Supplementary Information (SI) for Nanoscale Horizons.
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Materials and methods

1. Chemicals and Materials

The peptides were synthesized by Beyonpep Biotechnology Limited (China). All amino acids used in solid phase synthesis are L-amino acids. Details of peptides are shown in **Table 1** and **Table 2**.

Table 1. List of Peptide Modules and Module Abbreviations.

Peptide	Abbreviation
KKKRRGK	A
LLLLLL	B
HHHH	C
RGDK	X_1
GYQTI	X_2
PLGL-SSSSSSSSSSSSSSS	Y

Table 2. List of peptides obtained by solid phase synthesis.

Peptide	Abbreviation
KKKRRGKLLLLLLCHHHHRGDK	$ABCX_1$
KKKRRGKLLLLLLCHHHHGYQTI	$ABCX_2$
KKKRRGKLLLLLLHHHHGYQTI-PLGL-SSSSSSSSSSSSSSS	$ABCX_2Y$

siRNA targeting GFP, FITC-siRNA, siRNA targeting PTEN, amplification primers for GAPDH and PTEN were obtained from General Bio (Anhui) Co., Ltd. (China). GFP mRNA (Catalog No. R1001), Firefly Luciferase mRNA (Catalog No. R1005), Cy5-mRNA (Catalog No. R1010), Cell Counting Kit-8 (CCK-8) (Catalog No. K1018) and D-Luciferin potassium salt (Catalog No. C3654) were obtained from APExBIO Co., Ltd. (China). Dapi (Catalog No. C0065) and Lyso-Tracker Red (Catalog No. IF1840) were obtained from Beijing Solarbio Science & Technology Co., Ltd. (China). Goldview nucleic acid dye (BS357A) purchased from Biosharp (China) Commercial transfection reagent Lipofectamine 2000 was purchased from Thermo Fisher Scientific. Fetal Bovine Serum (FBS) was obtained from TransGen Biotech Co., Ltd. (China). The Eastep® Super Total RNA Extraction Kit (LS1040, Promega, Madison, WI, USA) and HiScript II One Step qRT-PCR SYBR Green Kit (Q221-01, Vazyme Biotech Co., Ltd., Nanjing, China) were used for RNA extraction and quantitative RT-PCR analysis.

2. Peptide assembly and peptide-RNA co-assembly

In the peptide-nucleic acid assembly method employed in this study, the N/P ratio calculation method referenced Kong's work[1] to quantitatively represent the dosage relationship between peptides and nucleic acids. The dosage ranges of peptides and nucleic acids in cell and mouse experiments referenced the work of Kong, Wang, Tang, et al.[1-4] Nanoparticles were assembled

in the absence and presence of nucleic acid. The peptides were dissolved in the neutral buffer (pH=7, 10 mM citrate buffer) to achieve a concentration of 1mg/mL. For the self-assembly of the complex by one-component peptides and RNA (C11 and C12), RNA was diluted in water first at a concentration of 1mg/mL, followed by the addition of the peptide solution (1mg/mL) and then mixed by vortexing. For the self-assembly of the complex by multicomponent peptides and RNA (C2 and C3), RNA was diluted in water first at a concentration of 1mg/mL, followed by the addition of premixed peptide solution (Peptides of different components were first dissolved in the neutral buffer at a concentration of 1 mg/mL, then mixed according to precalculated ratios to obtain a premixed peptide solution). Samples were usually incubated for 30 min at room temperature before further structure characterization. If not specified, the samples were diluted again with neutral buffer 30 minutes after assembly until the RNA concentration reached 150 μM. As the complexes are stabilized through dynamic electrostatic interactions, no further purification was performed, and the assemblies were used directly for subsequent experiments.

3. Determination of Free Peptide Concentration

Firstly, C2 formulation peptide solutions at total concentrations of 0.01, 0.05, 0.1, 0.5, and 1 mg/mL were introduced into the analytical chromatograph. The peak areas were then fitted to concentration data to generate a standard curve (**Fig. S1**). Subsequently, under the two N/P ratios 10 and 30, C2 formula peptides were assembled with random sequence siRNA. After 30 minutes, the mixture was centrifuged for 15 minutes at 14000g with a 10 kDa ultrafiltration membrane. The filtrate was then analyzed by analytical chromatography, and the peak area was substituted into the standard curve to determine the peptide concentration in the assembly system filtrate.

4. Cells Culturing and Transfections

HeLa, GFP-Hela and 293T cells were purchased from cellverse (Shanghai) Co., Ltd., pen/strep were purchased from Tianjin labbiotech Co., Ltd. HeLa and 293T cells were cultured in DMEM (10% FBS and 1% Pen/Strep) at 37 °C under 5% CO_2. Cells were grown in 24-well plates for about 12 hours before transfection experiments were carried out. Once the peptide-RNA complex has been assembled, dilute it to 500 μL with serum-free Opti-MEM and mix thoroughly. Before adding the Opti-MEM-diluted complex to the well plate, remove the DMEM complete medium from the well plate, then add the mixture to the well plate for approximately 4 hours. Subsequently, replace the Opti-MEM with DMEM complete medium. Regarding the use of Lipofectamine, Thermo Fisher Scientific's instructions were uniformly followed throughout this work. Positive control samples were prepared using a ratio of 1 μg of nucleic acid to 1 μL of Lipofectamine 2000.

5. Regulation of GFP expression by an antisense effect

GFP-HeLa cells were obtained by lentivirus infection. Cells were cultured in MEM supplemented with 10% FBS and 1% Pen/Strep at 37 °C under 5% CO_2. Prior to experimentation, purineomycin selection was performed to enhance expression efficiency, and GFP positivity rates were assessed by flow cytometry. Silencing assays were conducted only after positivity rates exceeded 95%. The cells (1×10^5) were seeded in 24-well plates and incubated overnight at 37 °C. Next, the cells were washed with PBS buffer, and the medium was changed to Opti-MEM. Then siRNA samples (150nM) were complexed with peptides, added to the wells, and incubated for 4 h. Then all the mediums were replaced with MEM supplemented with 10% FBS and incubated. 24 h later, the original MEM medium was again replaced with fresh MEM complete medium. After another 48 h, the fluorescence was observed by fluorescence microscopy. Then the cells were washed with PBS and trypsinized, followed by resuspension in PBS supplemented with 10% cell dissociation buffer and 1% FBS. The cells were analyzed by FACS Calibur (BD biosciences) using Flowjo software. During flow cytometry analysis, the gating strategy was supplemented in the supporting information(**Fig. S2)**.

6. In Vitro Cell Viability

Cytotoxicity parameters of the peptides were evaluated using Cell Counting Kit-8 (APE×BIO) in accordance with the manufacturer's instructions. Briefly, cells were plated at 5.0×10^3 cells/100 µL in a 96-well plate and incubated for 12 h at 37 °C. The cells were treated with peptides at varying concentrations and incubated at 37 °C for 48 h. Then 10 µL of Cell Counting Kit-8 solution was added to each well. After incubation for 2 h, the absorbance at 450 nm was measured using a microplate reader (EnSpire Multilabel Reader, PerkinElmer Inc.) at 25 °C. The assay was performed in three replicates for each sample.

7. Determination of the peptide-RNA complex uptake level

The peptides were dissolved in the neutral buffer to achieve a concentration of 1mg/mL. The delivered siRNA was labeled with FITC-fluorescent label and diluted in water first, followed by the addition of peptide solution, which was mixed by vortexing. It should be noted that the sequence of this siRNA is completely random and has no functional significance. In this experiment, it is used solely to detect nucleic acid uptake. Samples were usually incubated avoiding the light for 30 min at room temperature. And the uptake level of the complex to the cell was observed by fluorescence microscopy and flow cytometry after 4 h. siRNA concentration was fixed at 150 nM, and complexes with N/P of 0-40 were selected for the experiment.

8. Determination of the peptide-mRNA complex transfection level

The peptides were dissolved in the acidic citrate buffer solution with a pH of 7 and a concentration of 10 mM to achieve a final concentration of 1mg/mL. The delivered GFP-mRNA was diluted with acidic citrate buffer solution (pH=3 and a concentration of 10 mM) followed by the addition of peptide solution, which was mixed by vortexing. Samples were usually incubated avoiding the light for 30 min at room temperature and incubated for 4 h. Then all the media were replaced with DMEM supplemented with 10% FBS and incubated. After 48 h, the cells were washed with PBS and trypsinized, followed by resuspension in PBS supplemented with 10% cell dissociation buffer and 1% FBS. The cells were analyzed by FACS Calibur (BD Biosciences) using FlowJo software.

9. Circular Dichroism Spectroscopy

The secondary structure of samples was characterized by circular dichroism (CD) with a Jasco-810 CD spectrophotometer (Japan). A freshly prepared sample (25 µL, 3mg/mL) was loaded carefully into a cell with a path length of 0.1 mm (Precision Cells, Inc., USA), which was scanned at room temperature by subtracting the buffer background. The presented spectra represent an average of three scans between 350 nm and 180 nm with a step size of 0.2 nm and a speed of 100 nm/s.

10. Agarose gel electrophoresis

Agarose gel electrophoresis was used to detect the packaging effect of peptide carriers on nucleic acid drugs. Specifically, when preparing the gel, agarose with a concentration of 1.2% was added to the TAE buffer (1.2 g of agarose was added to 100 mL of 1×TAE buffer), and then it was heated in a microwave oven until it was completely dissolved. After the gel cools, GoldView nucleic acid dye is added, and the solution is shaken to ensure uniform dissolution of the dye. The gel is then poured into a mold and cooled until it solidifies completely. During gel cooling, prepare multiple N/P peptide-nucleic acid complexes at 2 µg of nucleic acid per sample. After 30 minutes of complex assembly, add DNA Loading Buffer to the complex solution, supplement with deionized water to achieve a total volume of 50 µL per sample, and thoroughly mix the samples. After the gel has solidified, add the peptide-nucleic acid samples to the wells of the gel and place the gel in the electrophoresis chamber. Add 1×TAE buffer to the electrophoresis chamber until the liquid level covers the agarose gel. Set the electrophoresis parameters: current 400 mA, voltage 125 V, electrophoresis time 25 minutes, and start the

electrophoresis. After completion, place the gel on an imaging system to observe and record the positions of the fluorescent bands representing nucleic acids. Finally, extract the grayscale data from each lane in the result image and calculate the nucleic acid encapsulation efficiency based on the grayscale values.

11. Zeta Potential Analysis

Zeta potential was analyzed on Malvern Panalytical Instruments Zetasizer nano ZS90. 1.2 mL freshly prepared sample at a peptide concentration of 40 μg/mL was directly added to a disposable fold capillary cell (DTS1070) for potential measurement. Finally, select the sample type as peptide, and the solvent as water. The test results are based on three repetitions of the experiment.

