Supporting Information

Phase-Separated Peptide Coacervates as Delivery Vehicles for mRNA Vaccines

Zhiyi Xu,[1,†] Chaiyaporn Kuwentrai,[2,†] Yaxin Hu,[2] Renhao Li,[2] Pengchao Wang,[2] Zhong Zheng,[1] Liqiang Feng,[7, 8] Jiang Xia,[1, *] Jian-Dong Huang [2-6, *]

[1] Department of Chemistry, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China

[2] School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China.

[3] Key Laboratory of Quantitative Synthetic Biology, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.

[4] Clinical Oncology Center, Shenzhen Key Laboratory for Cancer Metastasis and Personalized Therapy, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China

[5] Guangdong-Hong Kong Joint Laboratory for RNA Medicine, Sun Yat-Sen University, Guangzhou, China

[6] State Key Laboratory of Cognitive and Brain Research, The University of Hong Kong, Hong Kong SAR, China.

[7] China-New Zealand Joint Laboratory on Biomedicine and Health, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China.

[8] University of Chinese Academy of Sciences, Beijing 100049, China.

*Address correspondence to Jiang Xia (jiangxia@cuhk.edu.hk) and Jiandong Huang (jdhuang@hku.hk) † Z. X. and C. K. contributed equally to this work.

CONTENTS

Detailed experimental procedures

Materials and Instruments. Unless otherwise noted, all reagents were used without further purification. Boc-protected amino acids were obtained from Aladdin (Shanghai, China) and coupling reagents were obtained from GL Biochem (Shanghai, China). Cystamine Dihydrochloride and Nile Red were obtained from Macklin (Shanghai, China). siGENOME GAPD Control siRNA was obtained from Horizon Discovery (Cambridge, UK). Single-stranded DNA used was purchased from the BGI company (Shenzhen, China). CleanCap® Reagent AG (3' OMe) was purchased from TriLink Bio Technologies (California, USA). T7 High Yield RNA Transcription Kit and FastPure Cell/Tissue Total RNA Isolation Kit were purchased from Vazyme Biotech (Nanjing, China). E. coli poly (A) polymerase was purchased from Beyotime Biotechnology (Shanghai, China). Peptide characterization and purification were performed in RP-HPLC (Shimadzu, DGU-20A5, Japan). Peptide analysis was performed in an AutoFlex Speed LRF MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The gel images were captured by an ENDURO™ GDS Gel Documentation System (USA) or a Bio-Rad ChemiDoc Image System (USA).

Peptide synthesis. All peptides were synthesized in the liquid phase. Briefly, Boc-Lys(Fmoc)-OH (3.1 mmol) or Boc-Arg(Pbf)-OH (3.1 mmol), HBTU (2.94 mmol), and HOBt (2.94 mmol) were dissolved in DMF (10 mL) in a round-bottom flask, and the mixture was stirred with a magnetic stirrer. N, N-Diisopropylethylamine (DIPEA) (12.4 mmol) and cystamine dihydrochloride (CDC) (1.4 mmol) were added with an interval of 1 minute, respectively, and the reaction mixture was stirred for 16 h at room temperature. The reaction mixture was poured into 100 mL of water. White precipitate was collected by filtration and washed with water. The crude product was dried in a desiccator. The product from the first step, Boc-K(Fmoc)ssK(Fmoc)-Boc, was dissolved in DCM (3 mL) in a round-bottom flask. To deprotect the intermediate product, trifluoroacetic acid (TFA) (3 mL) was added, and the reaction mixture was stirred for 3 h. The solvent was evaporated on a rotary evaporator, yielding an oily residue. Diethyl ether (45 mL) was added to the flask, and the content was gently stirred. White precipitate was separated by centrifugation. The crude product was obtained after freeze-drying. 2.2 eq of Boc-Trp-OH or Boc-Phe-OH, 2.1 eq HOBt, 2.1 eq HBTU, and 8.9 eq DIPEA were dissolved in 6 mL DMF in a round-bottom flask, and the mixture was stirred with a magnetic stirrer for 16 h at room temperature. Continue until all amino acids are synthesized.

At last, 25% diethlyamine in DCM was added to the intermediate product, and the reaction mixture was stirred for 3 h. The solvent was evaporated on a rotary evaporator. Diethyl ether (45 mL) was added to the flask, and the content was gently stirred. White precipitate was separated by centrifugation. The crude product was obtained after freeze-drying.

Peptide purification and characterization.

Crude peptides were dissolved in DMSO. After being filtered through a 0.2 μm filter, the peptide solution was injected into RP-HPLC (Shimadzu, DGU-20A5, Japan) equipped with a semi-prep HPLC column (Vydac 218TP C18 LC Semi-Prep Column 10 μm, 250×10 mm ID). 0.1% TFA in H2O (v/v) and 0.1% TFA in ACN (v/v) were used as the mobile phases A and B, respectively. The total flow rate was set to be 3 mL/min (gradient: 0-7 minutes 25% B, 7-23 minutes 25-65% B, 23-25 minutes 65-95% B, 25-30 minutes 95% B). The peptide peaks were collected, lyophilized, and confirmed by MALDI-TOF mass spectrometry analysis (Bruker Daltonics, Germany). For peptide redox-responsive analysis, a C18 column (Vydac 218TP C18 LC Column 5um, 250×4.6mm ID) was equipped, and the flow rate was set to 1 mL/min. 0.1% TFA in H2O (v/v) and 0.1% TFA in ACN (v/v) were used as the mobile phases A and B, respectively.

Plasmid construction. E. coli DH5α strain was used for cloning and plasmid propagation and grown in selective Luria-Bertani medium or Luria Bertani plates with 1.5 wt% agar. 100 μg/mL ampicillin was added for selection. The primers were ordered from BGI. Plasmids were constructed using seamless cloning techniques and confirmed by Sanger sequencing.

Cell Culture. HEK293 cells and MDA-MB-231 cells (ATCC, USA) were cultured with DMEM (Gibco) media supplemented with 10% FBS and penicillin/streptomycin solution (Gibco) in a humidified atmosphere at 37℃ and 5% CO_2.

Coacervate Preparation for Imaging. Peptides were dissolved in DMSO at a concentration of 100 mg/mL as a stock solution. Cargoes like Cy5-labeled single-stranded DNA (Cy5-ssDNA) were mixed with 10 μL serum-reduced Opti-MEM medium (Gibco) at 37℃. 0.2 μL of peptide stock

solution were added and the medium turned to milky, indicating the formation of coacervates. Coacervates were visualized under a confocal microscope (Stellaris 8, Leica, Germany).

Redox reaction of WWK coacervates. 1 μL of peptide stock solution was added to 100 μL Opti-MEM medium and incubated at 37℃ for 15 min. Different concentrations of reduced glutathione were incubated with the mixture at 37℃ for 24 h. 25 μL of acetic acid was added to dissolve all the unreacted peptides, and all the samples were measured by RP-HPLC.

Measurement of turbidity and recruitment of nucleic acids in the coacervates. Phase separation of the peptides was monitored according to the turbidity of the solution using a microplate reader (Thermo Scientific™, USA). The plasmids were diluted with Opti-MEM to different concentrations, and 1 mg/mL of WWKssKWW peptide was added to 25 μL Opti-MEM with plasmids to form coacervates at 37℃. After 15 minutes of incubation, the mixture was spun at 10000 rpm for 5 minutes. The supernatant was loaded onto a 1% agarose gel with Gel-red staining. The samples were run on gel electrophoresis at 120V (constant voltage) for 20 minutes, and the Bio-Rad ChemiDoc Image System (USA) was used to detect the signal.

