Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting information

Fusogenic peptide modification to enhance gene delivery by peptide-DNA nano-coassemblies

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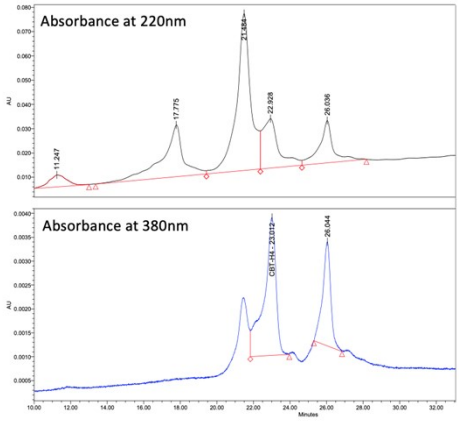
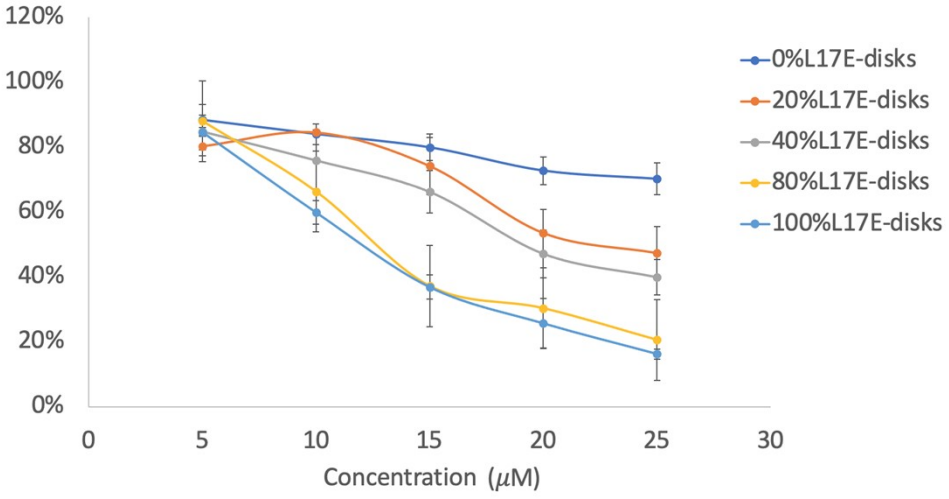
Supporting figures

S1 Click reaction between the two peptides monitored by UPLC and MALDI spectrometry mass. The absorbance was settled at 220nm for peptide detection and 380nm for the CBT ring. After reaction for 30min on ice, the reacting solution were diluted 10 times with 50% ACN in MQ water. At specific retention time, the samples were collected for MALDI mass detection for peptide confirmation.

