particles (Fiji ImageJ) from cryo images normalized by percentage distribution.

Circular dichroism measurements: CD spectra were recorded on a JASCO J-815 CD spectrometer fitted with a Peltier temperature controller. Unless otherwise specified, samples were measured at 20 °C in a quartz cuvette with a 2 mm path length. Spectra were recorded from 200 to 250 nm at 1 nm intervals, with a bandwidth of 1 nm, with the final spectrum consisting of the average of 5 sequentially recorded spectra. The mean residue molar ellipticity (θ, deg cm^2 $dmol^{-1}$) was calculated according to equation ($[\theta] = (100 * [\theta]obs)/(c * n * l)$), [θ]obs representing the observed ellipticity in mdeg, c the peptide concentration in mM, n the number of peptide bonds and l the path length of the cuvette in cm.

5. mRNA encapsulation efficiency.

The encapsulation efficiency (EE%) of EGFP-mRNA was measured using a Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen). For the determination of non-encapsulated EGFP-mRNA, LNPs after dialysis were diluted with 1 x TE buffer (RNase free) and treated with the RiboGreen™ reagent. For the determination of the total amount of EGFP-mRNA, LNPs after dialysis were treated with 1% Triton X-100 in TE buffer (RNase free) and incubated for 5 minutes followed by dilution with TE buffer and treatment with the RiboGreen™

reagent. The supplied RNA standards were used to generate a standard curve and changes in fluorescence was measured in 96-well plates using a TECAN Infinite M1000 Pro microplate reader. The percentage of mRNA encapsulation (EE%) was determined using the fraction of (Ftotal RNA – Ffree RNA)/Ftotal RNA * 100%.

6. Cell culture, and cell uptake study.

Cell culture: HeLa, CHO, NIH/3T3, and Jurkat cell lines purchased from ATCC were cultured according to ATCC guidelines. The DMEM and RPMI-1640 growth media (Sigma Aldrich) containing sodium bicarbonate, without sodium pyruvate and HEPES, were supplemented with 10% fetal bovine serum (Sigma), 1% L-glutamine (Thermo Fisher Scientific), and 1% penicillin/streptomycin (Thermo Fisher Scientific). HeLa, CHO, and NIH/3T3 were cultured with DMEM medium, and Jurkat was cultured with RPMI-1640 medium, at 37 °C in the presence of 5% CO_2.

Cell uptake (flow cytometry measurements): Flow cytometry analysis (FACs) of cellular uptake efficiency was performed to compare internalization efficiency differences. All lipids with a certain ratio (molar ratio DOPC:DOPE:cholesterol=2:1:1, 1 mol% of NBD-PE) were dried under N_2 flow, hydrated with PBS and sonicated at 55°C for 3 min. The CPE and CPK modified liposomes (CPE3-lipo, CPE4-lipo, CPK3-lipo, and CPK4-lipo) were made the same way while adding 1 mol% of CPE3, CPE4, CPK3, and CPK4 into the lipid mixture. CPK3, CPK4, CPE3, CPE4 lipid films were made, hydrated with complete DMEM, and sonicated for 10 min at room temperature. For cellular uptake efficiency tests, HeLa cells were pretreated with CPK3, CPK4, CPE3, CPE4 in DMEM for 2 h, then NBD labeled liposomes CPE3-lipo, CPE4-lipo, CPK3-lipo, and CPK4-lipo were added to the cells (15 min). After 15 min incubation, the medium was removed and cells were washed with PBS, digested with trypsin, washed, and resuspended in PBS, followed by flow cytometry measurements. For the cellular internalization efficiency of CPE3-LNP and CPE4-LNP, both LNPs were prepared as previously described by encapsulating Alexa488 nucleic acid, 1 mol% of CPE3 and CPE4 lipopeptides were added to the other lipids, and then proceeded to form LNPs, as described above, was followed.

Cell uptake (confocal imaging): Cells were seeded in an 8-well confocal slide at a density of $5*10^4$ cells/well and incubated at 37 °C in 5% CO_2, and after 18 h, the medium was removed and medium containing CPK4 (10 μM, 200 μL) and Hoechst 33342 (5 μM, 200 μL) was added and incubated for 2 h at 37 °C in 5% CO_2. Next, cells were washed with PBS (3X), and incubated with CPE4-LNP (200 μM, 200 μL) containing Alexa488 labeled nucleic acid for 15 min. The supernatant was removed and cells were washed with PBS, and DMEM free of phenol red indicator was added for confocal microscopy measurements using a Leica TCS SP8 confocal laser scanning microscope. For flow cytometry measurements, cells were seeded in 24-well plates at a density of $2.5*10^5$ cells/well, the rest of the procedure was the same as for the confocal measurements.

Endocytosis inhibitor assay (confocal imaging): HeLa cells were pretreated with nocodazole (40 μM), wortmannin (0.25 μM), dynasore (80 μM), pitstop2 (20 μM), genistein (200 μM), methyl-β-cyclodextrin (MβCD, 10 mM) or sodium azide (0.1% w/v) in DMEM medium for 1 h, after which the medium was replaced with medium containing lyso-tracker deep red (75 nM), CPK4 (10 μM), and fresh inhibitors and incubated for 2 h, then Alexa488 nucleic acid encapsulated CPE4-LNP (200 μM) were incubated in the presence of the inhibitors. After 15 min, the cells were washed three times, and phenol red indicator free DMEM was added for confocal microscopy imaging. When performing cellular uptake assays at 4 °C, cells were first incubated with lyso-tracker deep red (75 nM) and CPK4 (10 μM) for 2 h at 37 °C, then 1 h at 4 °C. The medium was removed and cells were washed and incubated for 15 min at 4 °C in the presence of CPE4-LNP (200 μM), followed by confocal imaging.

Endocytosis inhibitor assay (flow cytometry measurements): Cells were seeded on 24-well plates at a density of $2.5*10^5$ cells/well. After 18 h, the medium was removed and cells were incubated with inhibitors and CPK4 in medium (10 μM) for 2 h. Then Alexa488 nucleic acid encapsulated CPE4-LNP (200 μM, 15 min) was added in the presence of fresh inhibitors. The cells were washed, digested, and flow cytometry measurements using a Guava easyCyte machine (Luminex Corporation) were performed.

For the endocytosis pathway assay of unmodified LNPs, the cells were preincubated with endocytosis inhibitors for 2 h, then LNPs were added to the cells in the presence of fresh inhibitors and incubated for 4 h, and washed before the confocal imaging and flow cytometry measurements.

7. Lysosome colocalization study.

HeLa cells were seeded on 8-well confocal plates at a density of $5*10^4$cells/well. After overnight growth the cells were treated with lyso-tracker deep red (75 nM) and CPK4 (10 μM) for 2 h. The supernatant was removed, and Alexa488 nucleic acid encapsulated CPE4-LNP (200 μM) was added and incubated for 15 min. The medium was removed, and lyso-tracker deep red (75 nM) in DMEM was added and incubated at different times before confocal imaging.

