Supporting Information

A Modular Polymer Platform for Efficient mRNA Delivery in Cancer Immunotherapy

*Guanyou Lin†[a], Jianxi Huang†[a], Xinqi Li[a], Yunshan Liu[a], Taylor Juenke[a], Arthur Finstad[a] and Miqin Zhang[a]**

[a]Department of Materials Science and Engineering, University of Washington,
Seattle, Washington 98195, United States.
†These authors contributed equally.
E-mail: mzhang@uw.edu

Supplementary Figures

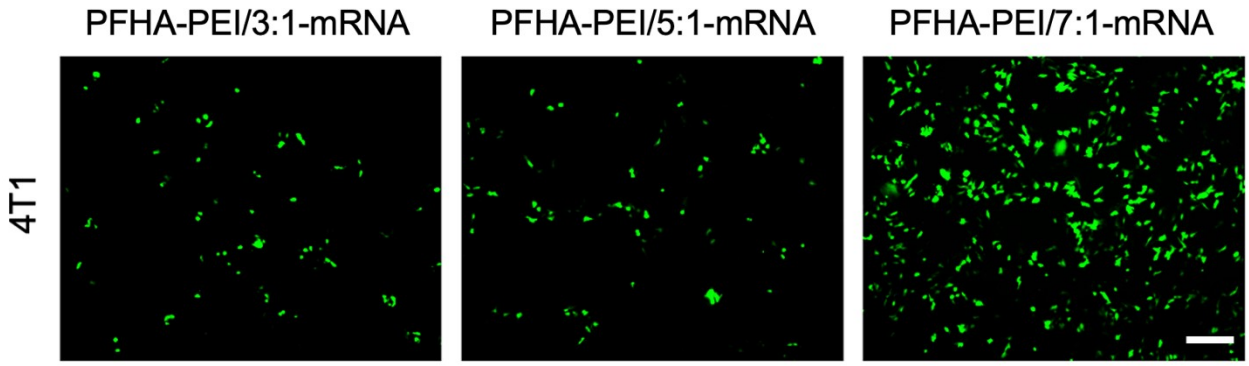


Figure S1. Optimization of PFHA:PEI ratio for transfection efficiency in 4T1 cells. Fluorescence images showing 4T1 cells transfected with PFHA-PEI-mRNA to determine the optimal PFHA:PEI ratio. PFHA:PEI of various ratios, including PFHA:PEI/3:1, PFHA:PEI/5:1 and PFHA:PEI/7:1, were synthesized and complexed with mRNA at a PFHA-PEI:mRNA weight ratio of 10:1. The transfection was performed on 4T1 cells at 2 μg/mL mRNA dose, followed by 48-hour incubation before imaging. The scale bar is 200 μm. The results indicate that PFHA:PEI/7:1 exhibits the highest transfection efficiency among the tested ratios. Although higher PFHA:PEI ratios could potentially enhance transfection efficiency further, it is noted that excessively high ratios may compromise the aqueous solubility of PFHA-PEI. Therefore, PFHA:PEI/7:1 is identified as the optimized ratio for achieving the best balance between transfection efficiency and aqueous solubility.