12. Transmission Electron Microscopy

The morphology of peptide assemblies was imaged by TEM at room temperature. A 10 μL sample was applied on a copper grid and incubated for 3 min, followed by removing excess solution with filter paper and an addition of 10 μL staining solution (10mg/mL phosphotungstic acid, Sigma-Aldrich) for 15 s. After wicking away the staining solution, the sample grids were placed in a desiccator to dry under a vacuum overnight. A JOEL JEM-1400 Flash transmission electron microscope was used to image the samples at 80 kV.

13. Dynamic light scattering

Dynamic light scattering (DLS) was carried out on a Malvern Panalytical Instruments Zetasizer nano ZS90. Use Zetasizer Software to control the instrument for testing. Set the sample type to "protein" and the solvent to water. Since the sample is aqueous, a glass cuvette was selected for particle size measurement. All samples were prepared from aqueous peptide-RNA complex solutions and studied at 25 °C. Three repeat measurements were taken.

14. Confocal Laser Scanning Microscope

The samples were added in the same way as the uptake level experiments. The difference is that HeLa cells were added to the Thermo Scientific Nunc petri dish with a glass bottom at a concentration of 1×10^5/mL 12 h before experiments. 4 h later, cells were washed 3 times with 1× PBS and treated with complete medium containing Lyso-Tracker Red. According to the reagent manufacturer's instructions, the original concentration of Lyso-Tracker Red is 1 mM. When using it, take 1 μL and add it to 15 mL of DMEM complete culture medium to dilute it, resulting in a final concentration of 66.7 nM. When staining, take 1 mL of the diluted Lyso-Tracker Red and add it to the dish for 1h. Finally, fix the cells with 4% polyformaldehyde for 30 minutes. Cells were then washed 3 times with 1×PBS and nuclei were stained with DAPI for 5 min. Cells were washed 3 times with 1×PBS before adding complete medium. Finally, the uptake status of FITC-siRNA was observed on Super-resolution confocal microscopy (Leica SP8) with a 63× oil lens.

15. Encapsulation kinetics

All kinetic measurements were carried out at room temperature in deionized water. For each experiment to determine k_{on}, the fluorescence emission of a 200 μL solution of Atto488-labelled random sequence siRNA (10 nM) was measured at 515 nm (excitation, 488 nm) and this value was taken as t = 0 s. Then, different volumes of deionized water were added to each experimental well. Finally, peptide solutions of equal concentration but different volumes were added to each experimental well, resulting in final peptide concentrations ranging from 25 to 125 nM in each well. The drop in emission over time was monitored at intervals of 10 s. The plot of emission vs. time was fit to a single exponential ($y = A*\exp(-x/t) + y_0$), providing k_{obs} for each concentration of peptides. These k_{obs} values were then plotted against the concentration of peptides and k_{on} was obtained from the slope of a linear fit.

For the determination of k_{off}, a 200 μL solution of Atto488-labelled siRNA (10 nM) and peptide (5 nM) was incubated until no further change in fluorescence was observed. Due to the high stability of the peptide/RNA complex, the complex is placed in the acidic buffer (10mM Citric acid buffer, pH=4) and an excess of unlabeled RNA was used to displace the encapsulated Atto488-RNA. The change in fluorescence emission at 515 nm was monitored at 10 s intervals after the addition of 1 μM of unlabeled RNA. The data was fit to a single exponential to generate k_{off}.

16. Hemolytic activity assay

The mouse blood was obtained from Xuhe (Tianjin) Pharmaceutical Technology Co., Ltd., and centrifuged (1200 rcf/g) for 10 min to achieve erythrocytes. The obtained erythrocytes were washed three times with PBS and then diluted to a final concentration of 5% (v/v). 500 μL of samples (peptide concentration:0.5mg/mL) were mixed with 500 μL of erythrocytes and incubated for one hour at 37°C. Solutions were then centrifuged at 1200 rcf/g for 10 min, and a volume of 100 μL of supernatant was taken into a 96-well plate, and the absorbance at 540 nm was read using a microplate reader (PerkinElmer, EnSpire, USA). 0.1% Triton X-100 was used as the positive control and PBS as the negative control.

Additionally, the standard curve for the centrifuge rotor is：

$$RCF = 7 \times 10^{-5} \times (RPM)^2$$

17. Xenograft tumor model

Animal experiments were performed at Xuhe (Tianjin) Pharmaceutical Technology Co., Ltd., and were approved by the company's Ethics Committee in compliance with the Laboratory Animal Law (LLSC-2024022001, LLSC-2026032401). BALB/c female nude mice (6 weeks old) were bought from Beijing Huafukang Bioscience Co., Ltd. (Beijing, China). The mice were maintained in a barrier facility with a 12 h light/12 h dark cycle, at ≈ 20 ℃ and 40% humidity. The mice were subcutaneously inoculated with 1×10^7 HeLa cells on the right side of the abdomen near the armpit. The control group was subcutaneously injected with 50 μL of normal saline.

18. In vivo distribution assay of peptide-nucleic acid complexes

Using a dose of 0.5 mg mRNA per kg of mouse weight, the formulation was employed to deliver untranslated Cy5-labeled mRNA to investigate its in vivo biodistribution. The aforementioned mice were randomly divided into 5 groups (2~3 mice per group). Saline, formulations C1, C2, C3, and Lipofectamine 2000 were diluted to 200 μL with PBS buffer and injected through the tail vein when the tumor volume reached 50-100 mm^3. Fluorescence images of the mice were taken by PerkinElmer IVIS Spectrum 8 h after the administration of the peptides.

19. In vivo delivery of peptide-nucleic acid complexes and drug expression assays

To assess luciferase expression in vivo, mice were randomly divided into 5 groups, and the peptide-FLuc mRNA complex was administered intravenously to the mice at a dose of 0.5 mg/kg mRNA. 24 h later, 200 μL of D-Luciferin potassium salt (15 mg/mL, ultrasonically dissolved in sterile water) was injected intraperitoneally in the mice, and whole-body luminescence imaging was performed 15 min later.

After in vivo imaging, the mice were immediately euthanized and dissected. Major organs, including tumor, heart, lung, spleen, renal and liver, were collected and placed in a black imaging plate for ex vivo bioluminescence imaging using the same imaging system. The luminescence signals from individual organs were recorded to evaluate the tissue distribution of luciferase expression.

20. qPCR analysis of in vivo gene silencing

BALB/c mice were intravenously injected with PTEN-siRNA@C3 complexes. At 72 h post-injection, mice were euthanized and major organs including heart, liver, spleen, lung, and

kidney were harvested. For each organ, approximately 10–15 mg of tissue was excised from the same anatomical region and homogenized on ice.

Total RNA was extracted using the Eastep® Super Total RNA Extraction Kit (Promega) according to the manufacturer's protocol. The RNA concentration was determined using a UV spectrophotometer and subsequently diluted to a concentration of 20 ng μL^{-1}.

Quantitative RT–PCR analysis was performed using the HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme) on an ABI QuantStudio 5 Real-Time PCR System, following the manufacturer's instructions. GAPDH was used as the housekeeping gene for normalization, and PTEN gene expression levels were analyzed using QuantStudio™ Design & Analysis Software. Relative gene expression levels were calculated using the $2^{-(\Delta\Delta Ct)}$ method.

21. Repeat dosing of mRNA@C3

BALB/c female nude mice were i.v. injected with mRNA@C3 (with a dose of 0.5 mg mRNA per kg of mouse weight) or PBS (n = 2 per group) every 48 h a total of three times. Weight measurements were taken and recorded daily for both treated and control mice within 14 days after the first administration.

22. Clinical chemistry and cytokine measurements

BALB/c female nude mice were i.v. injected with a dose of 0.5 mg mRNA per kg of mouse weight or PBS (n = 2 per group). After 4 h, serum was collected, and cytokine (IL-6 and TNF-α) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Mouse Interleukin 6 (IL-6) ELISA Kit, Jonlnbio, JL20268; Mouse Tumor Necrosis Factor Alpha (TNF-α) ELISA Kit, Jonlnbio, JLW10484) according to the manufacturer's instructions. After 48h, whole blood was collected in BD Vacutainer Heparin Tubes (Becton Dickinson) and the serum was separated. The liver function (AST and ALT) and renal function (UREA and CRE) were measured using the fully automated biochemical analyzer (MINDRAY，BS-360E).

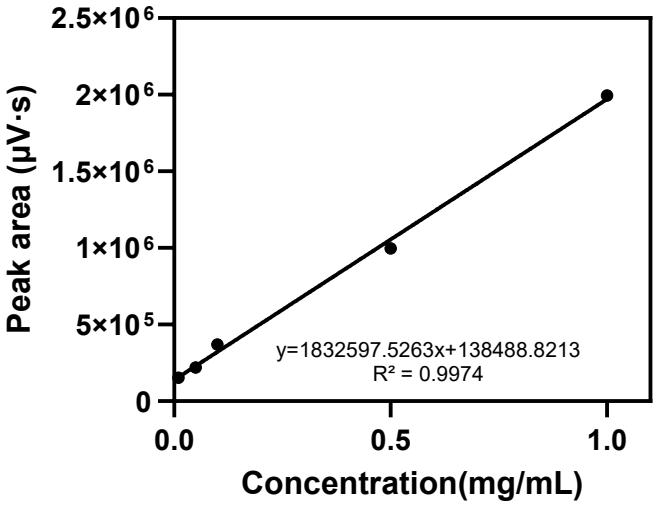

Fig. S1 Fitted standard curve for peptide concentration and analytical chromatographic peak area.

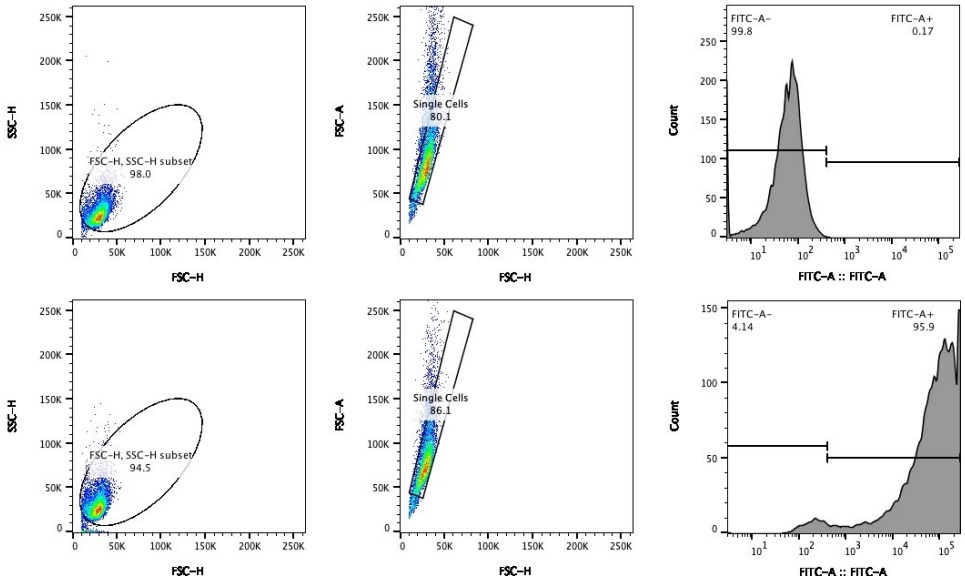

Fig. S2 Gating Strategy for Flow Cytometry Analysis (Negative control and positive control).