In-Vitro Cytotoxicity Assay. 1×10^5 HEK293 cells were seeded in a 96-well plate (NEST Scientific). Different concentrations of **WWK** coacervates were incubated with the cells for 24 h. The supernatant was removed and replaced with fresh medium. Cytotoxicity was measured based on the extracellular LDH using an LDH Cytotoxicity Assay Kit (Beyotime) according to the manufacturer's instructions.

Cellular Uptake Experiments. Cells were seeded in 20 mm confocal dishes (NEST Scientific, Wuxi, China) till ~60% confluency. 0.5 μL of Cy5-ssDNA (100 μM) was mixed with 100 μL Opti-MEM, and 1 μL of **WWK** peptide (100 mg/mL) was added. After incubating at 37℃ for 15 minutes, 400 μL Opti-MEM was added to the solution. The cell culture medium was replaced with the coacervate-containing medium. After 4 hours of incubation, the coacervate-containing medium was removed and replaced with fresh medium supplemented with FBS. The cells were incubated for

another 20 h and then washed with PBS twice before they were imaged under a confocal microscope or analyzed with a flow cytometer (FACSVerse, BD Biosciences, USA).

Nucleic acid delivery to HEK293 cells. HEK293 cells were seeded in a 24-well plate till ~60% confluency. 1 μg plasmids, or 50 nM siGAPDH, or 2-4 μg EGFP mRNA were mixed with 100 μL Opti-MEM first. For the **WWK** groups, 1 μL of **WWK** stock solution (100 mg/mL in DMSO) was added to the medium and incubated at 37℃ for 15 min. For Lipo3000 groups, nucleic acids were added to 50 μL Opti-MEM first, 1 μL Lipo3000 was added to another 50 μL Opti-MEM, and the two solutions were mixed and incubated for 15 minutes. For PEI groups, nucleic acids were added to 25 μL Opti-MEM first, and 3 μL PEI (1 mg/mL) was added to another 25 μL Opti-MEM and incubated for 5 minutes. Both were mixed and incubated for another 10 minutes. The cell culture medium was removed from the plate, and the mixture of nucleic acids and different delivery reagents was added to the wells.

Cell imaging for internalization mechanism study. HEK293 and MDA-MB-231 cells were seeded in the 20 mm glass-bottom dishes at a confluence of 60%. **WWK**/Cy5-labeled ssDNA coacervate mixture was incubated with the cells for 24 h. The cells were stained with Lyso-tracker Green, ER-Tracker Green, and Mito-tracker Green (Beyotime), respectively, for lysosome, ER, and mitochondria, following the instructions. For F-actin staining, cells were first fixed with 4% paraformaldehyde for 15 min at room temperature. After PBS washing for 3 times, Phalloidin-iFluor 488 (Abcam) was incubated with cells for 30 min at room temperature. We then washed the cells with PBS 3 times in the dark. For early and late lysosomes staining, cells were first fixed with 4% paraformaldehyde for 15 min at room temperature. After washing, 0.1% Triton X-100 in PBS was added to cells for 5 min at room temperature, followed by 5% BSA in PBST for 1 h. 100 μL of the primary antibodies RAB5A Rabbit pAb (Abclonal) and RAB7A Polyclonal pAb (Proteintech) were diluted 1:100 and added, respectively, to cells in the blocking buffer for overnight incubation at 4℃. After washing, 100 μL of ABflo® 647-conjugated Goat anti-Rabbit IgG (H+L) (Abclonal) diluted 1:500 in PBS was added to the cells for 1 h at room temperature. After final washing in the dark, cells were imaged using a confocal microscope (Stellaris 8, Leica, Germany).

Inhibitor treatment. 15 μM Dynasore, 1 mM amiloride, 10 μM chloroquine, 10 μM cytochalasin B, or 10 mM 2-deoxy-D-glucose (MedChemExpress) were incubated with HEK293 cells and MDA-MB231 cells for 2 hours, respectively, to inhibit the respective cellular uptake pathways. Then, **WWK** coacervates with Cy5-labeled single-stranded DNA was incubated with cells for 4 h. After washing and incubation in fresh medium for 20 h, the Cy5 signal in the cells was detected by a flow cytometer (FACSVerse, BD Biosciences, USA).

mRNA synthesis. Tobacco mosaic virus (TMV) 5' and 3' untranslated regions (UTR) cloned in pUC57 plasmid was modified by inserting poly-adenine (A) tail (130 bases in length) behind the 3'UTR. The gene of interest was then inserted into the plasmid backbone. *In vitro* transcription was performed using a T7-HiScribe mRNA synthesis kit (NEB). Uridine bases (UTP) were substituted with N-methyl pseudouridine triphosphate (TriLink BioTechnologies, USA). The mRNA products were purified by the lithium chloride precipitation method. 5′-capging was performed using the vaccinia-virus-capping system (NEB, USA). The final mRNA products were reconstituted in RNase-free water.

Western Blotting experiments. 10 μg of the RIPA buffer was added to lyse cells into SDS Sample Buffer (6 ×), followed by boiling the samples at 95℃ for 10 min. SDS PAGE was run on a pre-cast SDS polyacrylamide gel at 90V (constant voltage) for 15 minutes and 160V for 45-60 minutes until the dye reached the bottom of the gel. Gels were removed and soaked in 1 L of transfer buffer for 15 minutes. 0.22 μm PVDF membrane (Vazyme, China) was cut to a similar size to the transfer area of the gel, and protein gel was transferred in the fast transfer buffer (Beyotime Biotech, China) at 330mA for 35 minutes at a constant current. The membrane was removed from the blotting cassette, rinsed briefly with TBST, blocked with freshly prepared 5% nonfat dried milk for 1 h on a shaking platform, and incubated with primary GAPDH rabbit pAb (Abclonal), α-Tubulin rabbit mAb (Abclonal), rabbit anti GFP-Tag mAb (Abclonal), TRIM21/SS-A rabbit pAb (Abclonal), or mouse anti His-Tag mAb (Abclonal), respectively, diluted in 5% nonfat milk in TBST at 4℃ overnight. After washing, the membrane was incubated with anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology) or HRP-conjugated goat anti-rabbit IgG (H+L) (Abclonal), in TBST-5% nonfat milk for 1 h at room temperature. For WB detection, Ultra High Sensitivity ECL Kit

(MedChemExpress) was prepared according to the manufacturer's instructions. The membrane was incubated with the detection solution for 1 min and imaged using a Bio-Rad ChemiDoc Image System (USA).

***In vitro* imaging of luciferase luminance.** Luciferase mRNA (1 μg/well) was mixed and briefly vortexed with LipoMessengerMax (2.5 μl/well, according to manufacturer's instructions), PEI, InstantFECT cationic liposome, protamine, silica nanoparticles, or **WWK** peptide (0.9 mg/ml). The dosage of the mRNA transfection reagents used in this study was adopted from our previous work.[1] For **WWK**/mRNA formulation, the mixture was incubated at 37°C in DMEM for 15 minutes before transfection. Human embryonic kidney 293 cells (HEK293 cells) were seeded in a 48-well plate (approximately 2.5 × 10^4 HEK293 cells/ well), and different mRNA formulations were added dropwise into wells. Cells were incubated for 24 h (37 °C in 5% CO2 atmosphere). Subsequently, luciferin substrate (2 μl at 30 mg/ml, Gold Biotechnology) was added to the respective wells, and plates were imaged by IVIS luminescence imager (Perkin Elmer IVIS spectrum 2020, USA) to detect luciferase signals under well-plate settings.