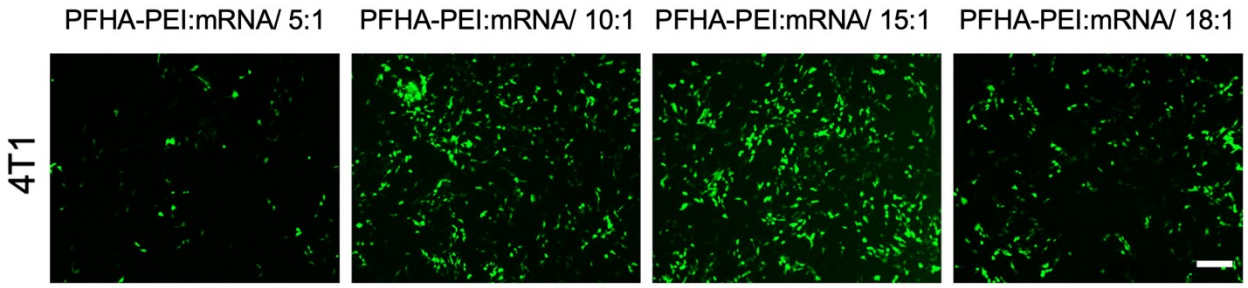


Figure S2. Optimization of PFHA-PEI:mRNA ratio for transfection efficiency in 4T1 cells. Fluorescence images illustrating 4T1 cells transfected with PFHA-PEI-mRNA to determine the optimal PFHA-PEI:mRNA ratio. PFHA:PEI/7:1 was complexed with mRNA at various PFHA-PEI:mRNA weight ratios, including 5:1, 10:1, 15:1, 18:1. The transfection was carried out on 4T1 cells at 2 μg/mL mRNA dose, followed by a 48-hour incubation before imaging. The scale bar is 200 μm. The results indicate that PFHA-PEI:mRNA/15:1 wt/wt achieves the highest transfection efficiency among the tested ratios. Both higher or lower PFHA-PEI:mRNA weight ratios resulted in lower transfection efficiency on 4T1 cells. Therefore, PFHA-PEI:mRNA/15:1 wt/wt identified as the optimized ratio for achieving the best transfection efficiency.

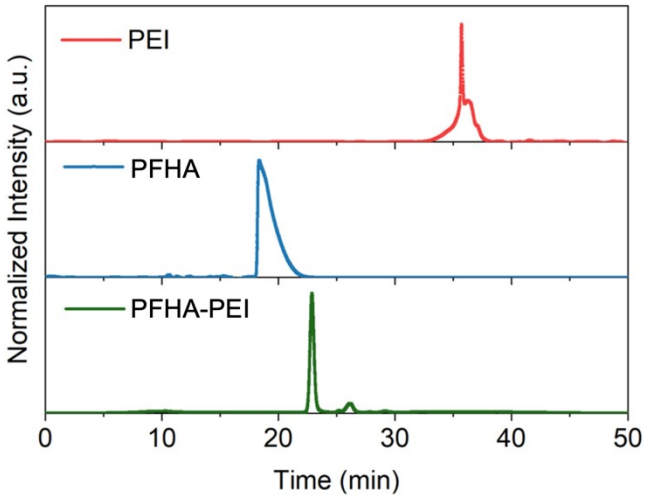


Figure S3. HPLC analysis of PEI, PFHA and PFHA-PEI to assess the purity of PFHA-PEI. The mobile phase compromised two solvents: A 85% Acetonitrile and B 15% DI-H2O. The separation of PEI, PFHA and PFHA-PEI was achieved using a linear gradient of A–B (v/v), with a constant flow rate of 0.2 mL/min. Absorbance was monitored at 220 nm. The retention times for PEI, PFHA and PFHA-PEI were 36 mins, 18 mins and 23 mins, respectively. The absence of characteristic peaks of PEI and PFHA in the PFHA-PEI spectrum indicates that PFHA-PEI is free of unconjugated PFHA and PEI impurities, confirming its purity.

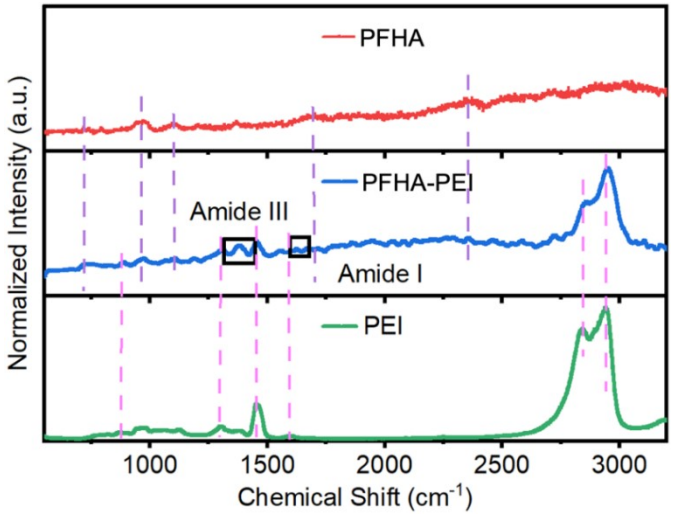


Figure S4. Raman spectra of PEI, PFHA and PFHA-PEI for validating the linkage between PFHA and PEI. Purple vertical dashed lines highlight the common peaks shared by both PFHA and PFHA-PEI, while pink vertical dashed lines highlight the common peaks shared by both PEI and PFHA-PEI. The PFHA-PEI spectrum reveals the presence of amide III and amide I, as indicated by the left and right square boxes, respectively. These findings confirm the successful linkage between PFHA and PEI in PFHA-PEI.