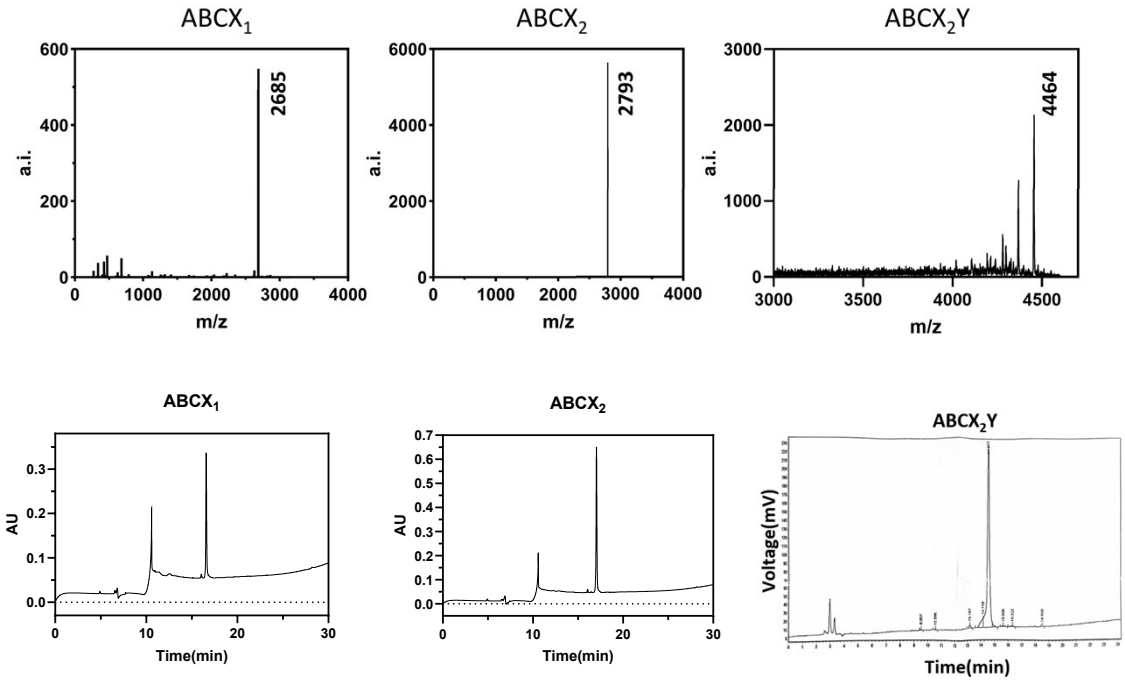

Fig. S3 Mass spectrometry (upper) and chromatograms (lower) of peptides.

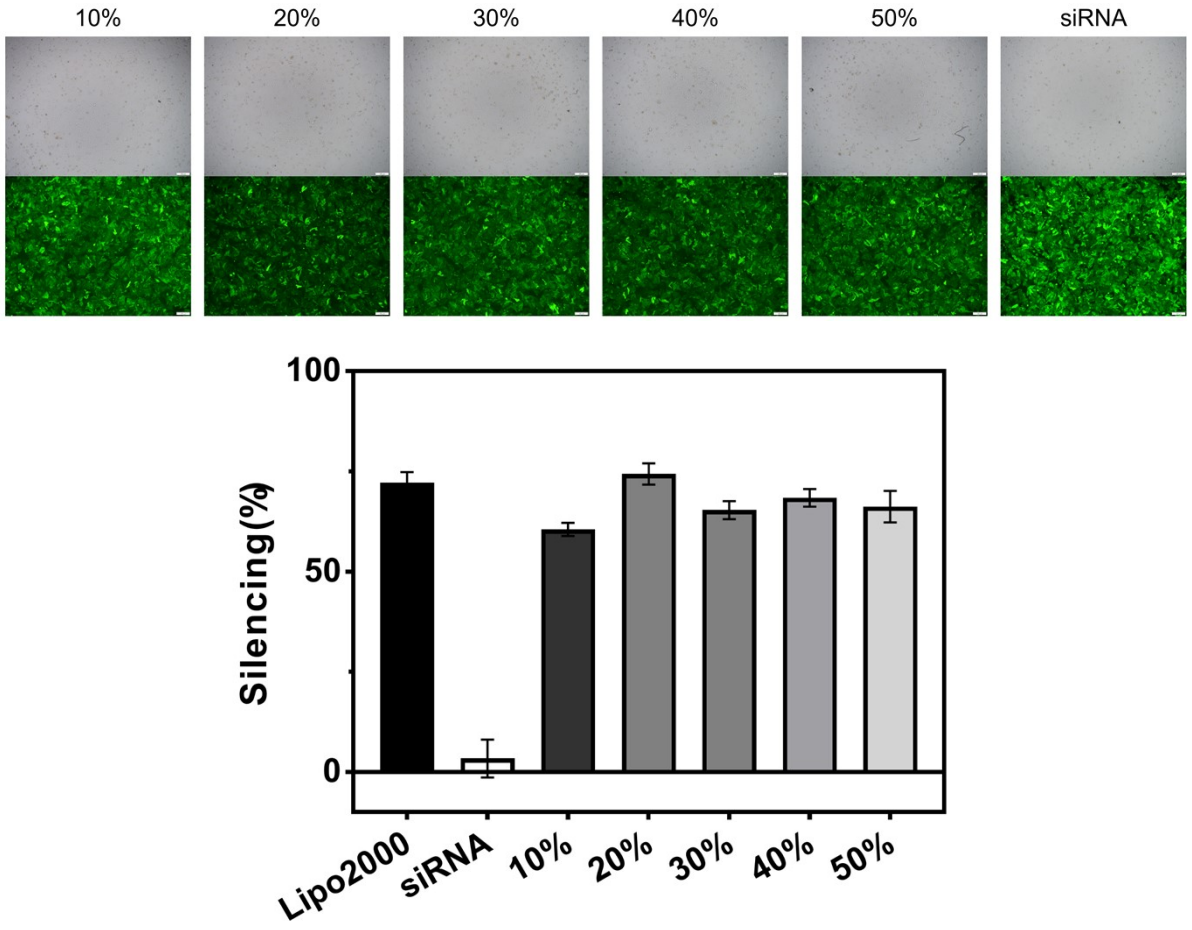

Fig. S4 Test results of the molar ratio of $ABCX_2$ in the C2 formulation. The upper picture shows the microscopy results, and the lower bar chart shows the silencing efficiency. Significance analysis comparing the results of other experimental groups with the siRNA group showed $p < 0.0001$ in all cases.

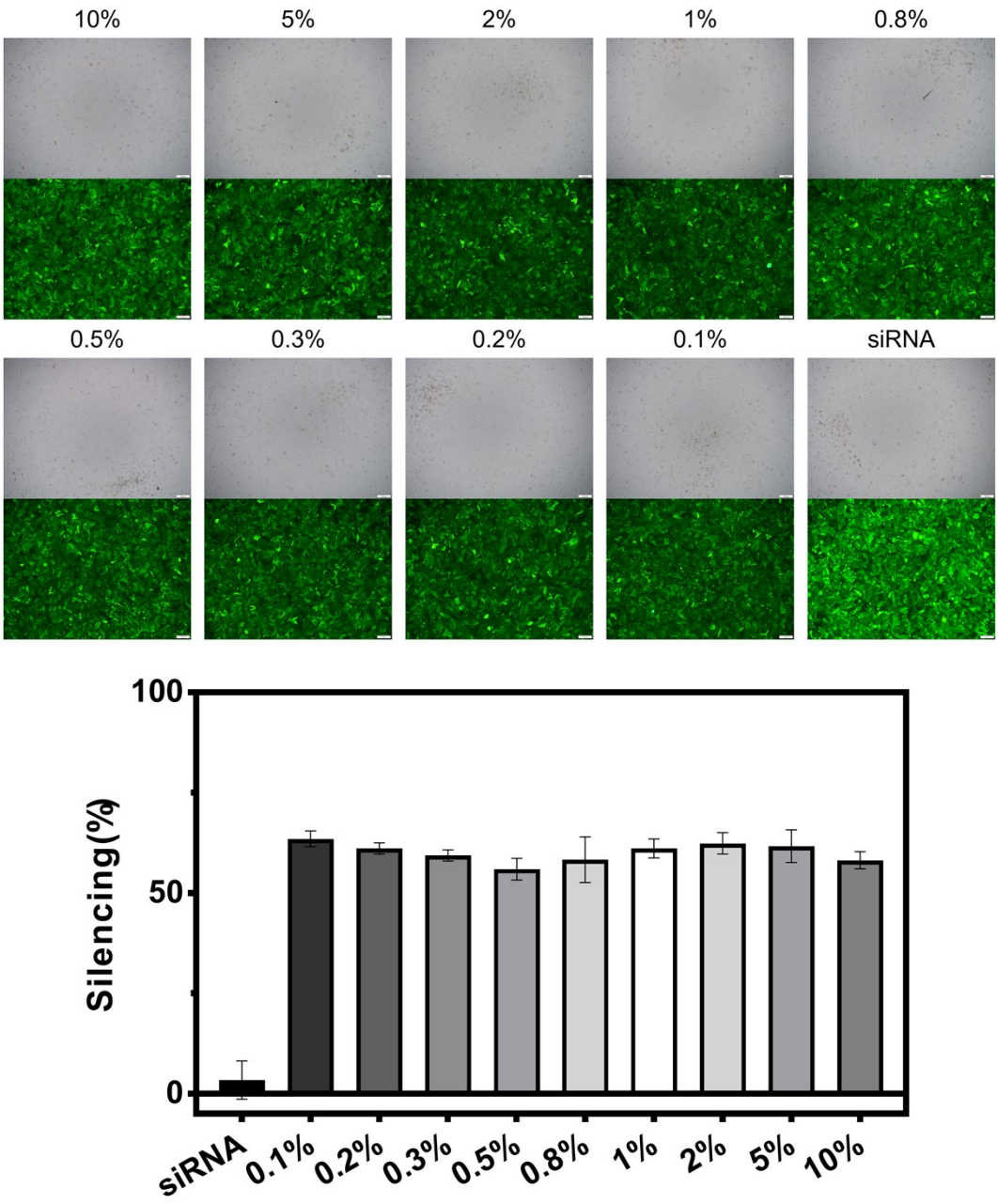

Fig. S5 Test results of the molar ratio of $ABCX_2Y$ in the C3 formulation. The upper picture shows the microscopy results, and the lower bar chart shows the silencing efficiency. Significance analysis comparing the results of other experimental groups with the siRNA group showed $p < 0.0001$ in all cases.