***In vivo* imaging**. Luciferase mRNA (10 μg/mouse) was added to **WWK** coacervates (0.9 mg/ml), PEI, InstantFECT Cationic liposome, or lipofectamine (6 μl/mouse) before subcutaneous injections into the hind flank of mice. The dosage of the mRNA transfection reagents used in this study was adopted from our previous work.[1] 24 hours after injection, C57BL/6 mice were anesthetized with ketamine and xylazine. Luciferin substrate (100 μl at 30 mg/ml from Gold Biotechnology) was injected intraperitoneally. After 15 minutes, all mice were placed into the IVIS imager (IVIS® Spectrum, Thermo Fisher Scientific, USA) for detecting luminescent signals. Region of interest (ROI) signals were quantified on the IVIS software to determine luminescence intensities.

IL-1β ELISA. Purified human PBMCs were cultured in RPMI medium supplemented with 10% FBS for 24 hours before being added to 96-well U-shaped plates. Various mRNA formulations (naked mRNA, LipoMessengerMax/mRNA, InstantFECT/mRNA, Protamine/mRNA, PEI/mRNA, Comirnaty/mRNA, and **WWK**/mRNA) were added respectively (1 μg/well) and incubated for an additional 17 h. Culture supernatants were extracted from each well and mixed with human IL-1β

ELISA reagents according to the manufacturer's protocol (Abcam, USA). Briefly, biotin-conjugated anti-human IL-1 β antibody was added to each well, followed by a 2-h incubation at room temperature. After washing, streptavidin-HRP was then added and incubated for 1 h. Lastly, TMB (100 µl) was added for the colorimetric reaction, and after 15 to 20 min, the stop solution was added before absorbance reading at 450 nm.

Animal Experiments. Six-week-old C57BL/6 mice were provided by the Centre of Comparative Medicine (CCMR) of the University of Hong Kong. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching & Research (CULATR), the University of Hong Kong. The murine-derived B16-OVA cell line (Sigma-Aldrich, SCC420, source information can be provided by the supplier upon request) was cultured under mycoplasma-negative conditions in DMEM with 10% FBS. A total of 2×10^5 cells in 100 µL DMEM were subcutaneously injected into the flank of female mice using a Hamilton syringe and a size 25S gauge bevel tip needle at day 0. **WWK**/OVA mRNA (peptide, 0.9 mg/ml; mRNA, 20 µg), LipoMessengerMax/OVA mRNA (20 µg), and vehicle controls were subcutaneously administered at days 3, 5, and 7. At the tumor endpoint (day 25), mice were sacrificed, and primary and metastatic tumors were surgically harvested for tumor size analysis using electronic calipers. Spleens were surgically harvested and processed for further analysis at day 11 by resuspending strained splenocytes in an FBS-containing RPMI medium, adding RBC lysis buffer, and washing in filtered PBS. For survival studies, tumor-bearing mice were closely monitored up to the day 40 endpoint and euthanized according to the humane endpoint guidelines and judged based on the veterinarian's observation for signs and symptoms of pain and distress to minimize harm to tumor-bearing animals. The percentage of weight loss data allowed is 20% of the original body weight for the humane endpoint criteria of all animal tumor experiments. The animal experiments in this study were approved by the Committee on the Use of Live Animals in Teaching & Research, the University of Hong Kong (#CULATR 23-034).

IFN-γ ELISpot assay. On day 11, mice were sacrificed, and splenocytes were harvested for IFN-γ ELISpot analysis. Briefly, 100 µl of splenocytes were incubated on the IFN-γ ELISpot plate, which was preactivated for 30 minutes using 200 µl FBS-containing DMEM media. Subsequently, 5 µg of the peptide (OVA and the positive inducer) were added, and cells were incubated for 24 hours at 37℃

in 5% CO_2. Cells were washed with the washing buffer and incubated with the secondary biotinylated antibody for 1 h at room temperature. Finally, the cells were incubated with the biotin substrate, followed by washing and drying to generate visible spots in positive wells. ELISPOT plates were scanned and analyzed by ImmunoSpot software to count the respective wells.

ELISA. At 1 month and 2 months after the final vaccination, blood obtained from the tail vein was centrifuged at 3000 rpm for 30 min. All mouse serum was stored at −80 ℃ for further characterization. A 96-well ELISA plate was coated with 10 μg/mL of antigen (spike protein) diluted in coating medium. 100 μL of the coating solution was added to each well and kept overnight at 4 ℃. After 12 h, the solution was discarded, and the ELISA plate was blocked with blocking buffer (5% milk in TBST) for 2 h at room temperature. The wells were then washed with TBST six times. Next, the serum collected above was serially diluted in milk-TBST solution at the following ratios: 1:24, 1:72, 1:216, 1:648, 1:1944, 1:5832, 1:17496, 1:52488. The diluted serum was added to the wells and incubated for 1 h at 37 ℃. The plate was washed and then incubated with mouse IgG, mouse IgG1, or mouse IgG2a secondary antibody diluted in milk-TBST solution at a ratio of 1:3000. After 1 h of incubation at 37 ℃, the plate was washed. Next, 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB substrate) was added to each well and incubated at 37 ℃ for 30 min. Finally, the reaction was stopped using H_2SO_4 (50 μL/well), and the absorbance was read at 450 nm by a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, USA).

Neutralization Assay. At 2 months from the final vaccination, mouse serum was collected for the SARS-CoV-2 Neutralization Assay (Thermo Scientific). Briefly, the wells were washed 2 times with a Wash Buffer, then 100 μL of positive control or pre-diluted samples were added to the appropriate wells. Assay Buffer was added for negative controls. The plate was covered and incubated for 30 minutes at room temperature with shaking. The wells were washed 3 additional times before 100 μL of Biotin Conjugate solution was added to each well. The plate was covered and incubated for an additional 30 minutes at room temperature with shaking. After aspiration and washing, Streptavidin-HRP Conjugate was added and incubated for 15 min at room temperature, followed by 3 additional washes. Then, 100 μL Substrate Solution was added to each well and incubated for 15 min. Finally, 100 μL Stop Solution was added to each well. The solution in the wells changes from

blue to yellow. The absorbance was read at 450 nm by a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, USA).

Flow Cytometry. Murine subcutaneous tumors were processed into single-cell suspensions after passing through a 70-μm cell strainer (Thermo Fisher Scientific). At 4℃, 1–2 $\times 10^6$ cells were incubated with Block buffer (PBS, 2% FBS, 0.5% BSA) for 15 min, prior to the addition of 50 μl Master Mix surface marker staining (FITC anti-mouse CD3, PE/Cy7 anti-mouse CD4, BV-421 anti-mouse CD8a, 1:50, BioLegend) for 30 min. Cells were washed in filtered PBS and centrifuged at 2000 RPM before resuspension in 200 μL FACs buffer (PBS, 2% FBS, 0.002% sodium azide). For fluorescence-activated cell sorting, a NovoCyte Quanteon flow cytometer was used to analyze the samples.

Statistical analysis. All results were plotted in Graphpad Prism 7 (GraphPad Software Inc., USA). Statistical comparisons between groups were determined by an unpaired t-test using Prism 7. Mantel-Cox tests were used for the survival analysis. For all statistical tests, $p < 0.05$ was considered statistically significant.