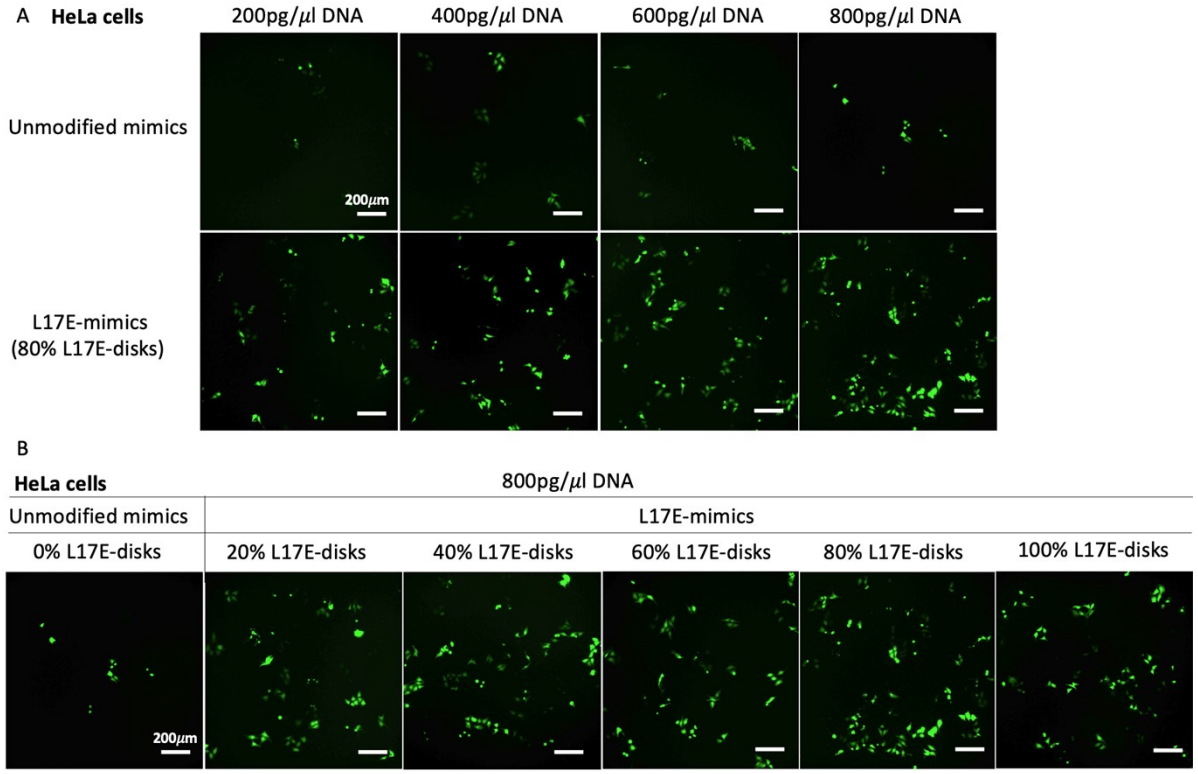


Peptide	UPLC retention time (min)	$[M+H]^+_{Cal.}$	$[M+H]^+_{Obs.}$
Cys-L17E	17	2962	2961.7
H4	21.5	2872	2872.6
CBT-H4	23	3129	3129.8
L17E-H4	26	6076	6076.8

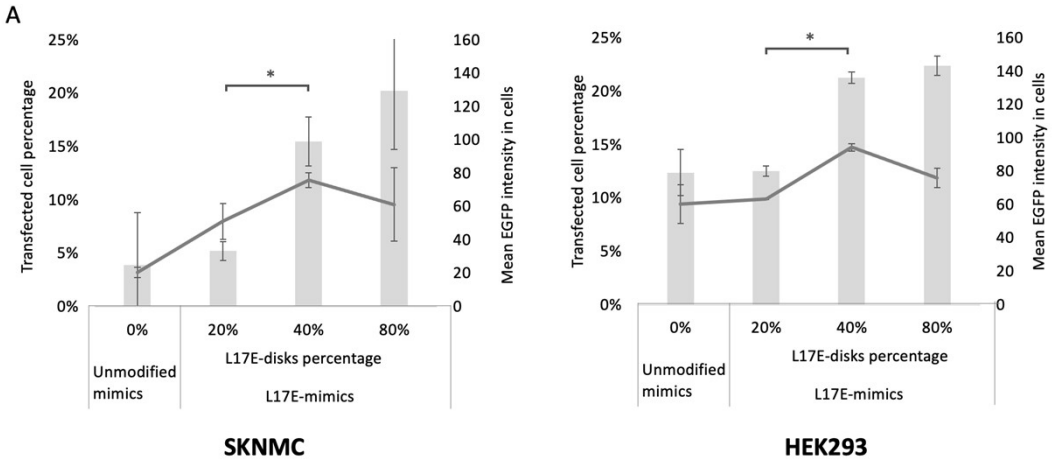
S2 MTT assay measuring the cell viability of different percentage of L17E-disks