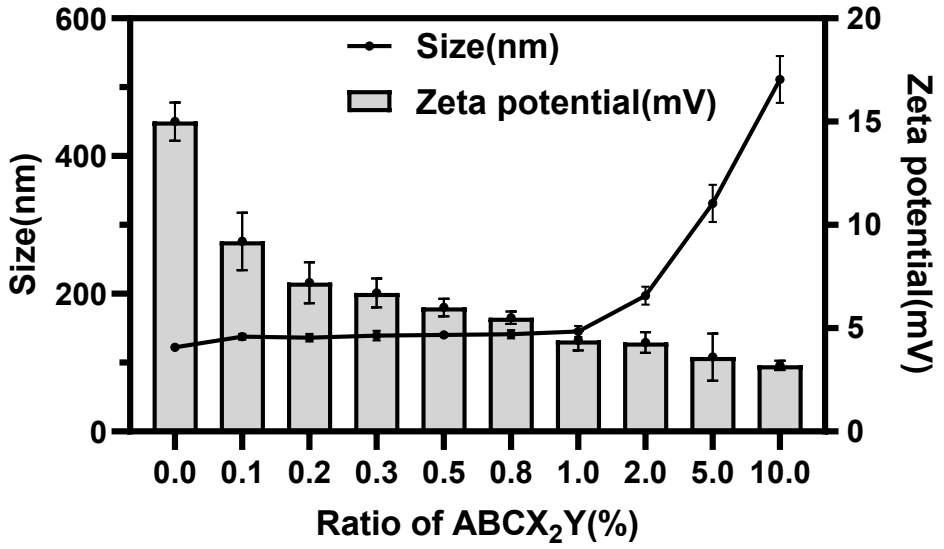

Fig. S6 Particle size and zeta potential results for peptide carriers with varying ratios of $ABCX_2Y$ (n=3), and the solution was supplemented with 20% fetal bovine serum.

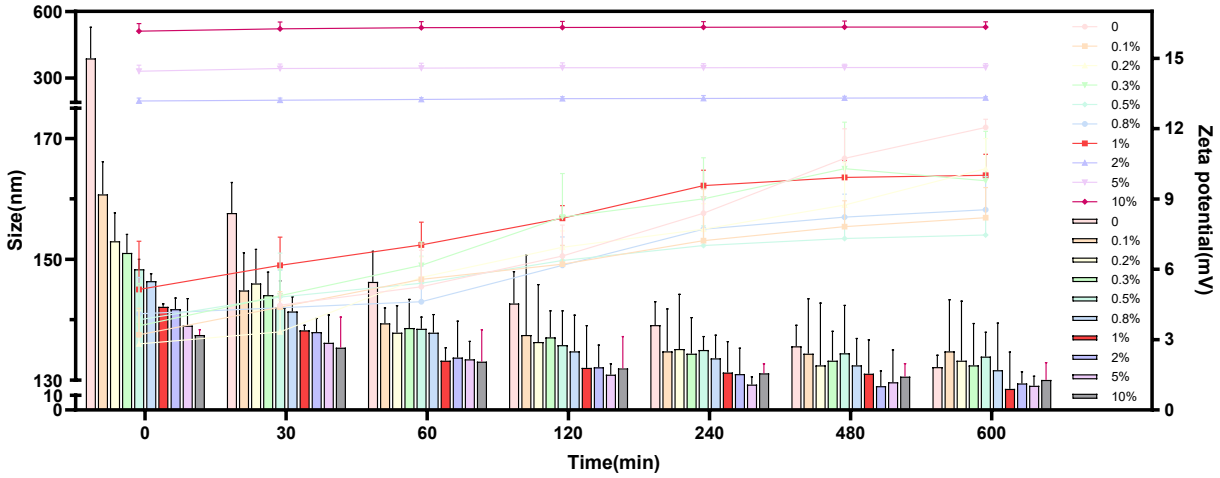

Fig. S7 Results showing the change in particle size and zeta potential over time for peptide carriers with varying ratios of $ABCX_2Y$ (n=3), and the solution was supplemented with 20% fetal bovine serum. Where, the line chart represents particle size variation, and the bar chart represents zeta potential variation.

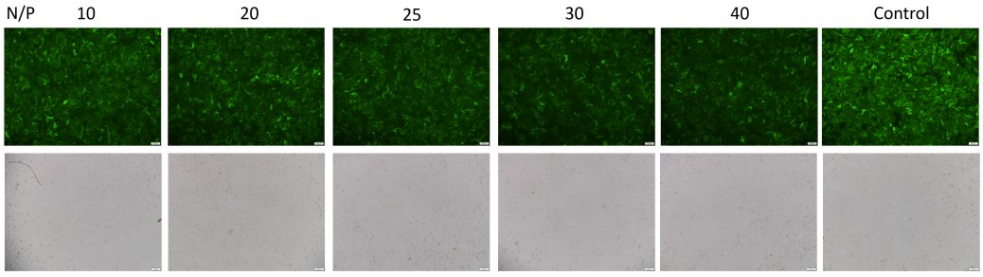

Fig. S8 Microscopic results of siRNA@C11 silencing of GFP-HeLa cells.

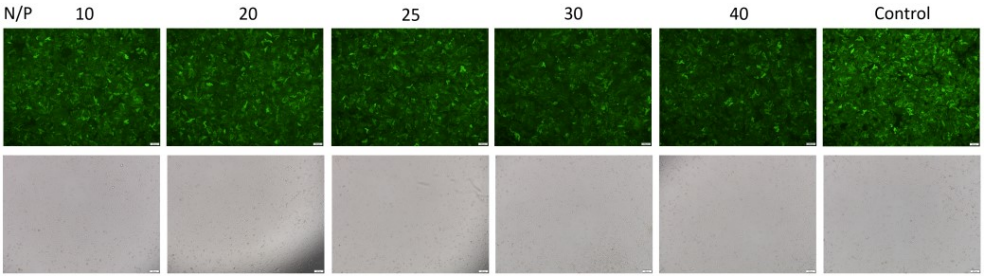

Fig. S9 Microscopic results of siRNA@C12 silencing of GFP-HeLa cells.

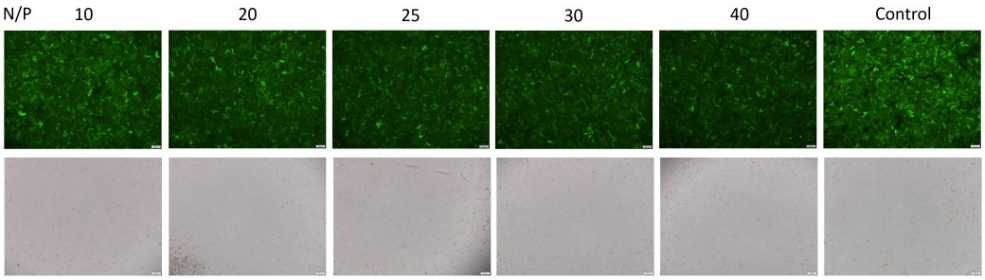

Fig. S10 Microscopic results of siRNA@C2 silencing of GFP-HeLa cells.

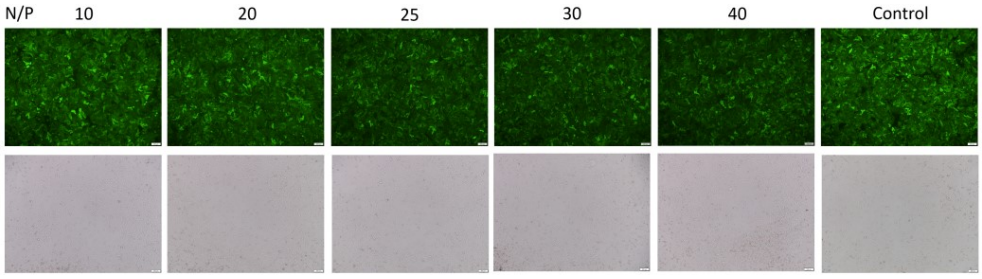

Fig. S11 Microscopic results of siRNA@C3 silencing of GFP-HeLa cells.

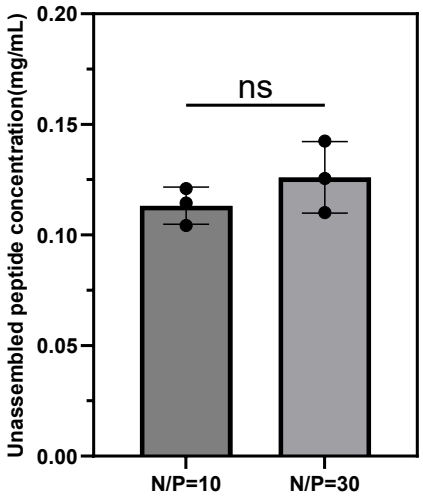

Fig. S12 Bar chart showing free peptide analysis results after C2 carrier and nucleic acid assembly at N/P ratios of 10 and 30 (n=3).