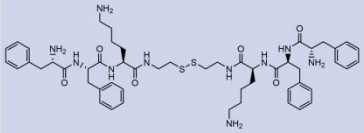
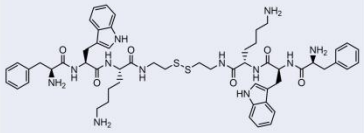
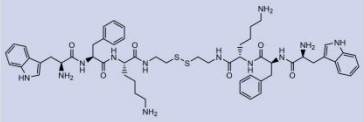
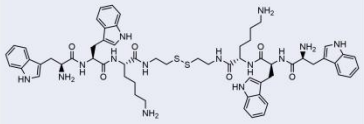
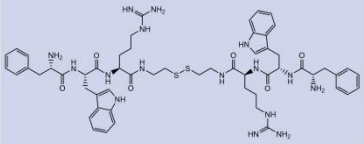
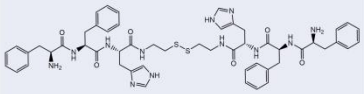
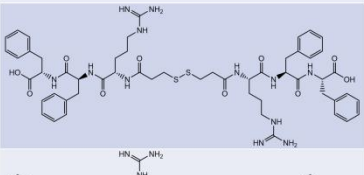
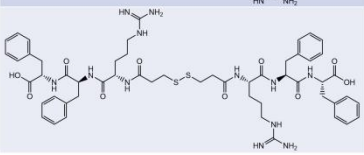
Sequence	Structure	Condensate Appearance
FFKssKFF		In PBS: Coacervates In Opti-MEM: Solution
FWKssKWF		In PBS: Aggregate In Opti-MEM: Aggregate
WFKssKFW		In PBS: Aggregate In Opti-MEM: gel-like particles
WWKssKWW		In PBS: Gel-like particles In Opti-MEM: Coacervates
FWRssRWF		In PBS: Gel-like particles In Opti-MEM: Gel-like particles
FFHssHFF		In PBS: Gel-like particles In Opti-MEM: Coacervates
HOOC-FFRssRFF-COOH		In PBS: Gel-like particles In Opti-MEM: Gel-like particles
HOOC-FWRssRWF-COOH		In PBS: Aggregate In Opti-MEM: Aggregate

Table S1. List of the synthesized peptides and their physical states.

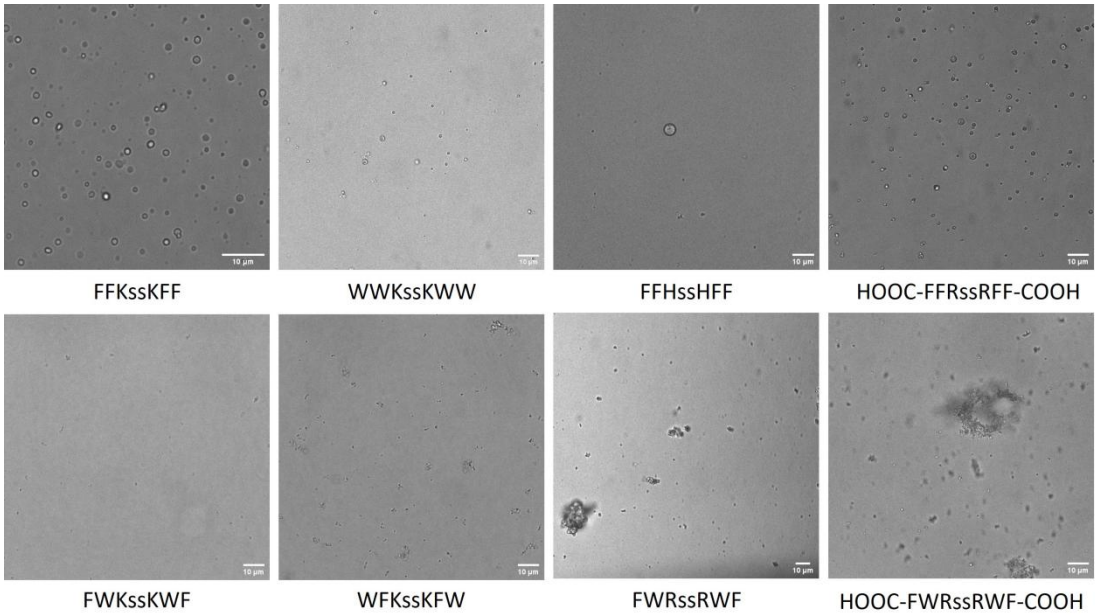


Figure S1. Screening peptides for coacervate formation in PBS.