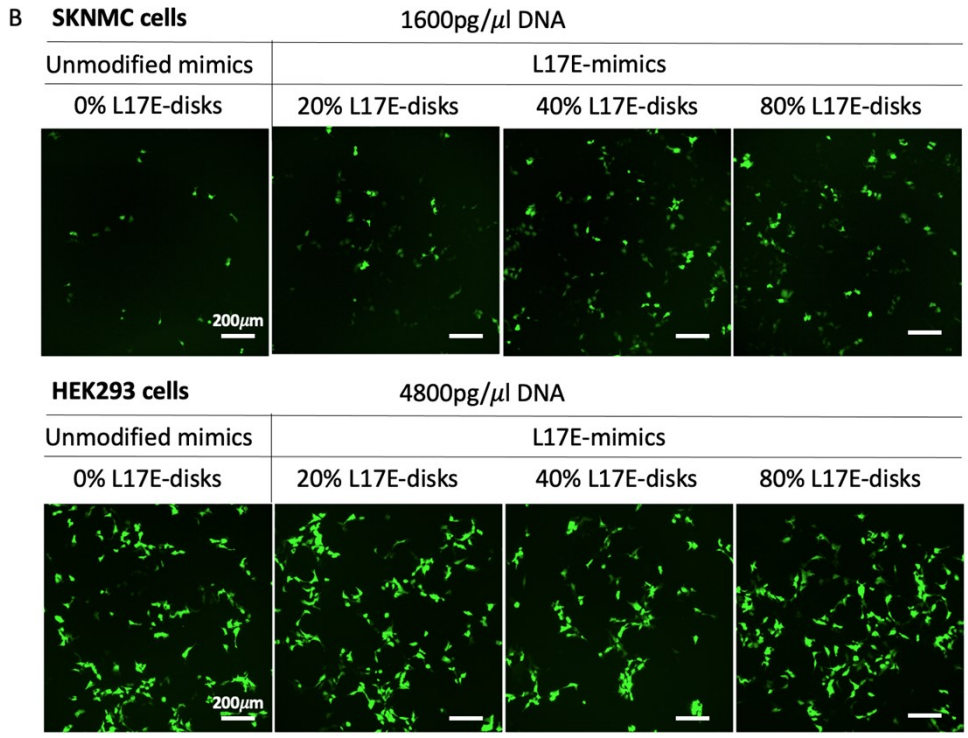


S3 Fluorescent microscope images of the Hela cells transfected with (A) unmodified and modified L17E-mimics (80%L17E-disks) at different DNA concentration 48 hours after sample addition; (B) Mimics with different L17E-disks percentage while same DNA concentration (800pg/μl). Scale bar: 200μm.



S4 Transfection efficiency of unmodified/modified mimics on SKNMC and HEK293 cells. (A) Flow cytometry data with bar indicating the percentage of EGFP-positive cells and lines indicating the mean fluorescence intensity. Data shown are mean ± SD, n ≥ 3. *Statistically significant with p-value ≤ 0.05. (B) Fluorescent microscope images. (DNA concentration: 1600pg/μl for SKNMC cells, 4800pg/μl for HEK293 cells) Scale bar: 200μm.





Method

Materials

All the Fmoc-protected amino acids and rink amide resins were purchased from GL Biochem (Shanghai, China). Peptide sequences: H4, HHHHKKKKK-C_{12}-LLHC'C'HLLGSPD-amide (C', S-(tert-Butylthio)-L-cysteine; C_{12}, 2-aminolauric acid); L17E, IWLTALKFLGKHAAKHEAKQQLSKL-amide; CBT-H4, CBT-HHHHKKKKK-C_{12}-LLHC'C'HLLGSPD-amide (CBT, 2-cyano-benzothiazole); Cys-L17E, C IWLTALKFLGKHAAKHEAKQQLSKL-amide. All the peptides (C-terminal amidated and N-terminal free) was synthesized in the lab on microwave-assisted peptide synthesizer (biotage, Sweden). 6-Amino-2-cyanobenzothiazole (CBT) was purchased from Bidepharm™. MTT, LysoTracker™ Red DND-99, CellLight™ Early Endosomes-GFP, Hoechst 33342 were purchased from ThermoFisher™. DNA labeling Cy5 kit was purchased form Mirus Bio™. Transfection reagent jetPrime was from PolyPlus™. The rest of chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Extraction of pEGFP and label with Cy5