8. Transfection assay.

CPE4-LNPs and LNPs encapsulating EGFP-mRNA were prepared as described previously. HeLa, CHO, NIH/3T3, and Jurkat were cultured in 8-well confocal plates at the density of $2*10^4$ cells/well overnight before cells were pretreated with CPK4-medium (10 μM) for 2 h, washed three times with PBS, then LNPs (1 μg/mL) were added to the cells and incubated for 2 h, then the medium was removed and washed three times, refreshed with fresh medium for continuous 18-24 h culturing before confocal imaging and flow cytometry measurements. The concentration of LNPs was determined by Quant-iT Ribogreen RNA assay. The commercial transfection agent lipofectamine 3000/EGFP-mRNA was prepared according to the manufacturers protocol using the same amount of EGFP-mRNA, and cells were transfected for 2 h and refreshed with medium before 18-24 h culturing.

9. Cell viability measurements.

HeLa cells were seeded on 96-well plates at a density of $1*10^4$ cells per well overnight, then the same procedure as previously described was followed but different concentrations of LNPs (0.25 μg/mL, 0.5 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL) were added. After 24 h incubation, cell proliferation reagent WST-1 solution (20 μL, Sigma-Aldrich) was added to the medium (200 μL) and cells were incubated for another 4 h at 37 °C. The absorbance at 450 nm was measured at room temperature using a Tecan infinite M1000. The cell viability was normalized with a control (blank HeLa cells), which was set at 100% cell survival.

10. Statistical analysis

All experiments were performed in triplicate (n=3) unless specified otherwise, and the significance was determined using an unpaired student t-test (Graphpad Prism) for all comparisons. *p≤0.05, **p≤0.01, ***p≤0.001, ****P<0.0001.

11. Safety statement

No unexpected or unusually high safety hazards were encountered.

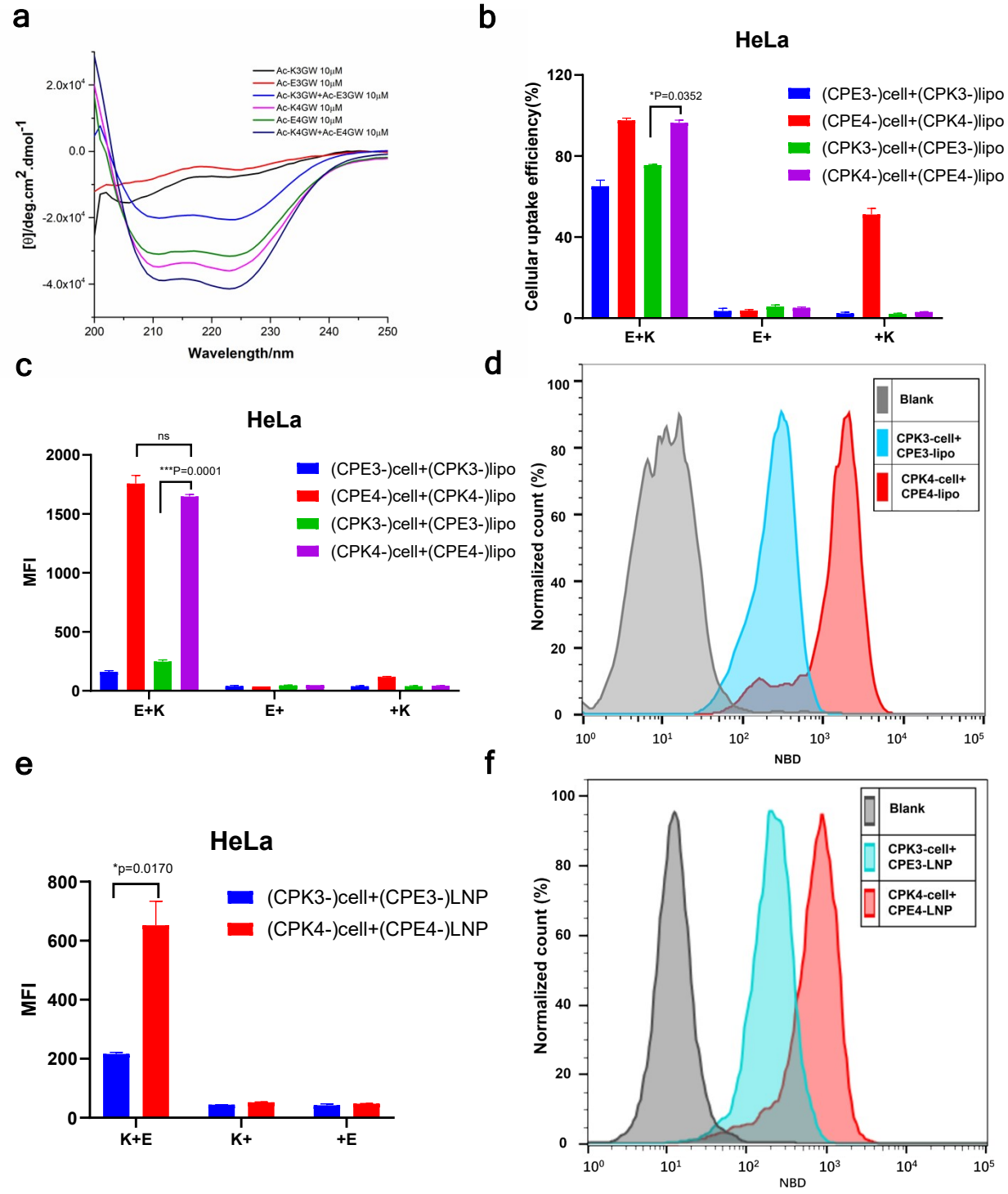


Fig. S1 Evaluation of coiled-coil peptide pair mediated uptake in liposomes and LNPs. (a) CD spectra of K3/E3 and K4/E4 pairs. Peptides were dissolved at a total concentration of 10 μM in PBS at pH 7.4, and spectra were measured at 20℃. (b) Cellular uptake of liposomes in HeLa cells. Uptake efficiency was calculated by quantifying the NBD-positive cells. (c-d) The fluorescence intensity (MFI) of cellular internalization of liposomes with HeLa cells. Lipid compositions of liposomes: DOPC/DOPE/cholesterol=2:1:1, 1 mol% of the NBD-PE served as the fluorescent dye, 1 mol% of the CPK (3 or 4) or CPE (3 or 4) were added for lipopeptide modified liposomes. E+K: both E and K peptide included; E+: only E peptide included; K+: only K peptide included. (e-f) The fluorescence intensity of cellular internalization of LNPs encapsulated nucleic acid by CPE3/4-LNP with HeLa cells pretreated with CPK3/4. Alexa488 labeled nucleic acid was encapsulated and served as the fluorescent dye. Unpaired t-test was used to determine the significance of the comparisons of data indicated in b, c, and d (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). In all panels, error bars represent mean ± s.d. (representative dataset from n=3 biologically independent samples).