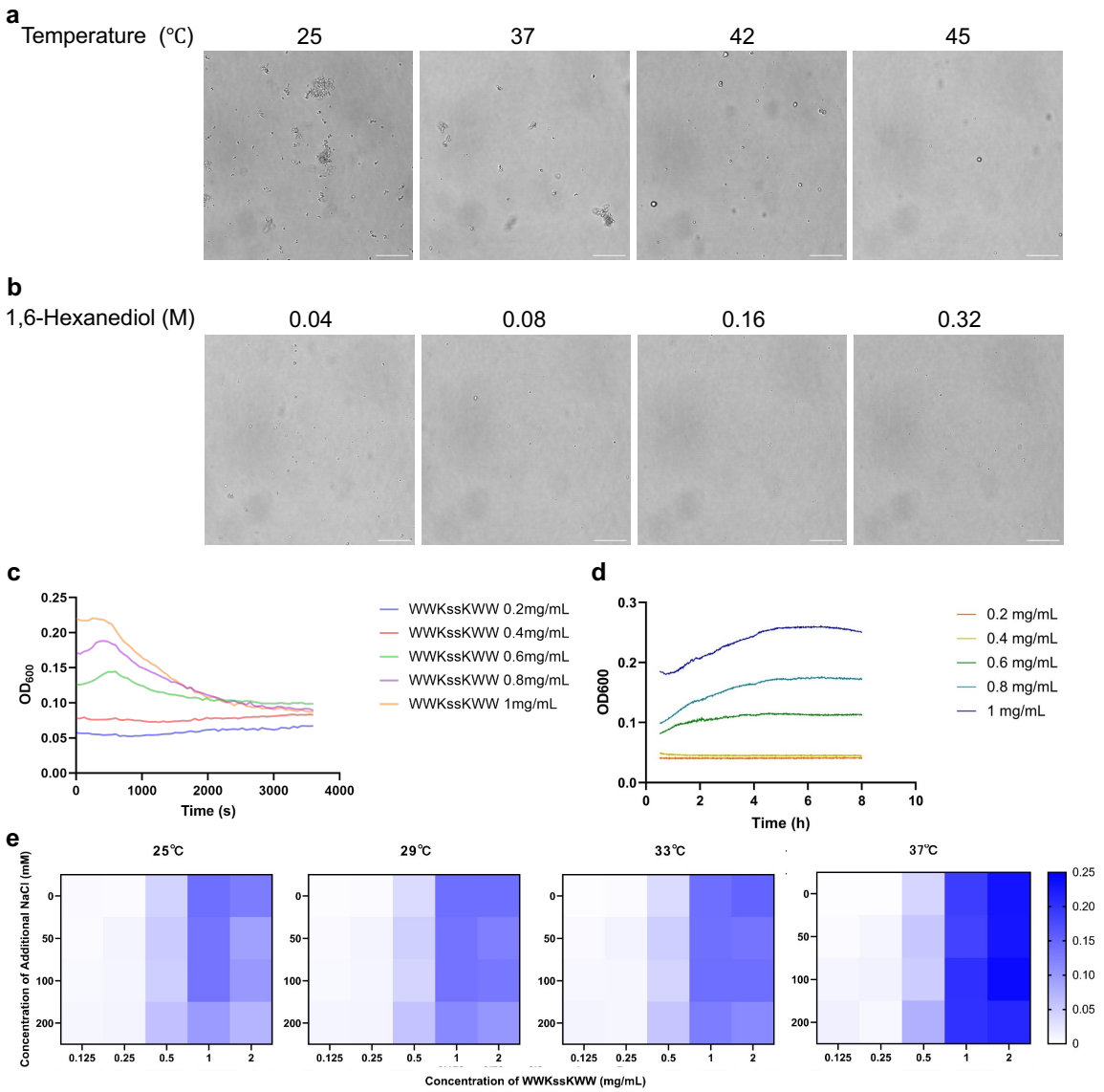


Figure S2. Characterization of WWK coacervates. a. Changes of **WWK** coacervates in PBS at different temperatures. **WWK** concentration, 2 mg/mL. **b**. Changes of **WWK** coacervates in different concentrations of 1,6-Hexanediol. **WWK** concentration, 2 mg/mL. **c**. Turbidity change of different concentrations of **WWK** in DMEM at 37℃ within 1 h. **d**. Turbidity change of different concentrations of **WWK** in DMEM at 37℃ within 8 h. **e**. Turbidity measurement of **WWK** coacervates under different concentrations, ionic strengths, and temperatures.

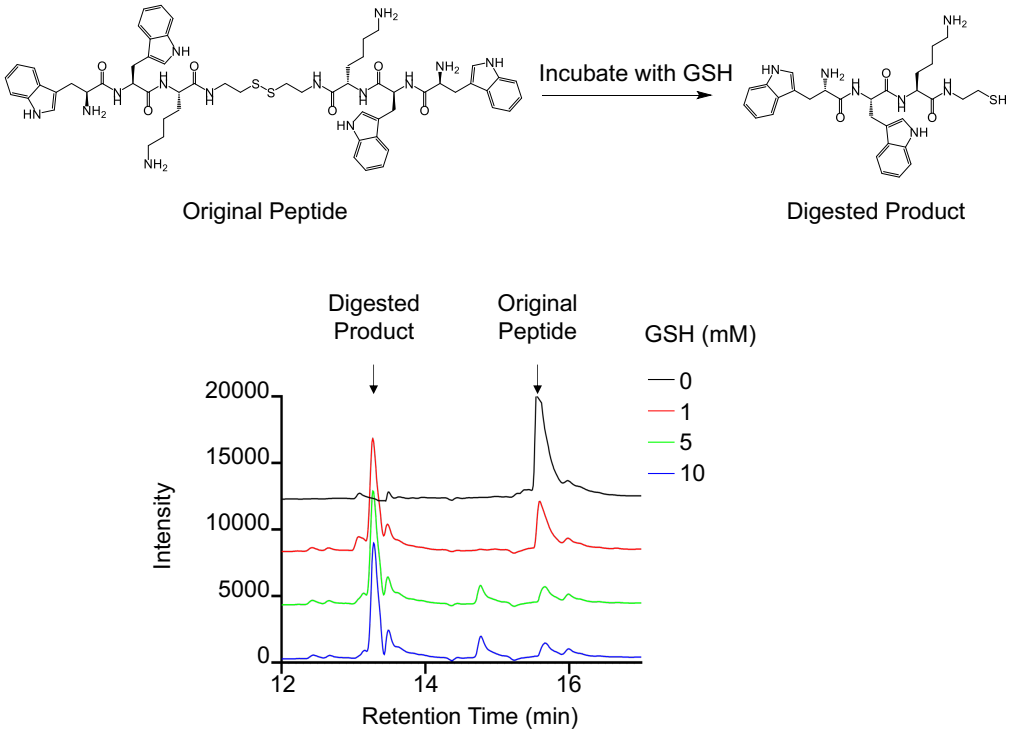


Figure S3. Reduction of WWK peptide by reduced glutathione (GSH). RP-HPLC spectra of the reaction mixture showing the decrease of WWK peptide and increase of reduced product in 24 h reacted with different concentrations of GSH.

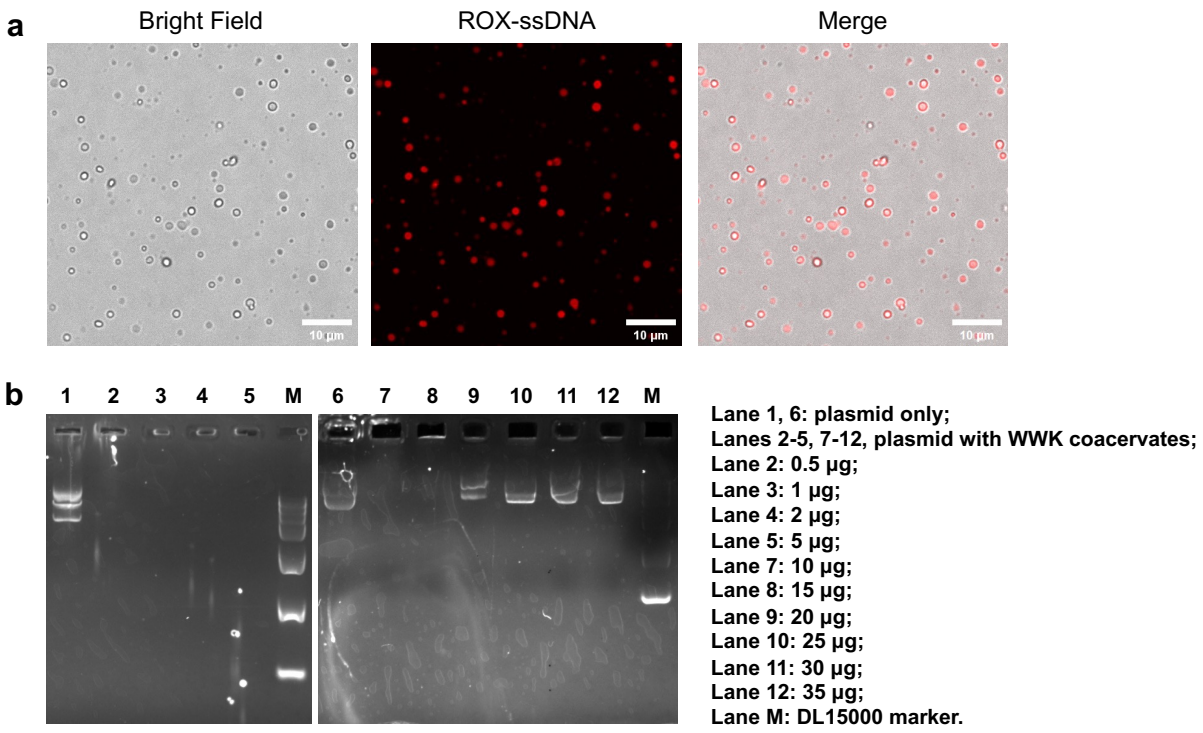


Figure S4. Nucleic acid recruitment in WWK coacervates and the delivery into HEK293 cells. a. Confocal microscopic images showing the encapsulation of a 22-nt DNA with 5' end modified with a ROX fluorescent molecule (ROX-ssDNA) in **WWK** coacervates. **b**. Encapsulation efficiency of different amount of a 7.6-kb plasmid within 1 mg/mL **WWK** coacervates at 37℃ in 25 μL Opti-MEM. Briefly, 0.5 (Lane 2), 1 (Lane 3), 2 (Lane 4), 5 (Lane 5), 10 (Lane 7), 15 (Lane 8), 20 (Lane 9), 25 (Lane 10), 30 (Lane 11), 35 (Lane 12) μg of plasmids (~7.6 kb) were dissolved in 25 μL Opti-MEM. Then, 2.5 μL 100 mg/mL WWK was mixed with the medium. The mixtures were incubated at 37℃ for 15 min and centrifuged after incubation. The supernatant was loaded on the agarose gel for electrophoresis. 1 μg plasmids without the addition of WWK were loaded as control groups (Lane 1 and 6).