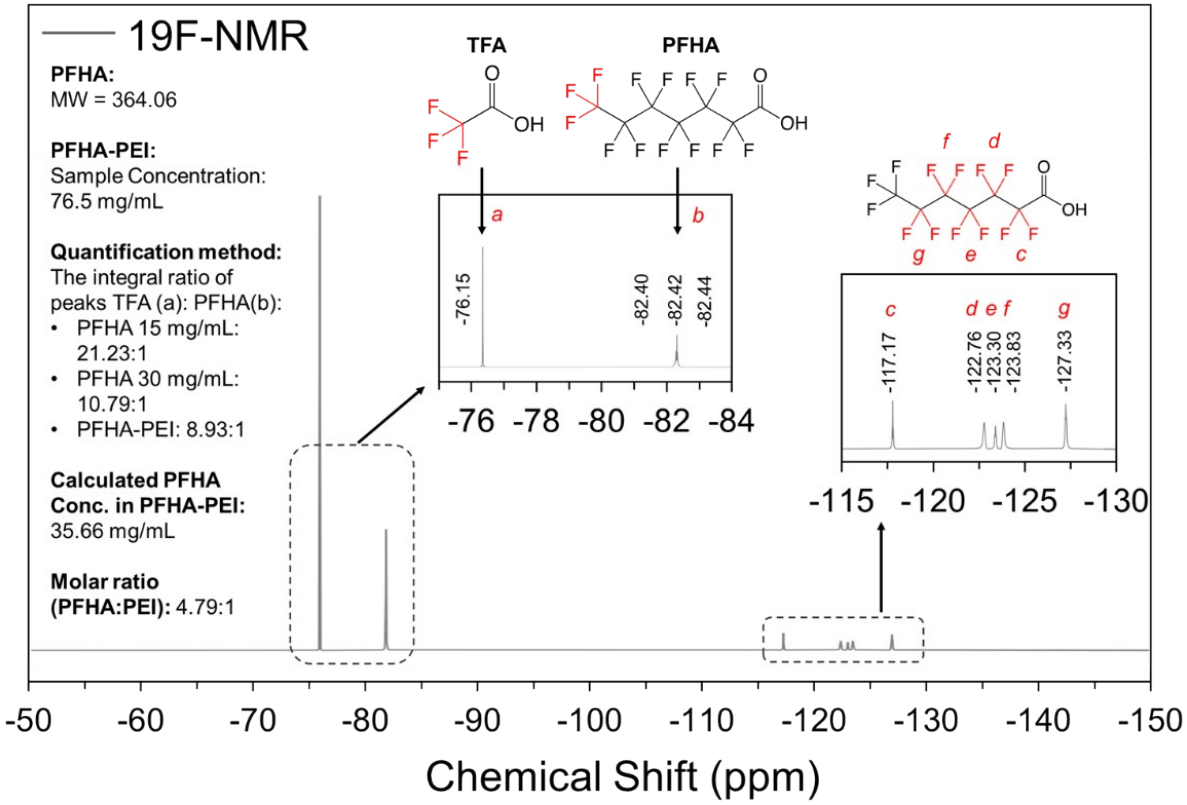
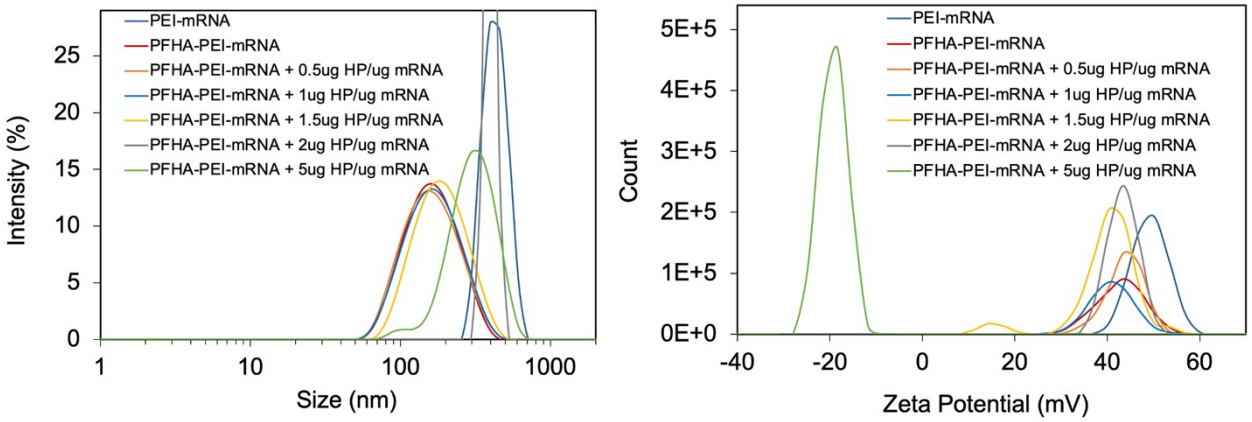