We used Green fluorescent protein encoded gene pEGFP (4.7kbps) as the reporter gene in this study. pEGFP transformed Escherichia coli was amplified in Luria-Bertani (LB) media overnight at 37°C. After the bacterial pellet was collected through centrifugation, the plasmid was extracted and purified with EndoFree Plasmid Maxi purification kit (QIAGEN, Valencia, CA) by following the manufacture's protocol. The purity and concentration of the purified plasmid was identified with UV absorbance measurement at 260nm and 280nm. The collected plasmids were divided into aliquots (1000ng/μl) and stored at -20°C until use. Partial of DNA was labeled with Cy5 for uptake study and later confocal imaging. We followed the manufacture's protocol for Cy5 labeling. The highest recommended labeling

intensity was used, which was 1 µl of Label IT Tracker™ Reagent per µg of DNA (1:1 (v:w)) followed by 3 hours reaction. After purification using ethanol precipitation, we quantify the purified, labeled nucleic acid on a NanoDrop 1000 spectrophotometer (ThermoFisher™) and diluted into 1000ng/μl.

Peptide synthesis

All the peptides were synthesized on microwave-assisted peptide synthesizer (biotage, Sweden) in our laboratory based on Fmoc-Solid-Phase Peptide Synthesis (SPPS). Once the synthesis was finished, partial of the peptide H4 was deprotected and simultaneously cleaved from dry resin with trifluoroacetic acid (TFA) for 2hrs. The peptide Cys-L17E was deprotected and cleaved with a cocktail containing 95% TFA and 5% ethanedithiol (EDT). Attachment of the 2-cyano-6-aminobenzothiazole (CBT) onto the peptide H4 was modified from the standard reported method.[31,32] Firstly, the un-cleaved H4 (~0.05mmol) was dried and added into THF (0.75ml) containing isobutyl chloroformate (IBCF, 0.05mmol) and 4-methylmorpholine (MMP, 0.1mmol). The reaction mixture was stirred for 20min at 0°C, then THF (0.75ml) containing succinic anhydride (0.8mmol) was dropped and followed further stirring for 2 hour at 0 °C. Then the mixture was stirred overnight at room temperature. After wash with DMF and pure acetone, the dried resin was again added into THF (0.75ml) containing IBCF and MMP and stirred for 20min at 0°C. The solution of 2-cyano-6-aminobenzothiazole (CBT, 0.05 mmol) in THF (0.25ml) was drop-added to the reaction mixture, followed by 2 hour at 0 °C and overnight room temperature stirring. After wash with acetone, the peptide CBT-H4 was deprotected and cleaved from dry resin with trifluoroacetic acid (TFA) for 2hrs. The filtrate was collected and precipitated with cold ether. The precipitate was washed twice with cold ether and air-dried for HPLC purification.

Peptide purification

The dried crude peptide was dissolved in 50% water/acetonitrile with 0.01% TFA mixture by sonication. The clear supernatant was collected after filtration with 220nm filter and purified on the reverse-phase HPLC (Angilent 1260 Infinity II) through the VYDAC protein & peptide C-18 preparative column (19 x 250mm) with a linear gradient at 6 ml/min. The collected fractions were evaporated on the rotavapor to remove acetonitrile, and the rest peptide aqueous solution was frozen in liquid nitrogen and freeze-dried on a freeze-dryer (FreeZone Benchtop Freeze Dryer, Labconco) to obtain the white peptide powder. The peptide H4 and CBT-H4 were stored at 4°C until use, while Cys-L17E was dissolved and aliquoted with MQ water at 5mM, frozen in liquid nitrogen and stored at -20°C. The peptides' identities were confirmed by MALDI-MASS (BRUKER) analysis.