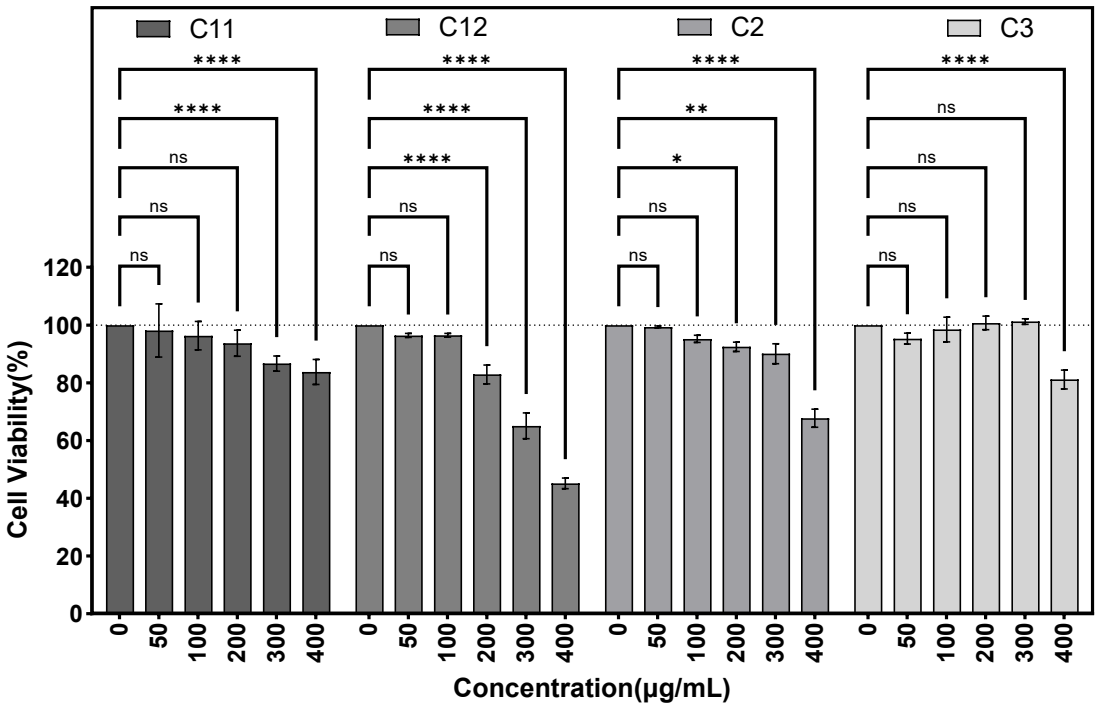

Fig. S13 In vitro cytotoxicity of peptide carriers, n=3 (HeLa). The horizontal axis represents the total peptide concentration, with N/P = 25.

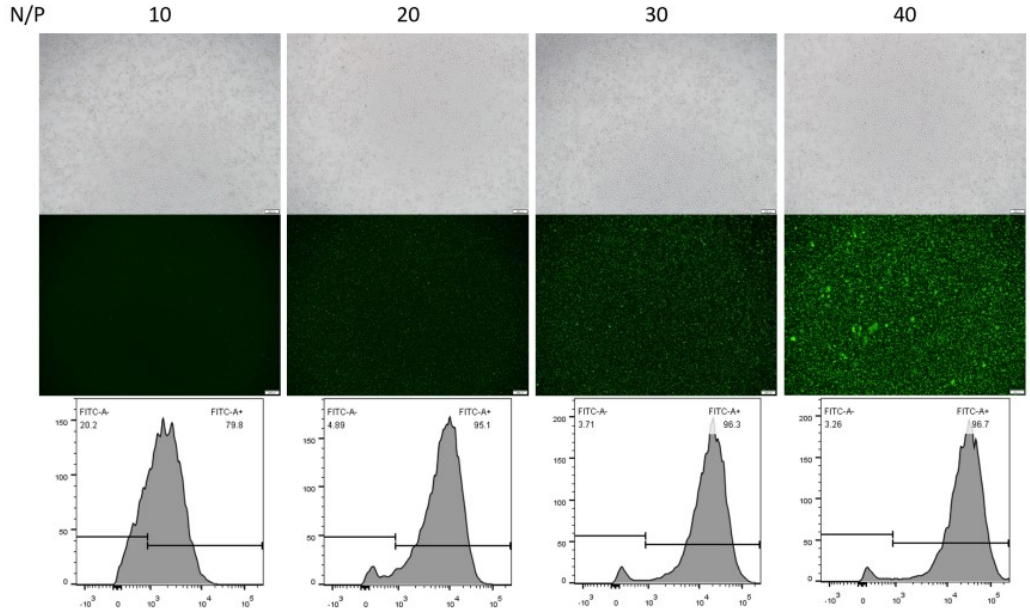

Fig. S14 Intake microscopic images of FITC-siRNA@C11 and flow cytometry characterization results (HeLa).

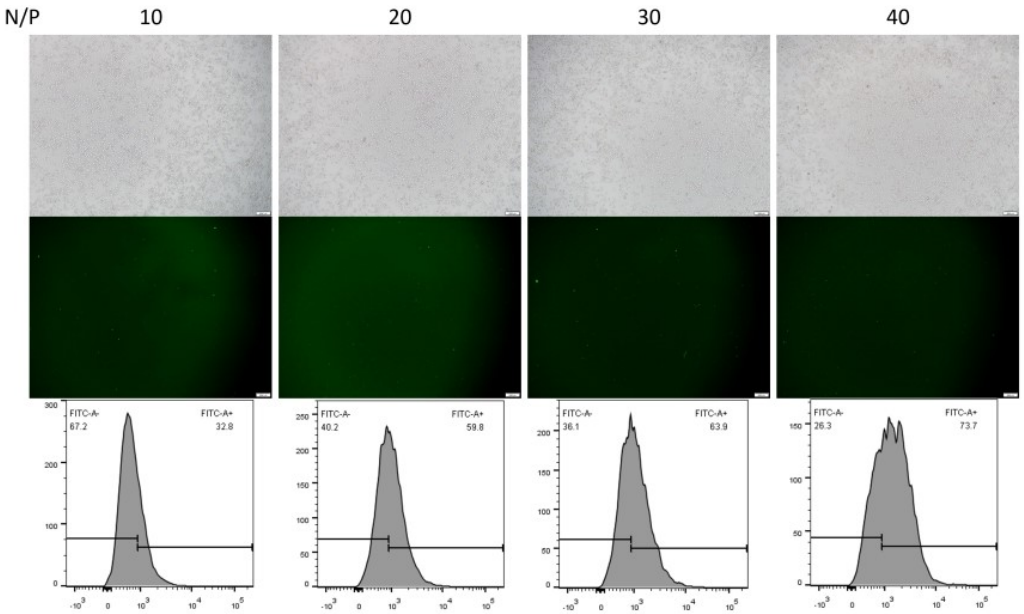

Fig. S15 Intake microscopic images of FITC-siRNA@C12 and flow cytometry characterization results (HeLa).

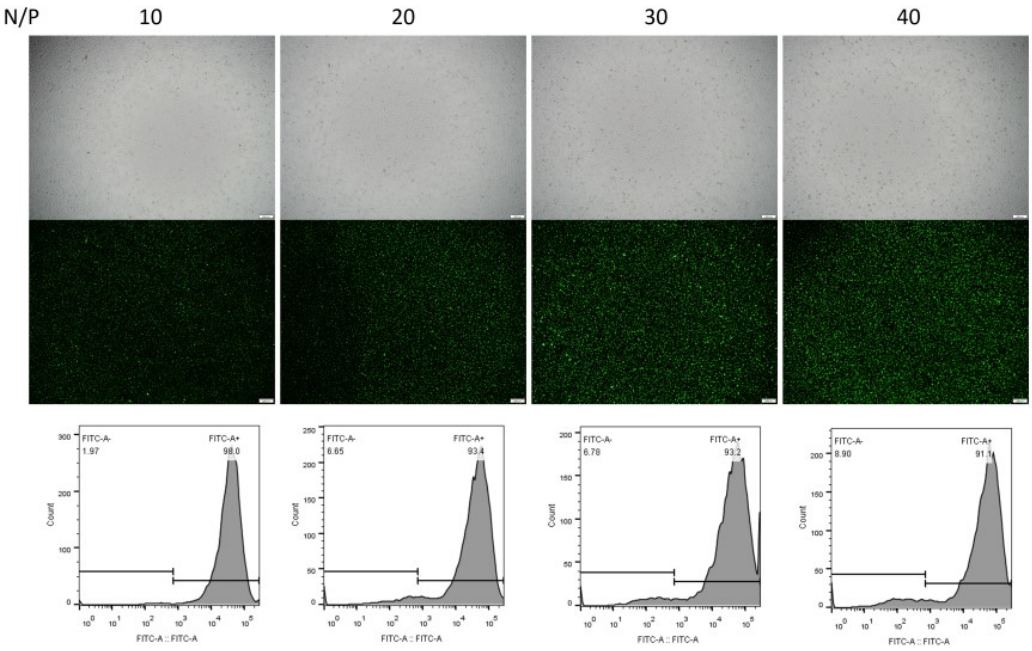

Fig. S16 Intake microscopic images of FITC-siRNA@C2 and flow cytometry characterization results (HeLa).

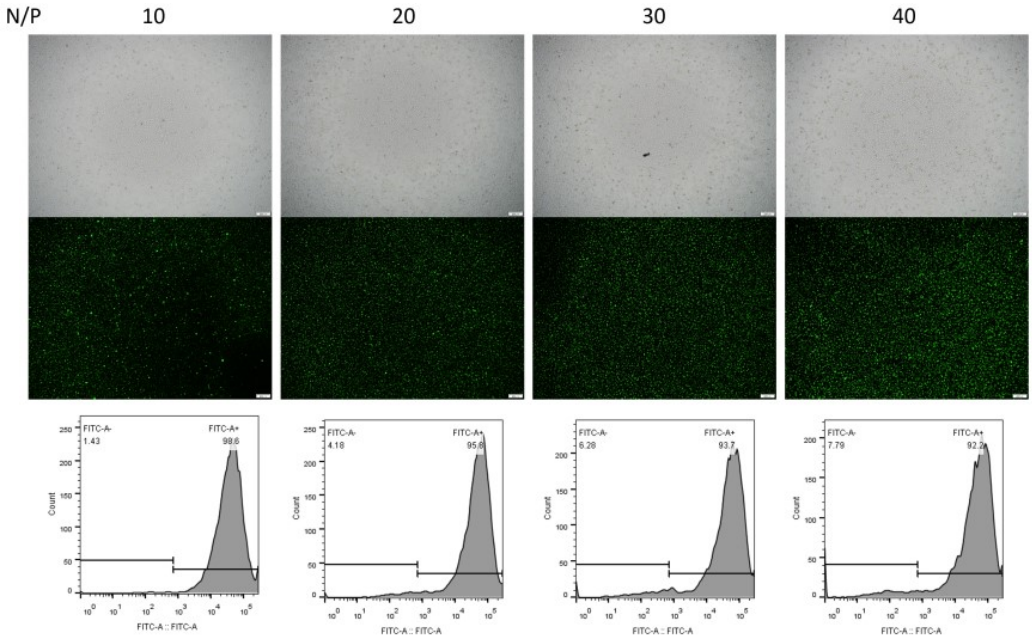

Fig. S17 Intake microscopic images of FITC-siRNA@C3 and flow cytometry characterization results (HeLa).