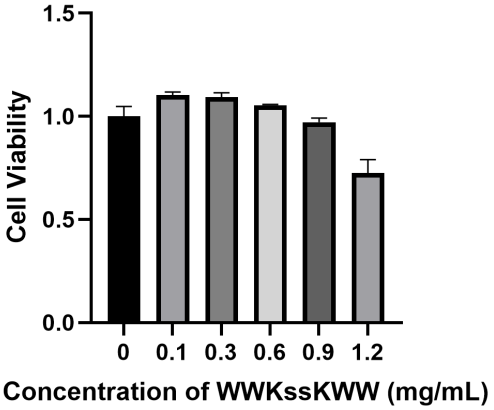


Figure S5. Cytotoxicity of WWK coacervates measured by the LDH assay. Briefly, HEK293 cells were incubated with different concentrations of **WWK** coacervates for 24 h before LDH assay was performed.

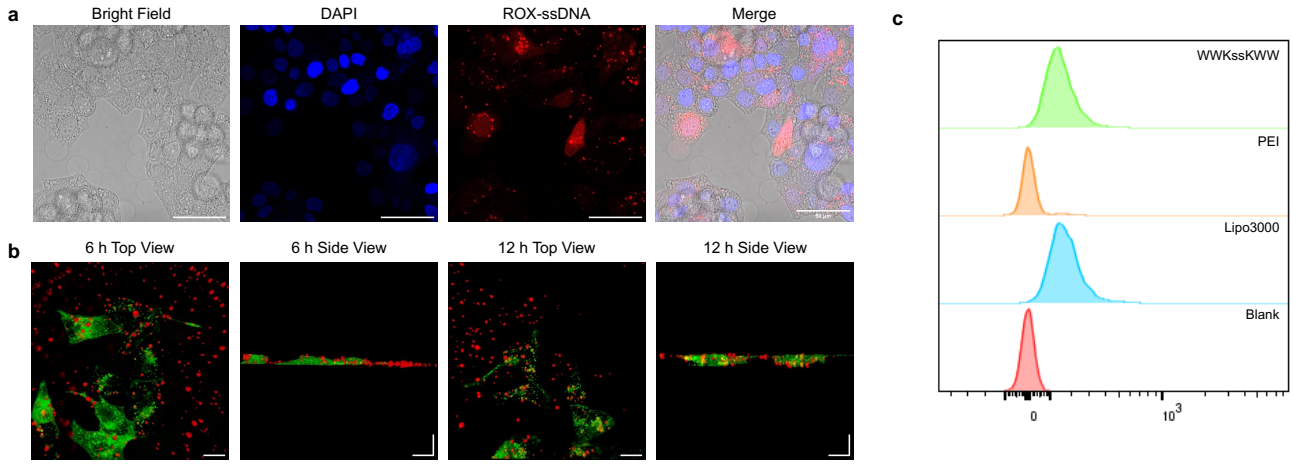


Figure S6. WWK coacervates mediate the delivery of ssDNA into HEK293 cells. a. Confocal microscopy images of HEK293 cells treated with ROX-ssDNA for 24 h, showing the distribution of ROX-ssDNA in the cytosol. **b**. 3D confocal microscopy images of HEK293 cells treated with **WWK**/ROX-ssDNA coacervates. Red channel, ROX-labeled ssDNA; green channel, DIO staining; scale bar, 20 μm. **c.** Flow cytometry data showing the fluorescence in HEK293 cells treated with **WWK**/ROX-ssDNA coacervates for 24 h, in comparison with ROX-ssDNA delivered by PEI and Lipo3000.

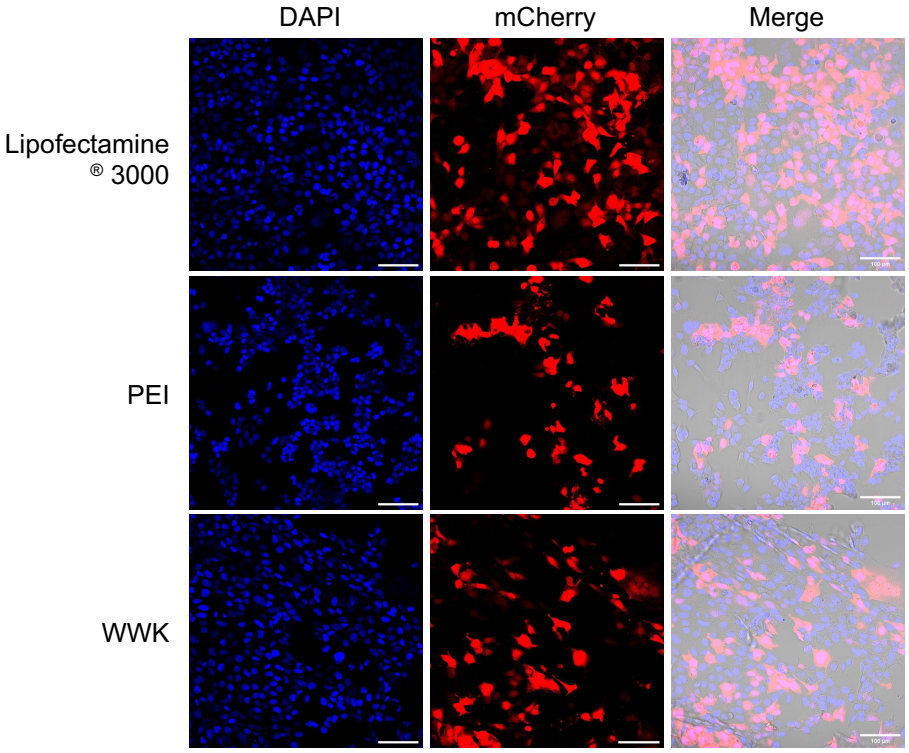


Figure S7. Confocal microscopy images showing the expression of mCherry in HEK293 cells transfected by pCMV-mcherry with different transfection reagents.