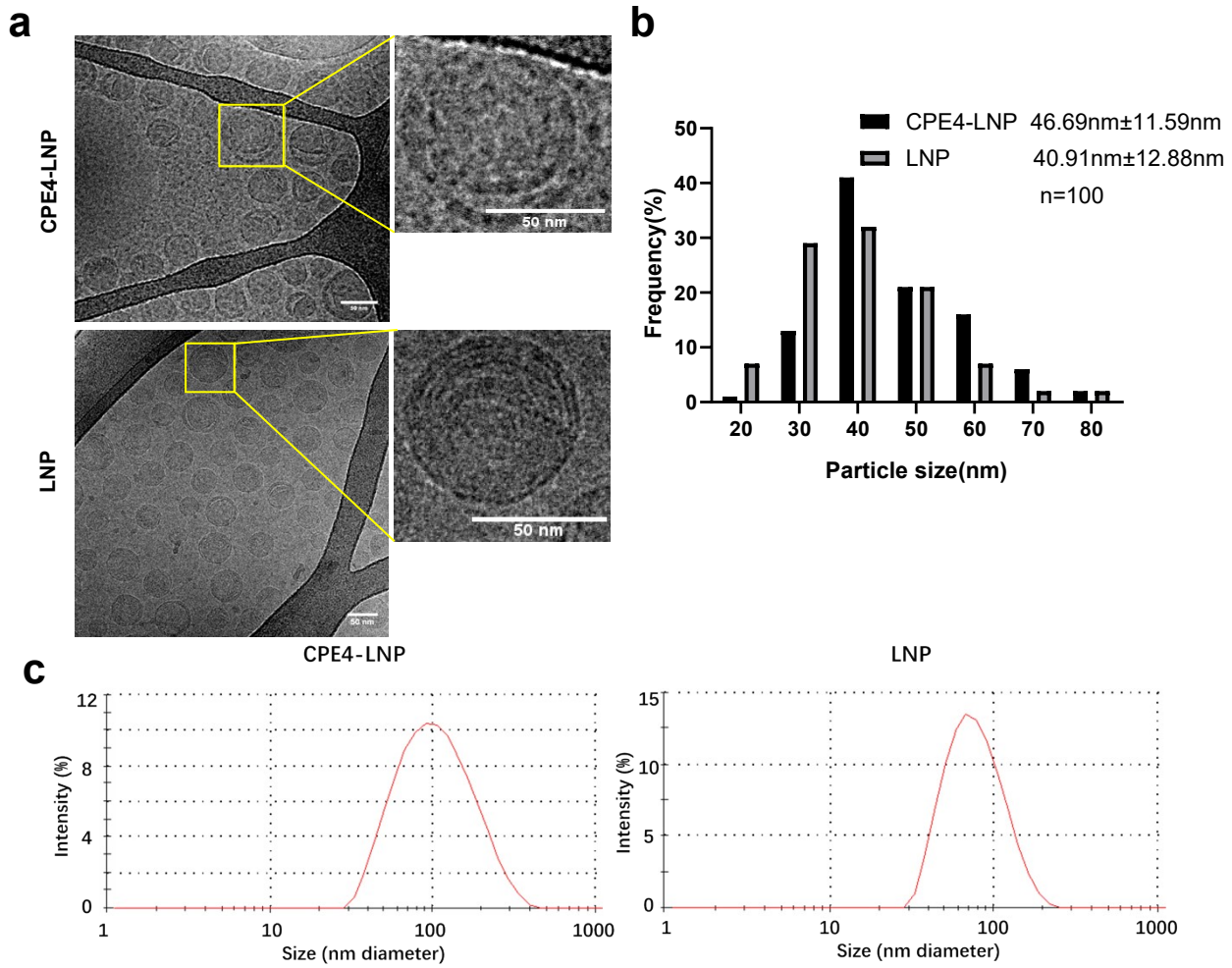
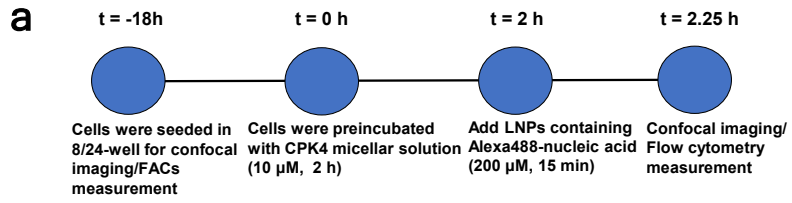
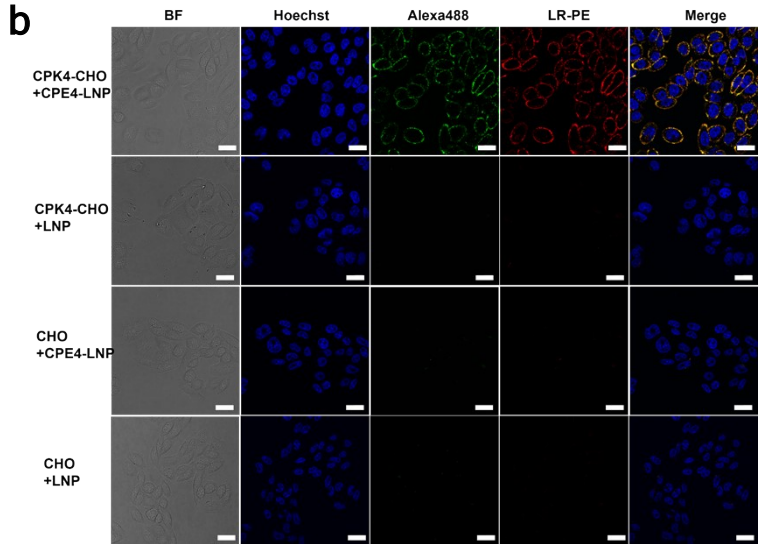
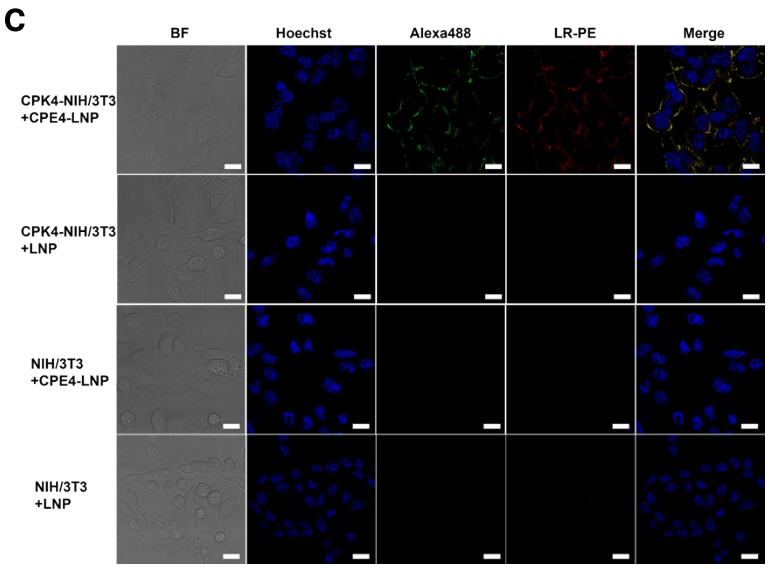