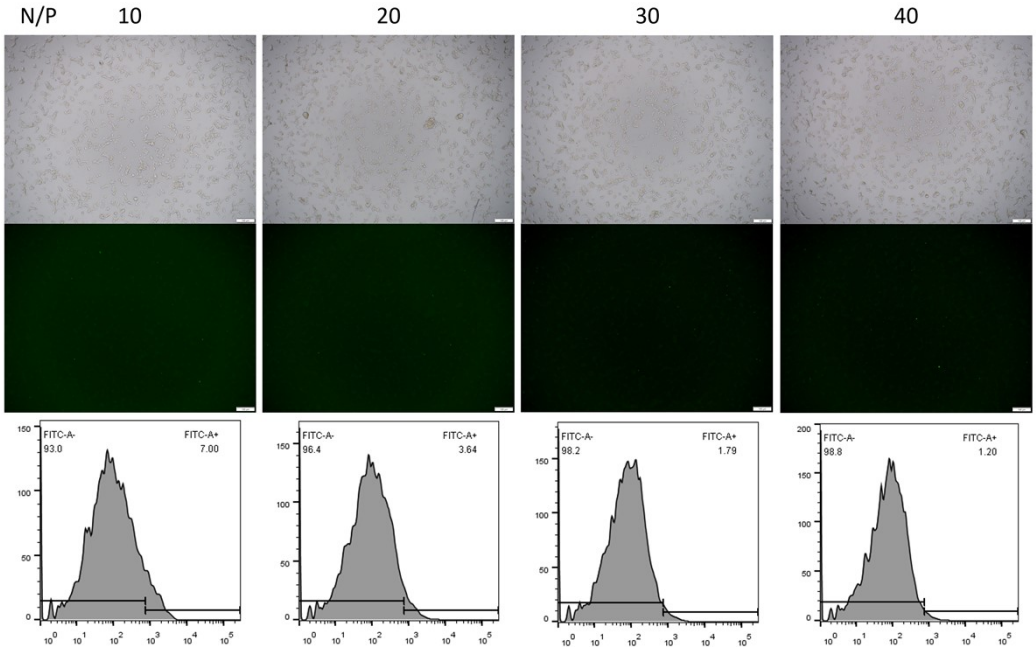

Fig. S18 Intake microscopic images of FITC-siRNA@C11 and flow cytometry characterization results (293T).

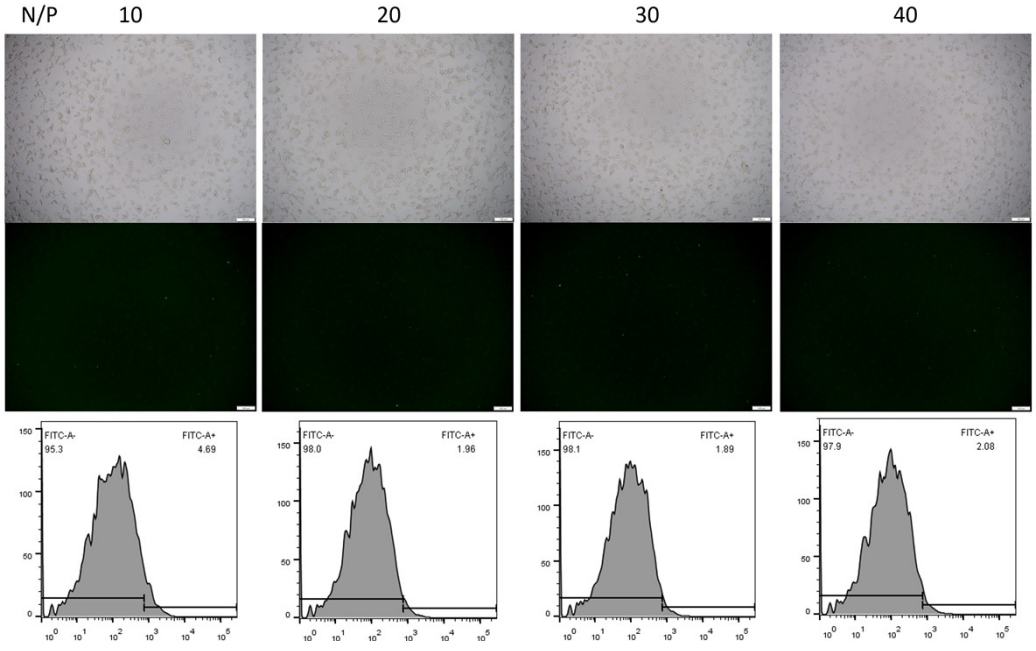

Fig. S19 Intake microscopic images of FITC-siRNA@C12 and flow cytometry characterization results (293T).

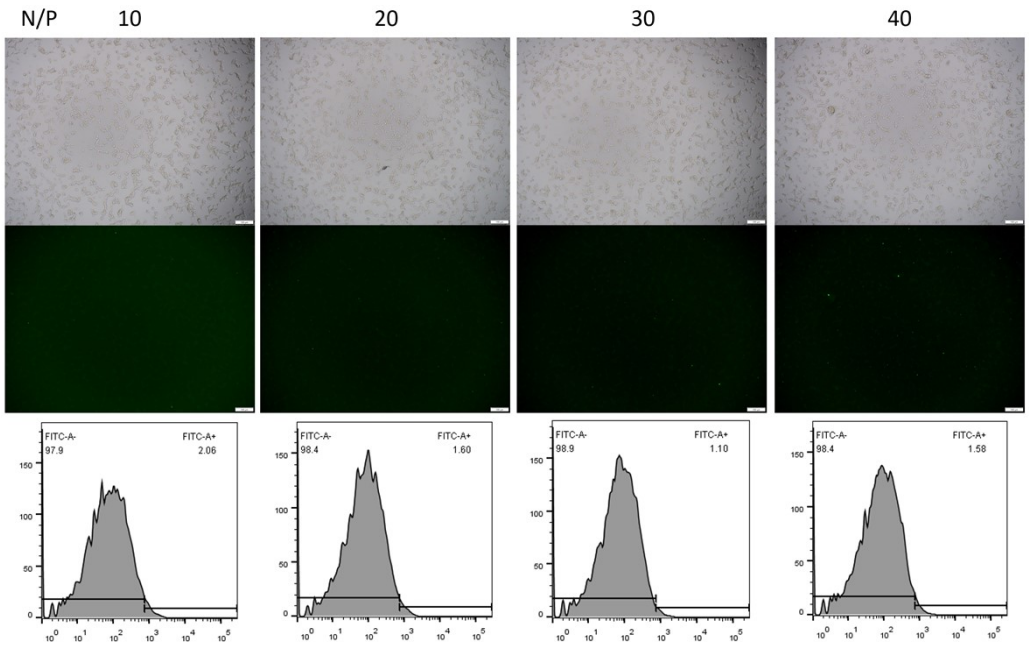

Fig. S20 Intake microscopic images of FITC-siRNA@C2 and flow cytometry characterization results (293T).

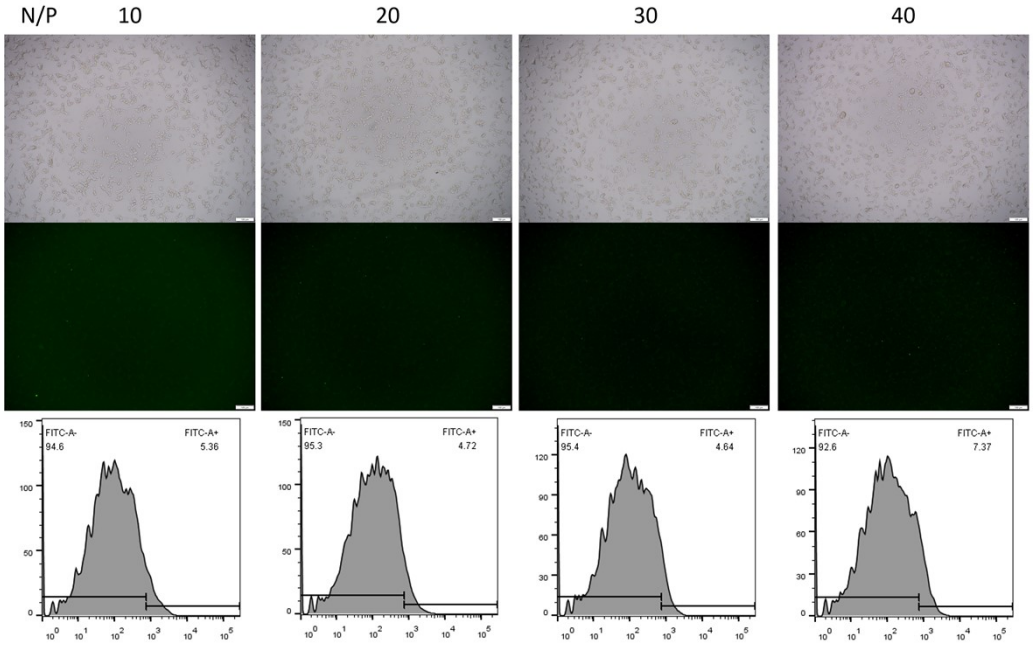

Fig. S21 Intake microscopic images of FITC-siRNA@C3 and flow cytometry characterization results (293T).

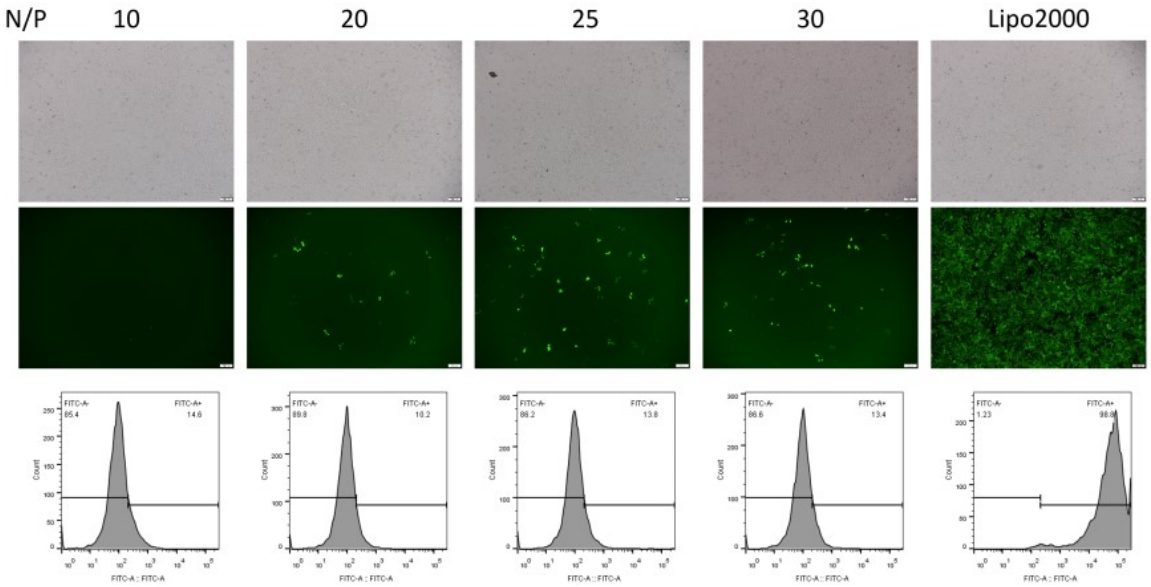

Fig. S22 mRNA transfection microscopic images of mRNA@C11 and flow cytometry characterization results