Figure S5. ^{19}F NMR spectra of PFHA, PFHA-PEI, and internal standard TFA (trifluoroacetic acid). PFHA samples (15 mg/mL and 30 mg/mL) were used to generate a calibration curve by calculating the ratio of integrated peak areas of TFA (−76.15 ppm, labeled **a**) to PFHA (−82.42 ppm, labeled **b**). These ratios were 21.23 and 10.79, respectively. Using the same approach, the integral ratio **a:b** of the PFHA-PEI sample was measured to be 8.93, from which the PFHA concentration in the PFHA-PEI sample was calculated to be 35.66 mg/mL. Given the total PFHA-PEI sample concentration (76.5 mg/mL), the molar ratio of PFHA to PEI was determined to be approximately 4.79:1. The zoomed-in spectral regions highlight the ^{19}F signals from TFA and PFHA fluorine atoms used for quantification.



Sample Name	Number-Weighted Size (d.nm)	Intensity-Weighted Size (d.nm)	PDI	Zeta (mV)
PEI-mRNA	371.37 +/- 31.81	551.60 +/- 15.31	0.402 +/- 0.064	49.9 +/- 0.726
PFHA-PEI-mRNA + 0 ug HP/ug mRNA	96.72 +/- 13.81	144.53 +/- 6.05	0.162 +/- 0.013	39.07 +/- 2.79
PFHA-PEI-mRNA + 0.5 ug HP/ug mRNA	86.47 +/- 5.24	143.50 +/- 5.89	0.195 +/- 0.011	40.73 +/- 1.44
PFHA-PEI-mRNA + 1.0 ug HP/ug mRNA	95.99 +/- 2.39	146.30 +/- 1.37	0.166 +/- 0.011	36.70 +/- 0.86
PFHA-PEI-mRNA + 1.5 ug HP/ug mRNA	96.36 +/- 18.90	169.13 +/- 0.760	0.161 +/- 0.009	38.60 +/- 2.89
PFHA-PEI-mRNA + 2.0 ug HP/ug mRNA	242.97 +/- 116.46	2603.00 +/- 949.100	0.999 +/- 0.001	40.50 +/- 0.37
PFHA-PEI-mRNA + 5.0 ug HP/ug mRNA	230.03 +/- 28.26	446.53 +/- 32.85	0.458 +/- 0.033	-33.87 +/- 0.34

Figure S6. Hydrodynamic size and zeta potential distribution profiles. Hydrodynamic size and zeta potential distribution profiles are presented for PEI-mRNA, PFHA-PEI-mRNA, and PFHA-PEI-mRNA-HP. The mRNA:HP weight ratio explored include 0, 0.5, 1, 1.5, 2, and 5. The accompanying full data table provides detailed information for each condition.