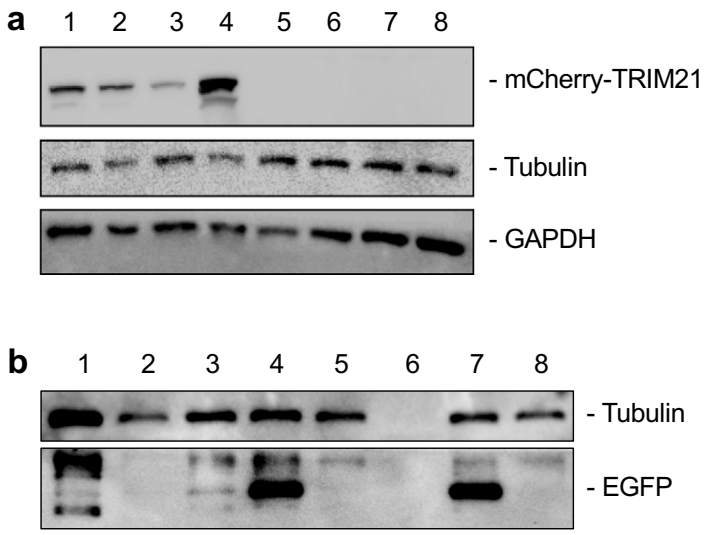


Figure S8. siRNA and plasmid transfection with different transfection agents in MDA-MB-231 cells. a. Transfection of plasmid pCI-neo.mcherry-TRIM21 and siGAPDH at different concentrations of **WWK** coacervates, Lipo3000, and PEI. Lanes 1-4, pCI-neo.mcherry-TRIM21 transfection: Lane 1, 0.2 mg/mL **WWK**; Lane 2, 0.3 mg/mL **WWK**; Lane 3, Lipo3000; Lane 4, PEI; Lane 5-7, siGAPDH transfection: Lane 5, 0.2 mg/mL **WWK**; Lane 6, Lipo3000; Lane 7, PEI; Lane 8, blank. **b**. Transfection of an EGFP mRNA in MDA-MB-231 cells by different transfection agents. Lane 1, blank; Lane 2, Lipo3000 + 4 μg mRNA; Lane 3, Lipo3000 + 2 μg mRNA; Lane 4, 0.2 mg/mL **WWK** + 2 μg mRNA; Lane 5, 0.5 mg/mL **WWK** + 2 μg mRNA; Lane 6, 0.9 mg/mL **WWK** + 2 μg mRNA; Lane 7, 0.2 mg/mL **WWK** + 4 μg mRNA; Lane 8, 0.5 mg/mL **WWK** + 4 μg mRNA; Lane 9, 0.9 mg/mL **WWK** + 4 μg mRNA.

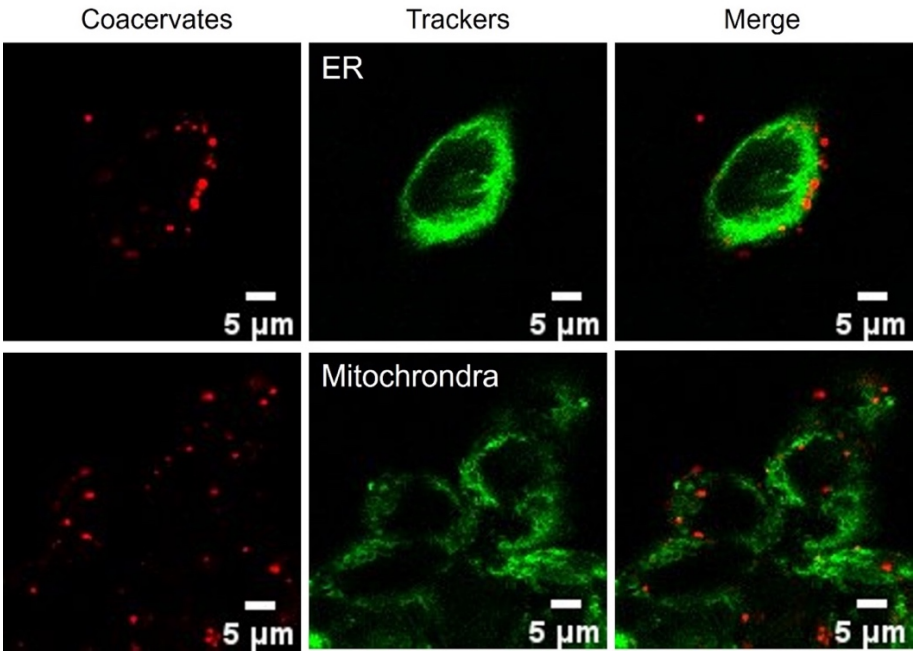


Figure S9. Confocal microscopy images showing that WWK coacervates did not colocalize with ER or mitochondria.

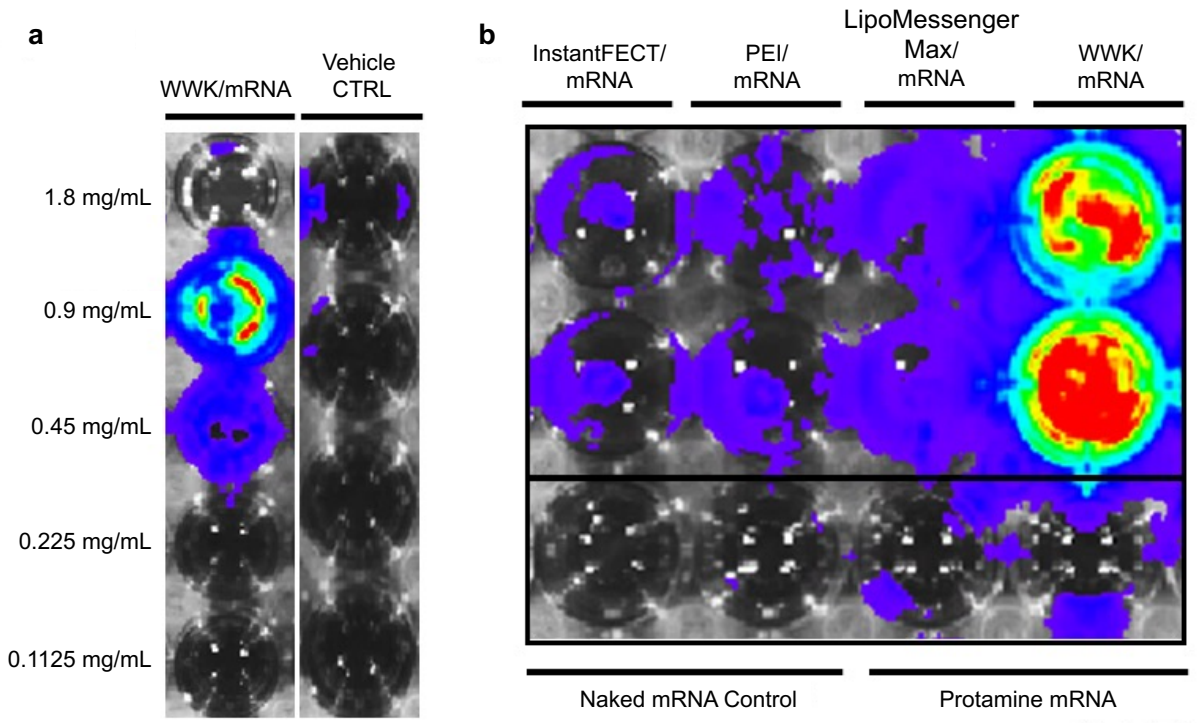


Figure S10. ***In vitro*** **transfection of luciferase mRNA into HEK293 cells by different transfection agents.** a) Luciferase activity in HEK293 cells transfected with luciferase mRNA and different concentrations of **WWK** coacervates. b) Comparison of luciferase activity in HEK293 cells transfected with luciferase mRNA and 0.9 mg/ml (selected as the optimal dose) **WWK** versus InstantFECT, PEI, and, LipoMessengerMax.