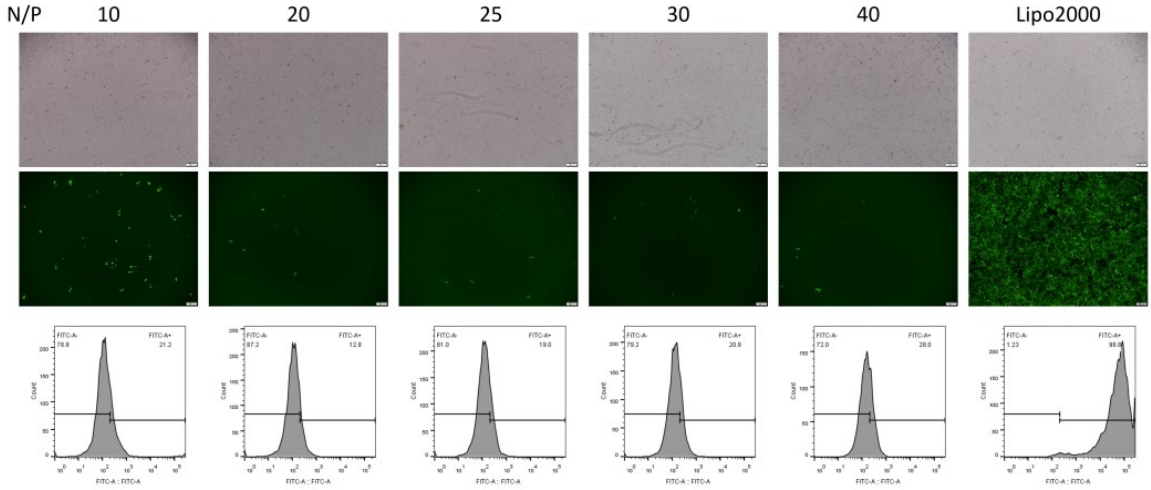

Fig. S23 mRNA transfection microscopic images of mRNA@C12 and flow cytometry characterization results

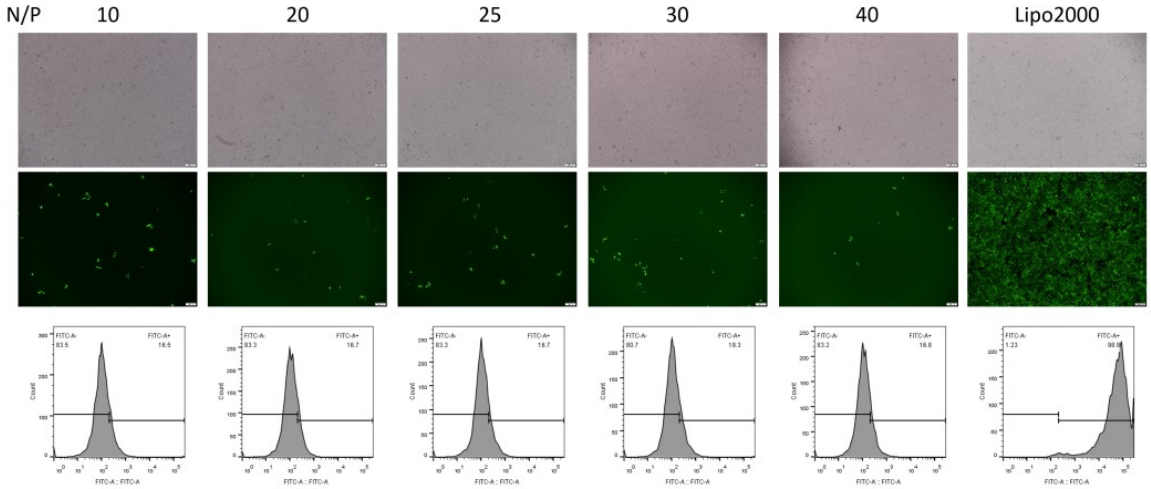

Fig. S24 mRNA transfection microscopic images of mRNA@C2 and flow cytometry characterization results

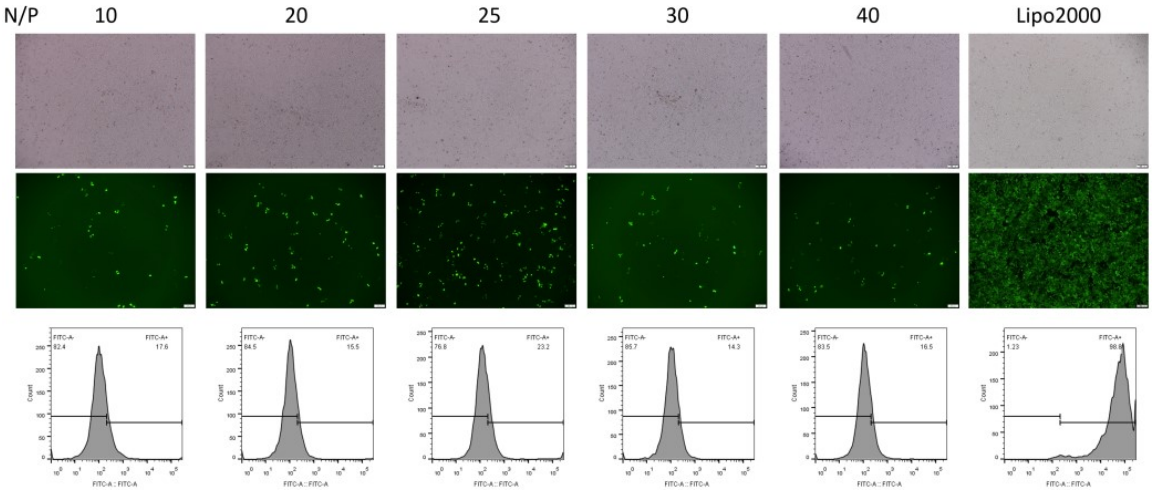

Fig. S25 mRNA transfection microscopic images of mRNA@C3 and flow cytometry characterization results

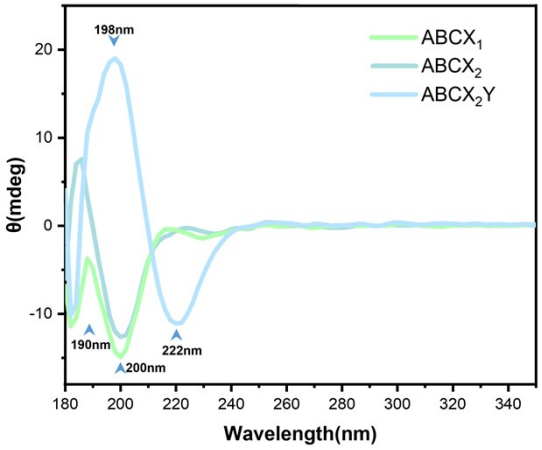

Fig. S26 Circular dichroic spectrogram of peptides.

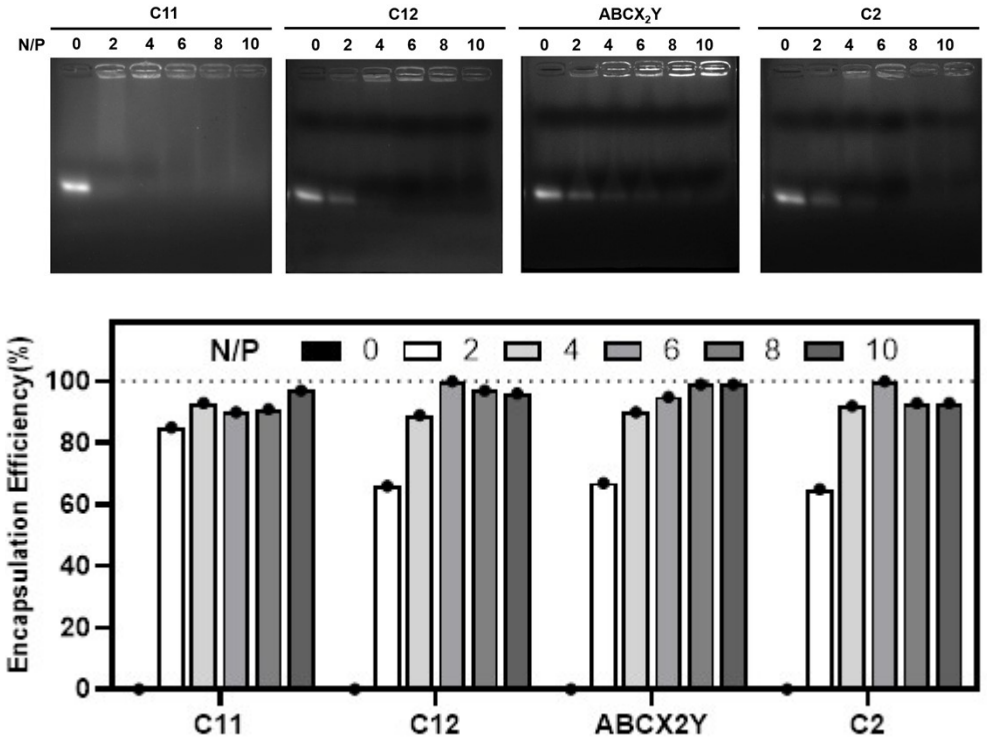

Fig. S27 Agarose gel electrophoresis of nucleic acid encapsulation capacity of peptide carriers. The bar chart displays the encapsulation efficiency across each lane of the gel electrophoresis. For calculations, N/P=0 serves as the positive control, while the blank areas of the gel function as the blank control.

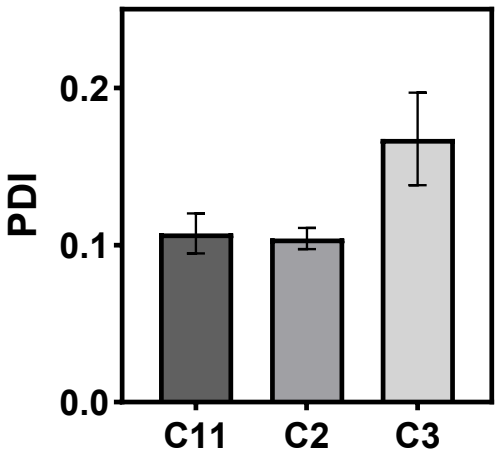

Fig. S28 Particle size PDI bar chart for C1, C2, and C3.