Fig. S2 (a) Cryo-EM images of Alexa488-nucleic acid encapsulated CPE4-LNP and LNP. (b) Size distribution of Alexa488-nucleic acid encapsulated LNPs as determined by cryo-EM. The values derived from the frequency distribution graphs represent mean ± s.d. (n=100). Scale bar is 50 nm. (c) Size distribution of Alexa488-nucleic acid encapsulated LNPs according to DLS.


a
t = -18h
t = 0 h
t = 2 h
t = 2.25 h
Cells were seeded in 8/24-well for confocal imaging/FACs measurement
Cells were preincubated with CPK4 micellar solution (10 μM, 2 h)
Add LNPs containing Alexa488-nucleic acid (200 μM, 15 min)
Confocal imaging/ Flow cytometry measurement


b
BF
Hoechst
Alexa488
LR-PE
Merge
CPK4-CHO +CPE4-LNP
CPK4-CHO +LNP
CHO +CPE4-LNP
CHO +LNP


c
BF
Hoechst
Alexa488
LR-PE
Merge
CPK4-NIH/3T3 +CPE4-LNP
CPK4-NIH/3T3 +LNP
NIH/3T3 +CPE4-LNP
NIH/3T3 +LNP

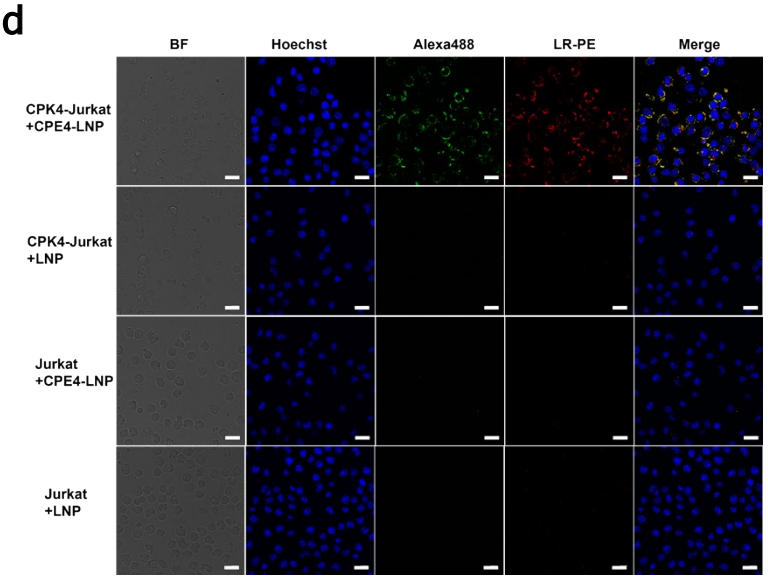

d
BF
Hoechst
Alexa488
LR-PE
Merge
CPK4-Jurkat +CPE4-LNP
CPK4-Jurkat +LNP
Jurkat +CPE4-LNP
Jurkat +LNP

Fig. S3 (a) Schematic representation of the LNPs uptake experiments with cells. (b) Confocal microscopic images of LNPs uptake with CHO cells. (c) with NIH/3T3 cells. (d) with Jurkat cells. Cells were preincubated with a micellar CPK4 solution (10 µM, 200 µL, 2 h). After removal of the medium, the LNPs containing Alexa488-nucleic acid were added (200 µM, 200 µL, 15 min), then cells were washed before imaging. Blue: Hoechst 33342; green: Alexa488-nucleic acid; red: LR-PE; BF: bright field. Scale bar is 20 µm.

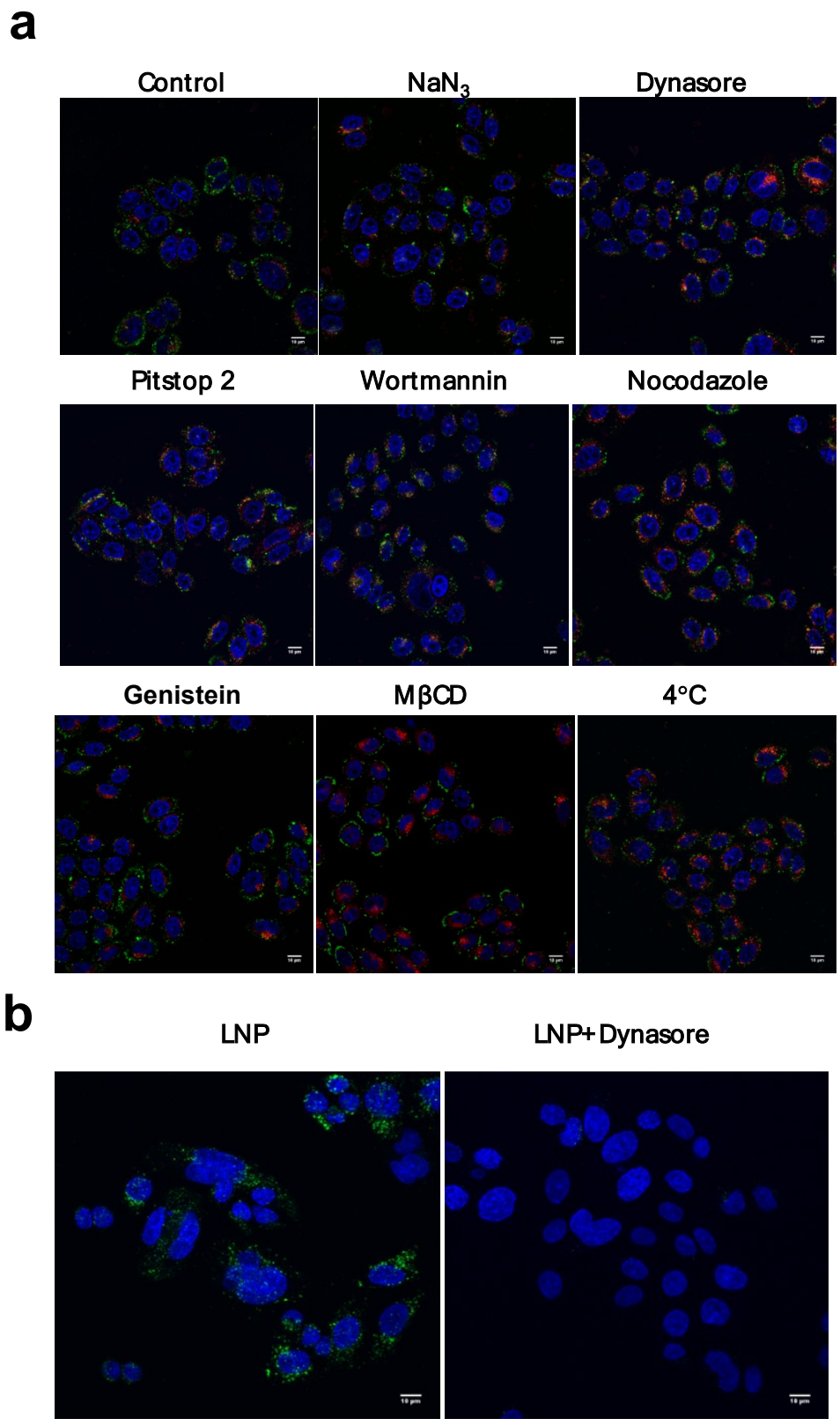


Fig. S4 (a) Confocal microscopic images of cellular uptake of CPE4-LNP with CPK4-HeLa cells in the presence of endocytosis inhibitors. HeLa cells were