Formation of peptide self-assemblies and peptide/DNA co-assemblies

The peptide CBT-H4 and H4 powder were dissolved in DMSO at 25mM as stock stored in room temperature. The H4-disks were formed by addition of the H4 peptide stock solution into the buffered solution (10mM HEPES, pH7.4) and waited for 30mins for the self-assemblies' formation. The concentration for H4-disks was settled at 0.75mM as our previous reports.[33] For L17E disks, CBT-H4 disks was firstly formed by pre-mixing the CBT-H4 and H4 peptide at 1:1 ratio and then added into the buffered solution with the same concentration as H4 disks. After 30mins, Cys-L17E equilibrium with CBT-H4 peptide (with a final concentration of 0.375mM) was added into the CBT-H4 disks solution. The click reaction of the Cys-L17E on CBT-H4 disks was monitored by measuring the absorbance of the

reaction solution by Ultra Performance Liquid Chromatography (UPLC, WATERS Aqcuity H-class). The L17E-disks were dissolved by diluting 10 times with 50% water/acetonitrile with 0.01% TFA. Absorbances were detected at 220nm and 380nm for peptide bonds and CBT rings respectively. 30mins after initiating the click reaction, 50% of the Cys-L17E was reacted with the CBT bond and formed L17E-H4 which was confirmed by MALDI-MASS detection. (Supporting figure S1) Overnight reaction showed no obvious increase of the L17E-H4 peak, thus the click reaction time was settled as 30mins. After obtaining the H4-disks and L17E-disks, the two disks solution were mixed at 20%, 40%, 60%, 80% of L17E-disks. The formation of the unmodified mimics was carried out by adding the concentrated DNA solution (1000ng/μl) into the H4-disks solution. Similar as the unmodified mimics, the L17E-mimics were formed by adding the concentrated DNA solution into the mixture of H4-disks and L17E-disks. The samples were mixed by vortexing for 5 seconds and incubated under room temperature for 30min for the co-assemblies' formation.

Structural characterization with Transmission Electron Microscopy (TEM)

Carbon supported TEM copper grids (400-mesh, TED PELLA, Inc., USA) were pre-treated with plasma cleaner for 1min to clean the grid surface and increase the sample attachment. The sample solution (2.5µl) was loaded on a parafilm, followed by covering the copper grid face-down on the sample drops. After 2min incubation, the excess solution on the copper grid was removed with a filter paper, followed by covering it on 2% uranyl acetate staining solution (2.5µl, Sigma-Aldrich). After another 2min incubation, the excess staining solution was dipped away with filter paper, and the sample grids were placed in a grid holder and air-dry overnight before imaging. A JOEL 2010 transmission electron microscope was used to image the samples.

Cell viability by MTT

Hela cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. To obtain the cell viability, Hela cells in a density of 1.5×10^4 cells/well were seeded in a 96-well plate plate (0.1ml DMEM) one day before the MTT test for reaching 80% confluency. On day 2, complete DMEM medium was replaced with Opti-MEM, followed by addition of disks' mixture with varying percentage of L17E-disks. For each percentage, viability tests included total peptide concentrations ranged from 5 to 25µM. 24 hours later, the MTT assay was conducted following the manufacturer's protocol. Generally, 10 µl MTT stock solution (12 mM) was added to each well and incubated for 4 hours. Then, all the cell mediums were replaced with 100µl DMSO to dissolve the formazan crystals. After 10-min incubation at room temperature, the absorbance was measured at 540 nm and the cell viability was calculated according to the controls.