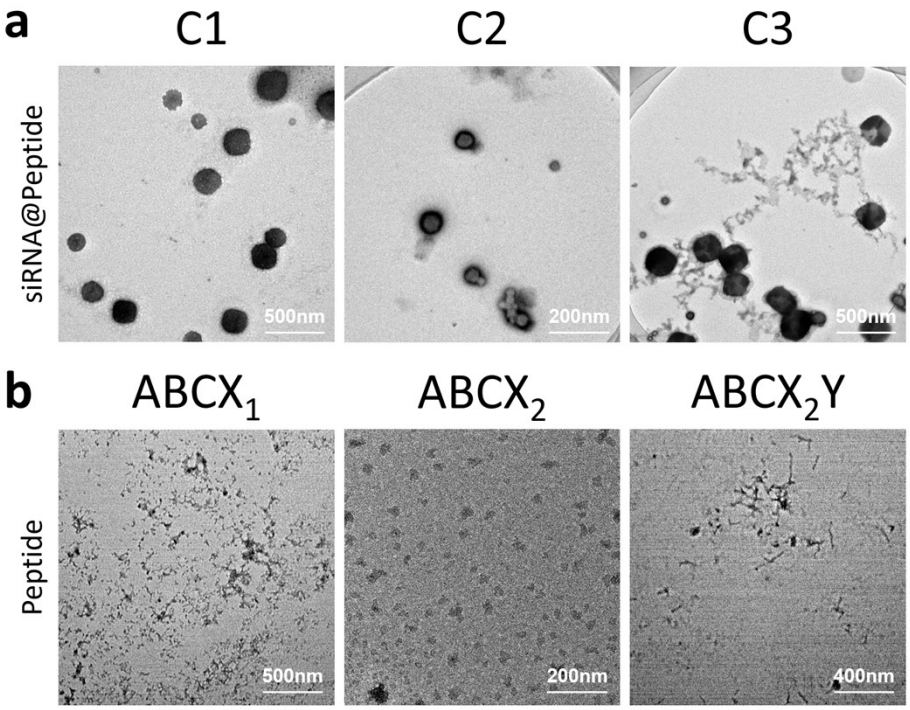

Fig. S29 a) Additional TEM images of C1 ($ABCX_1$), C2 and C3 after co-assembly with siRNA at N/P of 25; b)Supplementary TEM image of pure peptide.

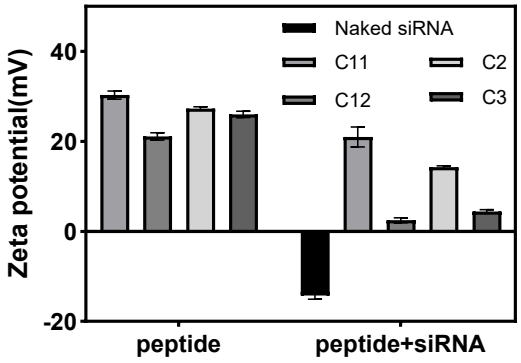

Fig. S30 Histogram of the potentials of peptides compared to peptide-siRNA complexes, n=3.

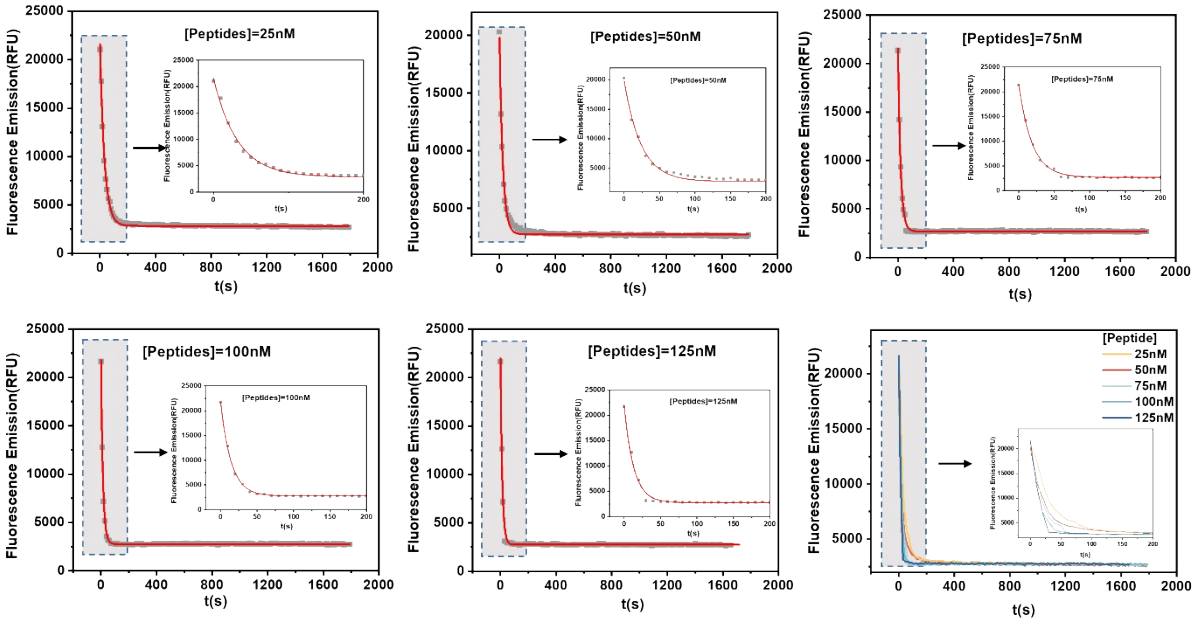

Fig. S31 Assembly kinetics of C11 formulation.

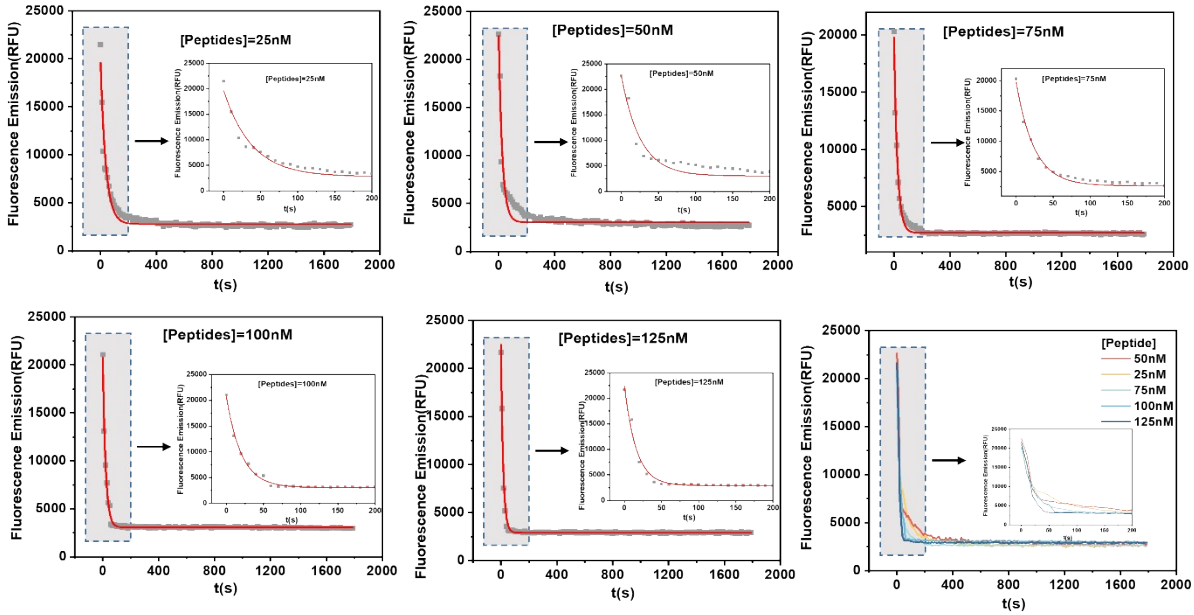

Fig. S32 Assembly kinetics of C2 formulation.

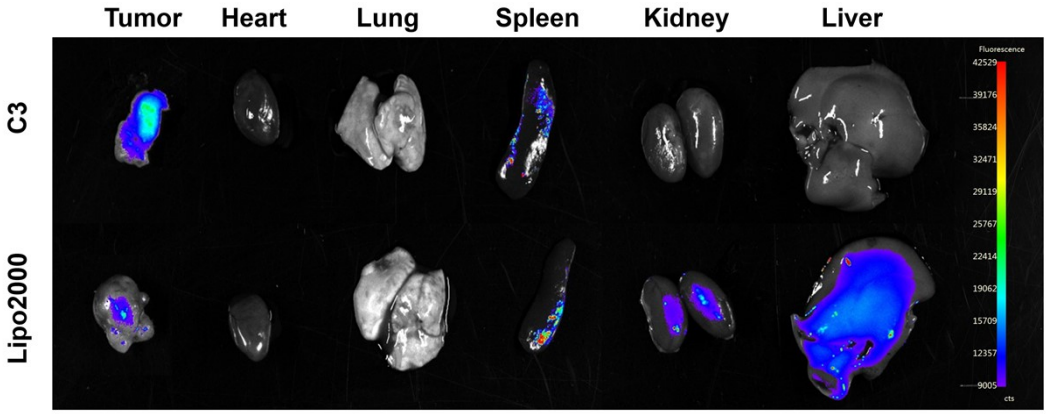

Fig. S33 Ex vivo bioluminescence imaging of major organs following systemic delivery of FLuc mRNA.

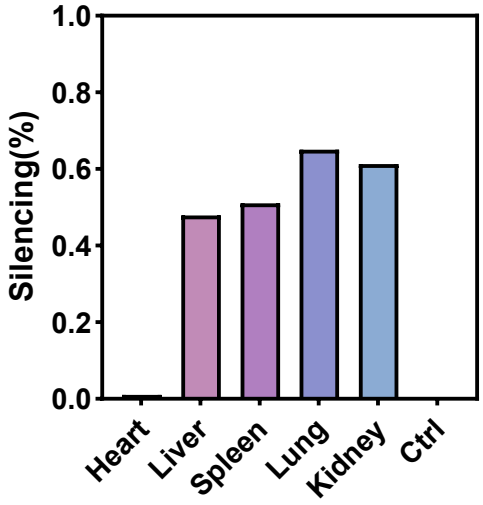

Fig. S34 Bar chart showing PTEN gene silencing efficiency in different organs 72 h after intravenous administration of PTEN-siRNA@C3 via tail vein injection in BALB/c mice.

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