first treated with different endocytosis inhibitors (1 h), followed by lyso-tracker deep red (75 nM, 200 µL) and CPK4 (10 µM, 200 µL, 2 h, in the presence of fresh inhibitors) incubation, then CPE4-LNP (200 µM, 200 µL, 15 min) were added together with fresh inhibitors, then cells were washed and added with phenol red free DMEM before imaging. Blue: Hoechst 33342; green: Alexa488-nucleic acid; red: lyso-tracker deep red. (b) Confocal microscopic images of cellular internalization of LNP with HeLa cells with endocytosis inhibitor dynasore. HeLa cells were pretreated with dynasore (80 µM, 200 µL, 1 h), then LNP (200 µM, 200 µL, 4 h) were incubated with the presence of fresh dynasore, and cells were washed before imaging. Blue: Hoechst 33342; green: Alexa488-nucleic acid. Scale bar is 20 µm.

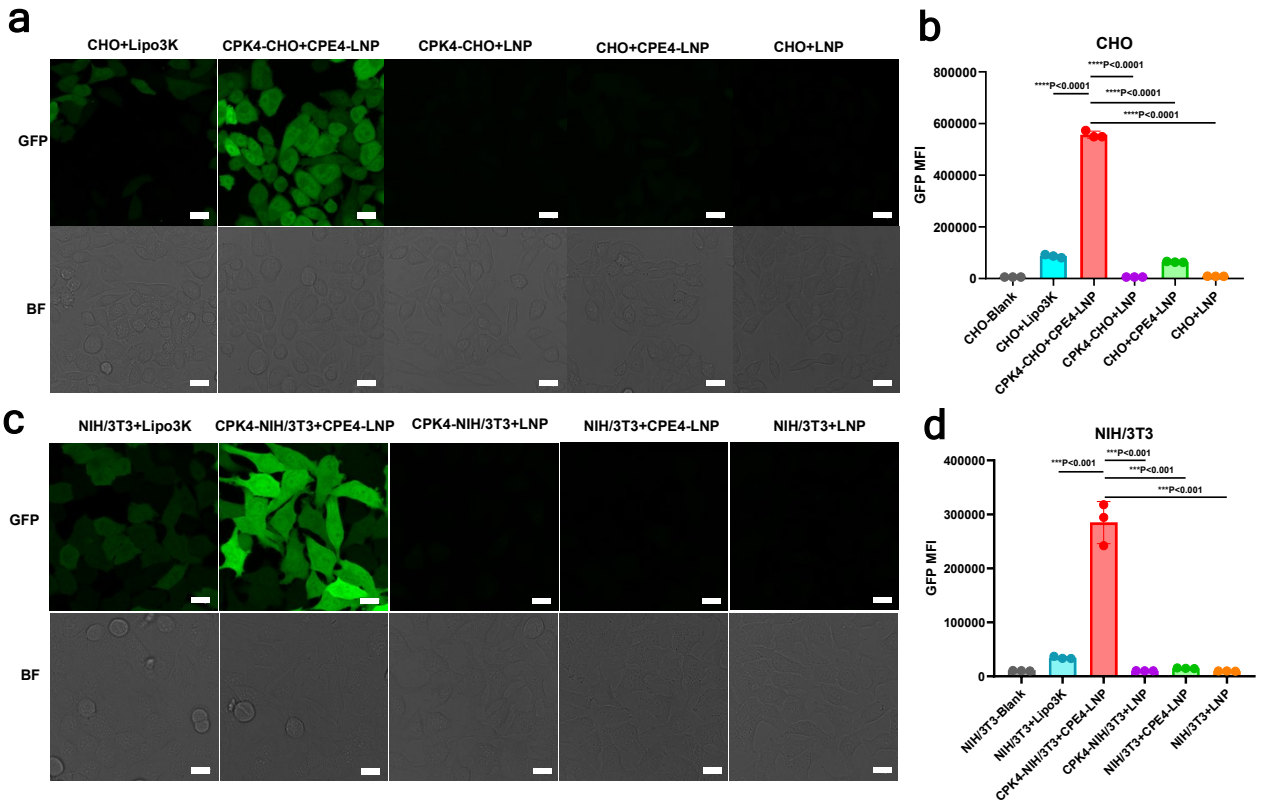


Fig. S5 (a) Confocal microscopic images of the EGFP-mRNA transfection of LNPs with CHO cells. Scale bar is 20 µm. (b) The GFP expression fluorescence intensity (GFP MFI) of LNPs with CHO cells measured by flow cytometry. (c) Confocal microscopic images of the EGFP-mRNA transfection of LNPs with NIH/3T3 cells. Scale bar is 20 µm. (d) The GFP expression fluorescence intensity (GFP MFI) of LNPs with NIH/3T3 cells measured by flow cytometry. Cells were pretreated with CPK4 solution (10 µM, 200 µL, 2 h), after removal of the medium, EGFP-mRNA encapsulated LNPs were added (1 µg/mL, 200 µL, 2 h), and then cultured for another 18-24 h before imaging and flow cytometry measurements. Lipo3K: lipofectamine 3000; GFP: green fluorescent protein; BF: bright field. Unpaired student t-test was used to determine the significance of the comparisons of data indicated in b, and d (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). In all panels, error bars represent mean ± s.d. (representative dataset from n=3 biologically independent samples).