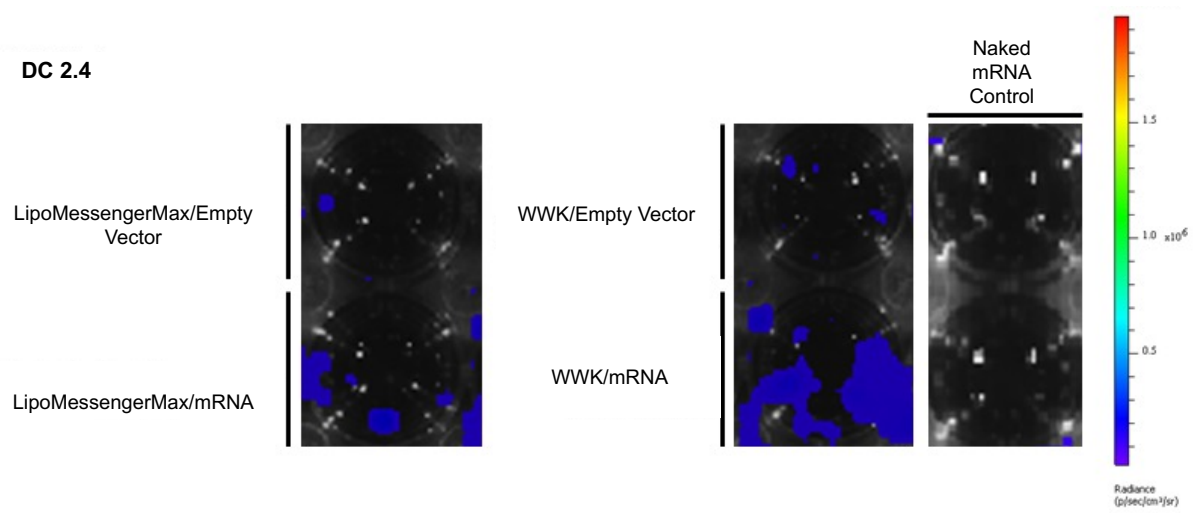


Figure S11. ***In vitro*** **transfection of luciferase mRNA into dendritic cells (DC 2.4) by WWK coacervates and LipoMessengerMax.**

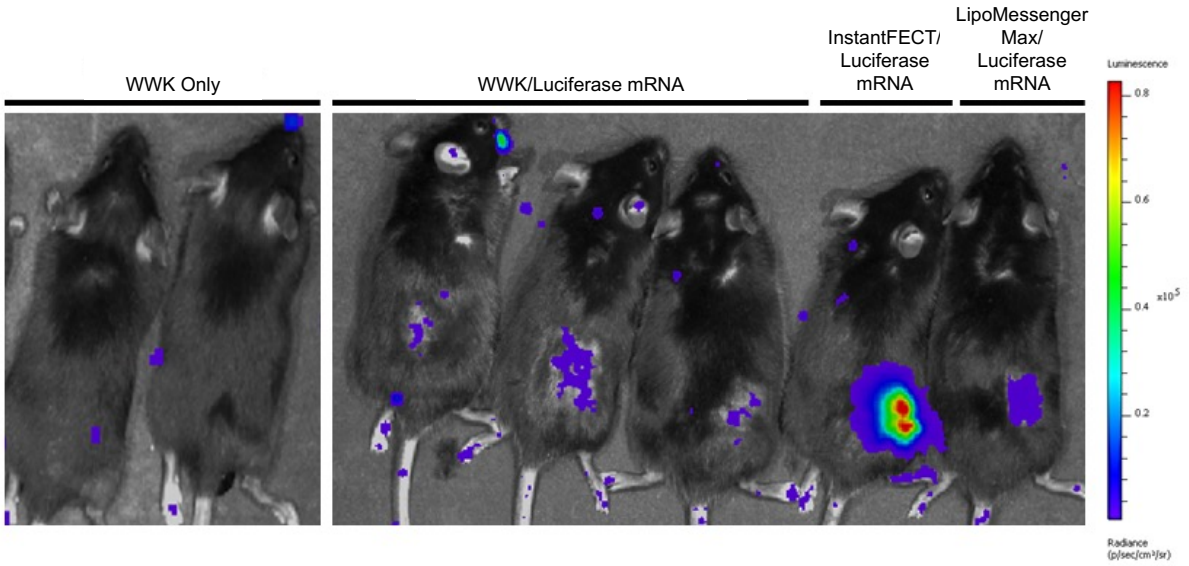


Figure S12. ***In vivo*** **imaging of mice subcutaneously injected with various luciferase mRNA formulations.** Luciferase mRNA (10 μg) was delivered by WWK coacervates, InstantFECT, and LipoMessengerMax, respectively.

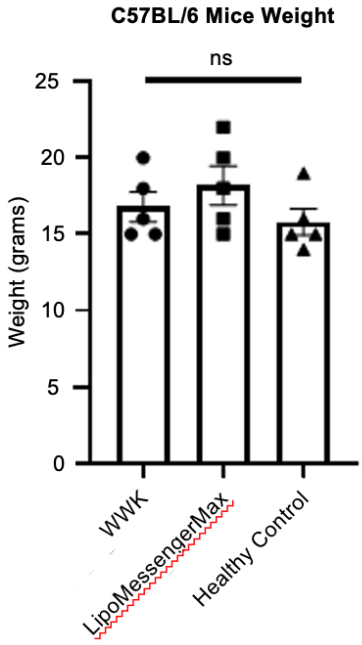


Figure S13. Body weights of C57BL/6 mice 48 hours after receiving injections of WWK coacervates or LipoMessengerMax.

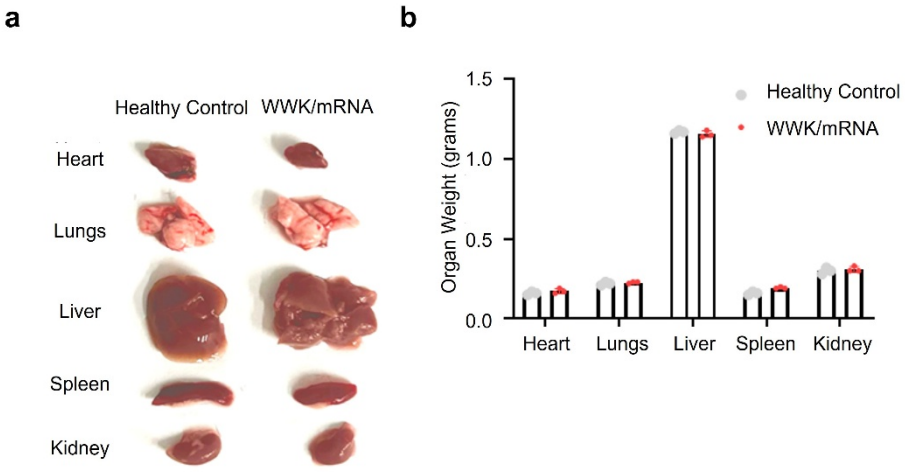


Figure S14. **Analysis of major organs of immunized mice.** 3 days after the final injection (third immunization) of the **WWK**/OVA mRNA formulation, mouse organs were harvested, imaged, and weighed for comparison with healthy controls. No difference was found between the two groups.

Reference:

1. Yu, J.; Kuwentrai, C.; Gong, H.-R.; Li, R.; Zhang, B.; Lin, X.; Wang, X.; Huang, J.-D.; Xu, C. Intradermal Delivery of mRNA Using Cryomicroneedles. *Acta Biomater.* **2022**, *148*, 133–141. https://doi.org/10.1016/j.actbio.2022.06.015.