Cellular uptake by flow cytometry

Similarly, Hela cells in a density of 1.5×10^4 cells/well were seeded in a 96-well one day before the cellular uptake test for reaching 80% confluency. Cy5-DNA was used as the fluorescence probe to form the unmodified mimics and L17E-mimics. The cell medium (0.1ml) was replaced with Opti-MEM (0.1ml) of low serum, followed by the addition of 0.67µl of samples solution. The final peptide concentration in the medium was 5µM. DNA concentrations in the medium ranged from 200 to 800pg/μl, corresponding to a DNA concentration of 30 to 120ng/μl before sample addition. After 4

hours of incubation at 37°C in the presence of 5% CO_2, the medium was changed, and cells were carefully washed with cold PBS twice. The cells were resuspended in complete DMEM (200μl) after treat with tryptase (50μl) for FACS analysis on a FACS Aria III flow cytometer (BD Biosciences, USA). For each sample, the minimal 10000 events were analysed and three tubes were averaged after subtraction of negative controls.

Transfection efficiency by fluorescent microscope and flow cytometry

All the conditions for transfection efficiency assessment on Hela cells were the same as for cellular uptake measurements. Generally, Hela cells were seeded with a density of 1.5×10^4 cells/well in a 96-well plate one day before transfection. DNA without label was used for measurement. After medium replacement by Opti-MEM (0.1ml), 0.67µl unmodified mimics or L17E-mimics were added to Hela cells. For SKNMC and HEK293 cells, seeding densities were the same as Hela at 1.5×10^4 cells/well. SKNMC and HEK293 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After optimization, the DNA concentration for SKNMC was settled as 240ng/μl, in correspondence with 1600pg/μl, and the final peptide concentration was at 5µM, in correspondence with 0.67µl sample solution per well. More sample solution was required for HEK293 cells to function. For HEK293 cells, the sample solution was 2ul/well (three times compared with SKNMC), with the final peptide concentration at 15µM and DNA concentration at 4800pg/μl. Commercially available reagent for DNA transfection, jetPrime, was used as the positive control according to the manufacture's protocol. The DNA amount for jetPrime was 400pg/μl in all three cell lines. After two days of incubation at 37°C in the presence of 5% CO_2, the cells of GFP-expression were imaged under the fluorescence microscope with ×10 objective. The transfection efficiency was quantified with flow cytometry on a FACS Aria III flow cytometer (BD Biosciences, USA). The above cells transfected with mimics were trypsinized and resuspended in complete DMEM for FACS analysis. For each sample, the minimal 5000 events were analyzed, and three repeats were averaged after subtraction of negative controls.

Intracellular localization by confocal microscope

Live cell imaging with an SP8 confocal microscope (Leica Microsystems, Germany) was used to assess the intracellular distribution of the delivered DNA. Hela cells were premixed with CellLight™ Early Endosomes-GFP at 20 PPC (number of particles per cell) and seeded in a density of 3.75×10^4 cells/well in a confocal dish (0.25ml DMEM) with a central glass disc on the bottom. The following day, the complete DMEM medium (0.25ml) was replaced with Opti-MEM (0.25ml), then 1.6µl freshly prepared Cy5-labeled mimics added and gently mixed into the cell culture medium. After 4-hour incubation, the cells were washed three times with complete medium and incubated for another 20 hours in complete medium. 1 hour before imaging, Hoechst 33342 was added to label cell nucleus at 2μg/ml. And the Lysotracker Red (DND99, 50nM) was added to stain the late endosomes/lysosomes 5min before imaging. The cells were directly observed under confocal microscope equipped with ×63 objective. For the co-localization study, cell images with the expressed EGFP-Rab5 proteins labelling early endosome, lysotracker labeling late endosomes/lysosomes and Cy5 labeling DNA with laser wavelengths of 488nm, 574nm and 633nm were collected by layers under the sequential model in SP8. All the laser intensity and gain value were set the same for unmodified mimics and L17E-mimics. Colocalization analysis was performed via JACoP plugin in Fiji by calculating the Mander's M1

coefficients for 30 to 40 cells per condition on confocal images. The EGFP-Rab5 over-expressed cells were not counted. The threshold for DNA signal in JACoP was determined according to the highest intensity in blank control.