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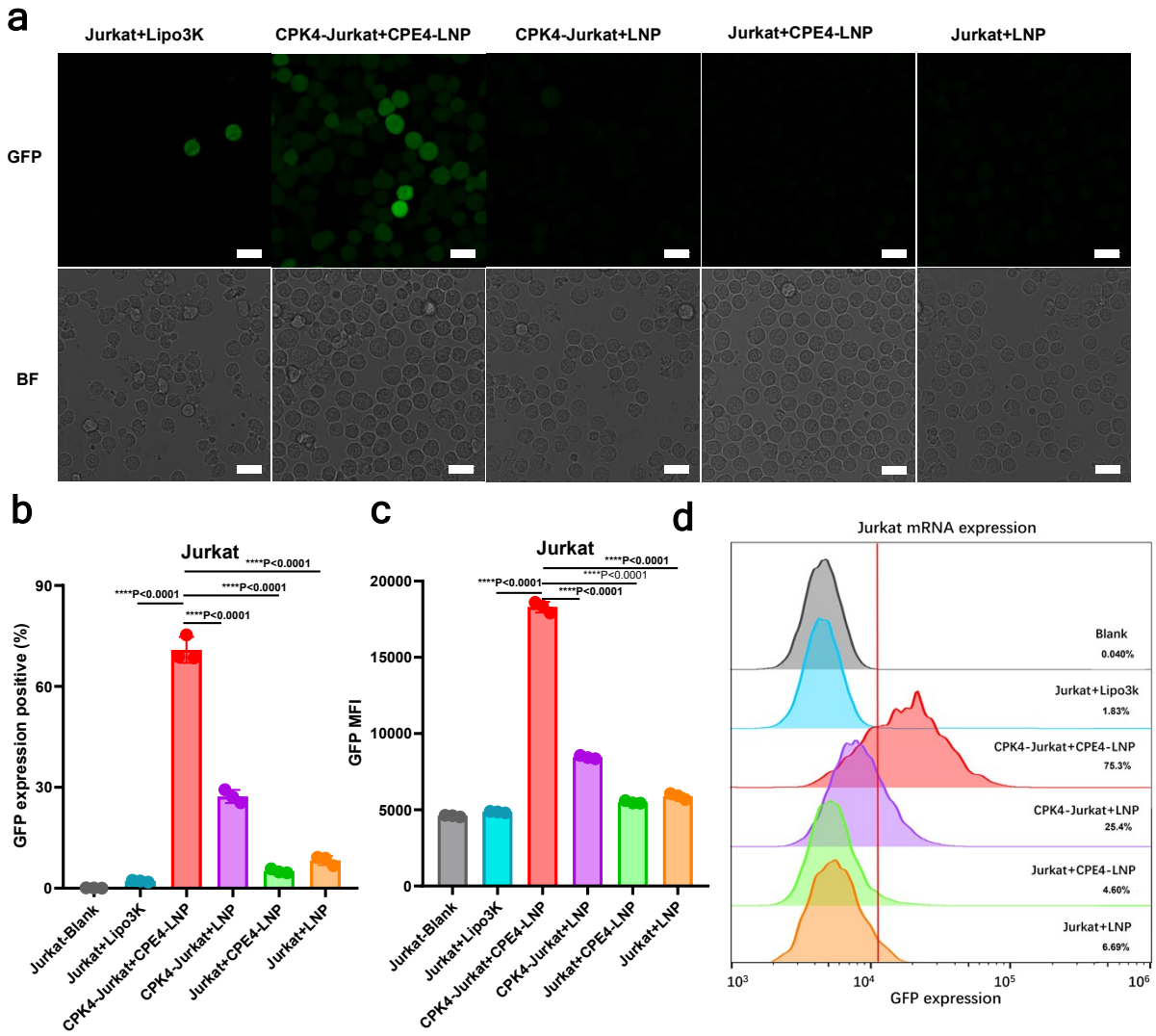

Fig. S6 Transfection efficiency of the fusogenic coiled-coil peptide system with Jurkat cells. (a) Confocal microscopic images of the EGFP-mRNA transfection of LNPs. Lipo3K: lipofectamine 3000; GFP: green fluorescent protein; BF: bright field. Scale bar is 20 µm. (b) The quantification of EGFP-mRNA transfection efficiency of LNPs. (c-d) The GFP expression fluorescence intensity (GFP MFI) of LNPs. Jurkat cells were first incubated with CPK4 (10 µM, 200 µL, 2 h), followed by EGFP-mRNA encapsulated LNPs were incubated (1 µg/mL, 200 µL, 2 h), after that, cells were washed 3 times and cultured for another 18-24h before imaging and flow cytometry measurements (representative dataset from n=3/group). Unpaired student t-test was used to determine the significance of the comparisons of data indicated in b, and c (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). In all panels, error bars represent mean ± s.d. (representative dataset from n=3 biologically independent samples).

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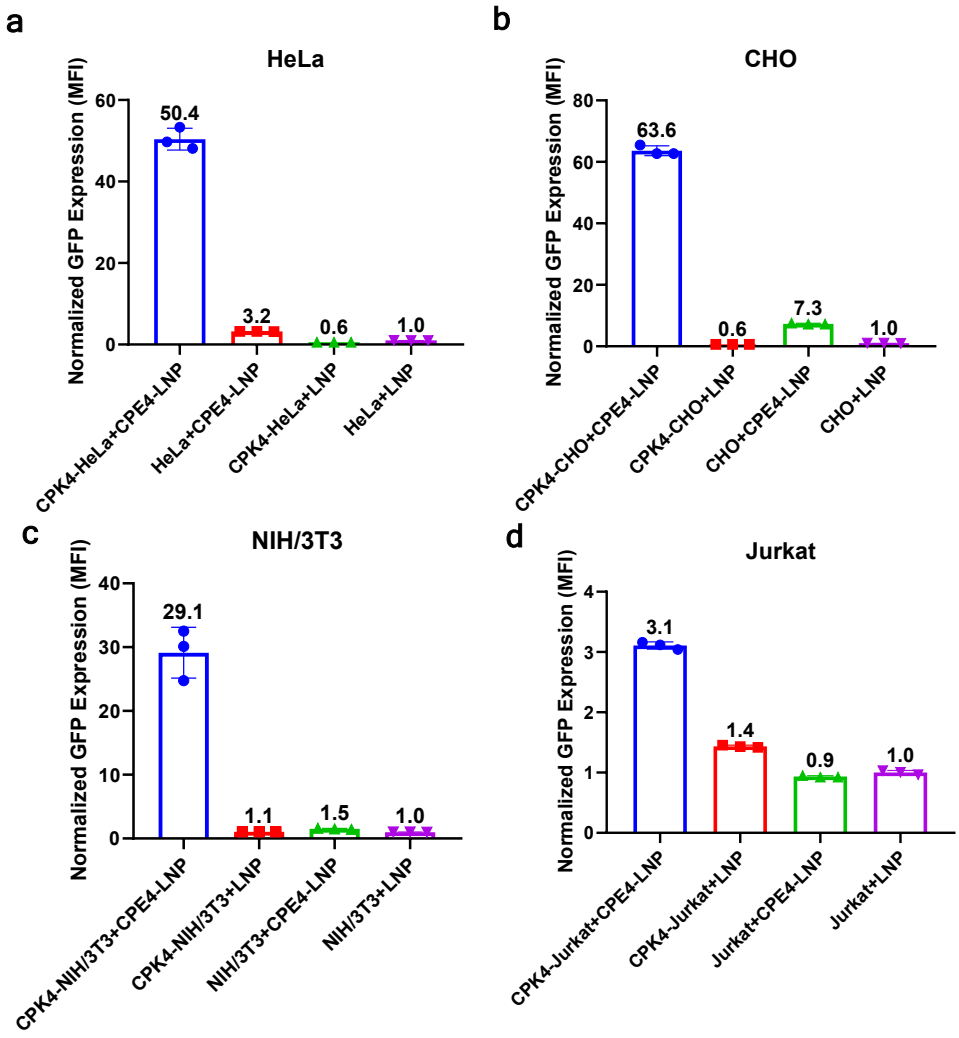


Fig. S7 GFP fluorescence enhancement. The protein expression fold number of GFP fluorescence intensity (GFP MFI) of groups normalized to plain LNP (a) with HeLa cells, (b) with CHO cells, (c) with NIH/3T3 cells, (d) with Jurkat cells. In all panels, error bars represent mean ± s.d. (n=3).

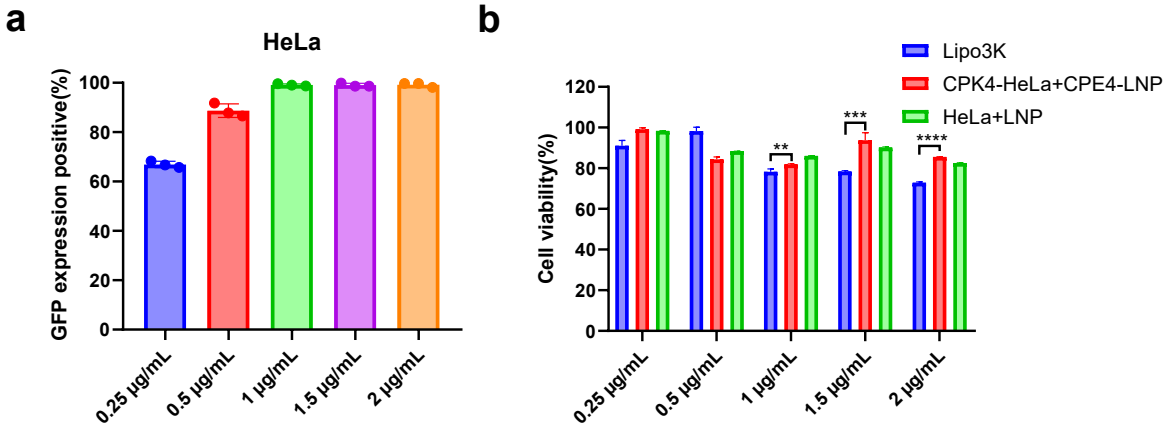
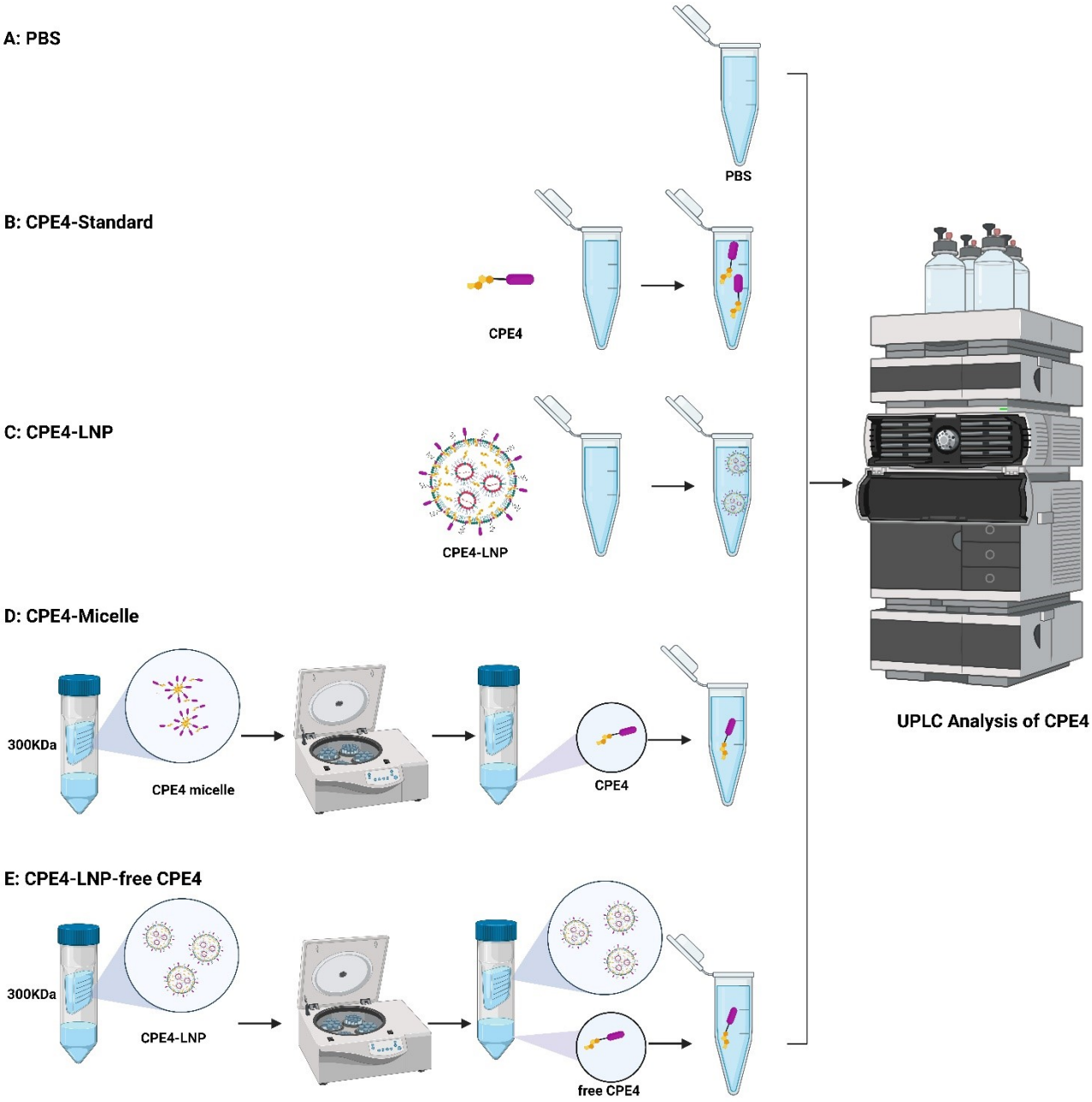


Fig. S8 (a) Transfection efficiency of different concentrations of EGFP-mRNA encapsulated CPE4-LNP with HeLa cells pretreated with CPK4. HeLa cells were first incubated with CPK4 (10 μM, 200 μL, 2 h), followed by different concentrations of EGFP-mRNA encapsulated CPE4-LNP incubation (2 h), then the medium was removed, cells were washed and cultured for another 18-24 h before flow cytometry measurements. (b) The cell viability evaluation of EGFP-mRNA encapsulated LNPs after transfection. HeLa cells were first incubated with CPK4 (10 μM, 200 μL, 2 h), followed by different concentrations of EGFP-mRNA encapsulated LNPs incubation (2 h), then the medium was removed, and cells were washed and cultured for another

24 h. After that, WST-1 solution (20 μL) was added to the medium (200 μL) and incubated for 4 h before measuring. Unpaired student t-test was used to determine the significance of the comparisons of data indicated in b (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). In all panels, error bars represent mean ± s.d. (representative dataset from n=3 biologically independent samples).



Scheme S1. Protocol of CPE4 detection using ultra-high-performance liquid chromatography.

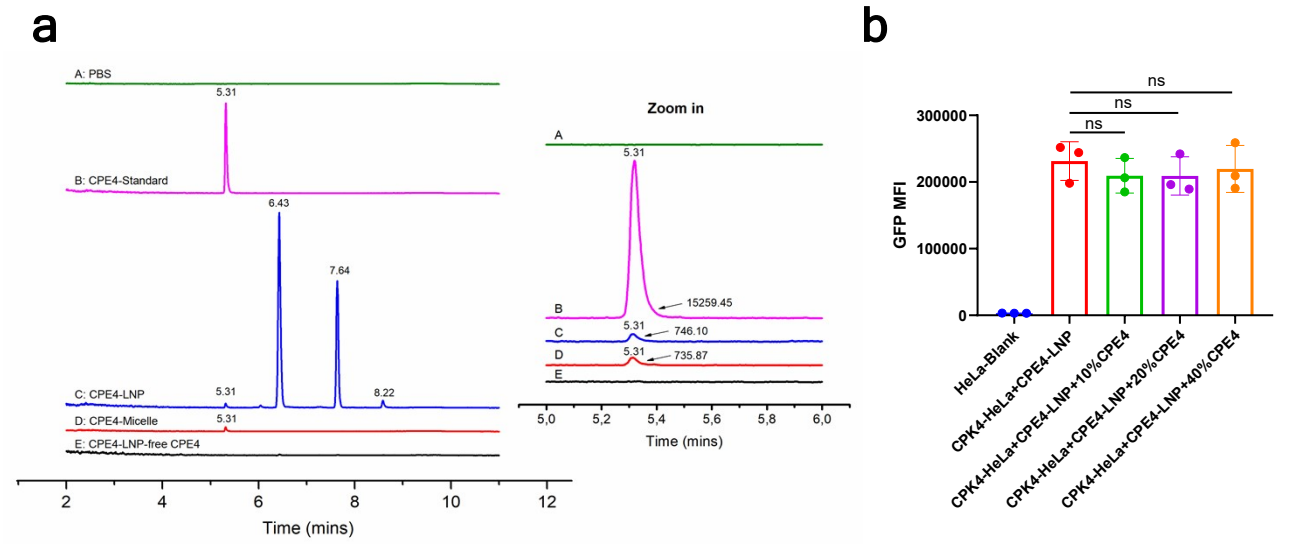


Fig. S9 (a) Ultra high-performance liquid chromatography (UHPLC) measurement of CPE4 lipopeptide. A: PBS; B: CPE4-Standard, pure CPE4 lipopeptide solution; C: CPE4-LNP formulation; D: CPE4-Micelle, the lower solution of CPE4-micelle after centrifugation with a 300KDa cut-off membrane; E: CPE4-LNP-free CPE4, the lower solution of CPE4-LNP after separation by centrifugation with a 300KDa cut-off membrane. The retention time of CPE4 is 5.31 min. Samples were diluted 5X after preparation, which was the same dilution factor as used in the in vitro cellular experiments. All measurements were completed three times, one representative measurement was shown. (b) The GFP expression fluorescence intensity (GFP MFI) of LNPs. HeLa cells were first incubated with CPK4 (10 μM, 200 μL, 2 h), followed by incubation with EGFP-mRNA encapsulated CPE4-LNP, and CPE4-LNP with extra 10%CPE4, 20%CPE4, 40%CPE4 (EGFP-mRNA, 1 μg/mL, 2 h). Cells were then washed 3 times and cultured for another 18-24 h before flow cytometry measurements. Unpaired student t-test was used to determine the significance of the comparisons of data (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns, not significant). In all panels, error bars represent mean ± s.d. (representative dataset from n=3 biologically independent samples).

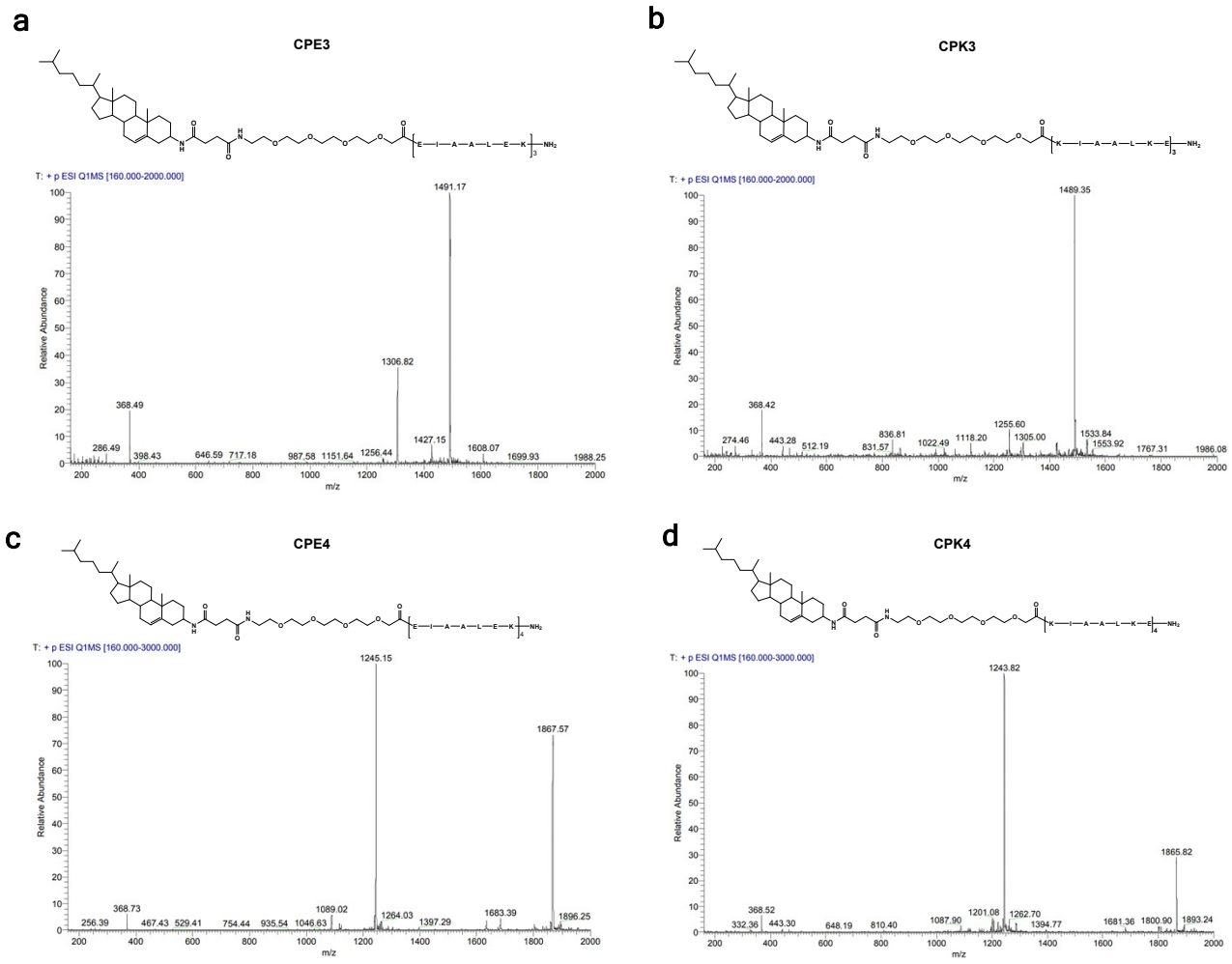


Fig. S10 LC-MS spectrum of (a) CPE3, (b) CPK3, (c) CPE4 and (d) CPK4.

References

1. J. Yang, A. Bahreman, G. Daudey, J. Bussmann, R. C. Olsthoorn and A. Kros, *ACS Cent Sci*, 2016, **2**, 621-630.