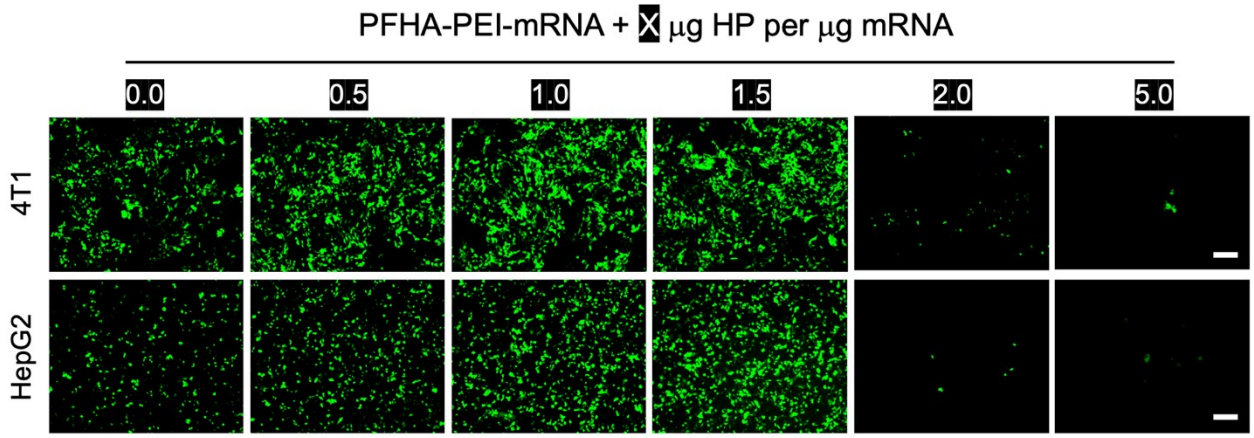


Figure S7. Optimization of Heparin (HP) Amount for Transfection Efficiency in Cancer Cells. Fluorescence images showing two types of cancer cells transfected with PFHA-PEI-mRNA-HP samples containing different amounts of HP, including 0, 0.5, 1.0, 1.5, 2.0, 5.0 μg HP per μg mRNA. The transfection was performed with 2 μg/mL mRNA dose for 48 hours to determine the optimal HP amount for transfection across different cell lines. The results indicate that 1.0 μg HP per μg mRNA yielded the best transfection results across all 3 cell lines. The scale bar is 200 μm.

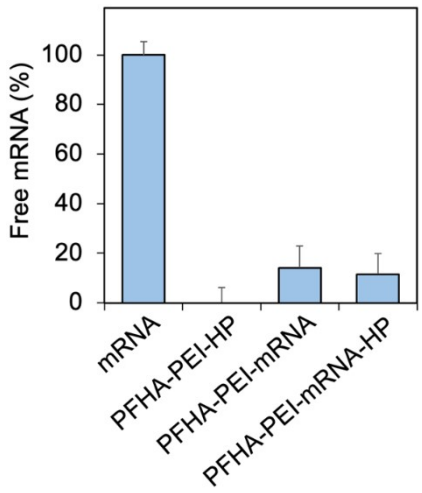


Figure S8. mRNA encapsulation efficiency of PFHA-PEI-mRNA and PFHA-PEI-mRNA-HP. The mRNA content in each sample was normalized against the pure mRNA positive control group (set at 100%). PFHA-PEI-HP without mRNA served as the negative control. The mRNA encapsulation efficiency was calculated by 100% – mRNA% in the supernatant.

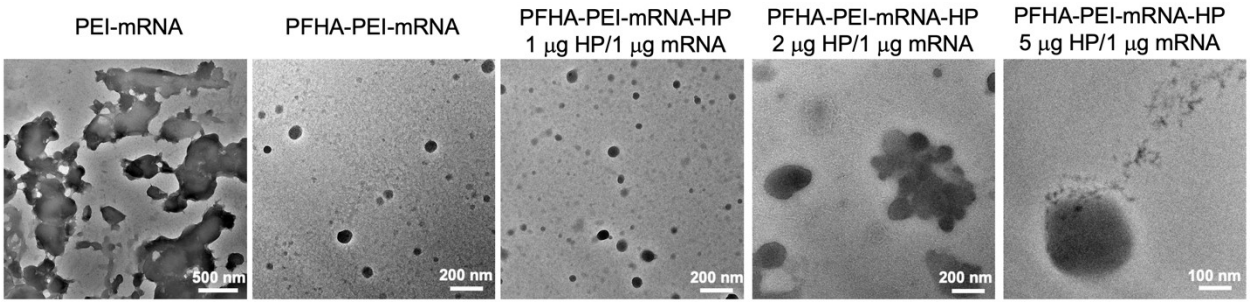


Figure S9. Additional TEM images of PEI-mRNA, PFHA-PEI-mRNA, and PFHA-PEI-mRNA-HP. Specifically, the TEM images of PFHA-PEI-mRNA and PFHA-PEI-mRNA-HP with 1 μg HP/1 μg mRNA showcase a wider field of view to highlight the relatively uniform sizes of these nanoparticles. The TEM images of PEI-mRNA and PFHA-PEI-mRNA-HP with 2 μg HP/1 μg mRNA show reveal the presence of significant aggregates. The TEM images of PFHA-PEI-mRNA-HP with 5 μg HP/1 μg mRNA illustrate the breaking apart of PFHA-PEI-mRNA-HP.

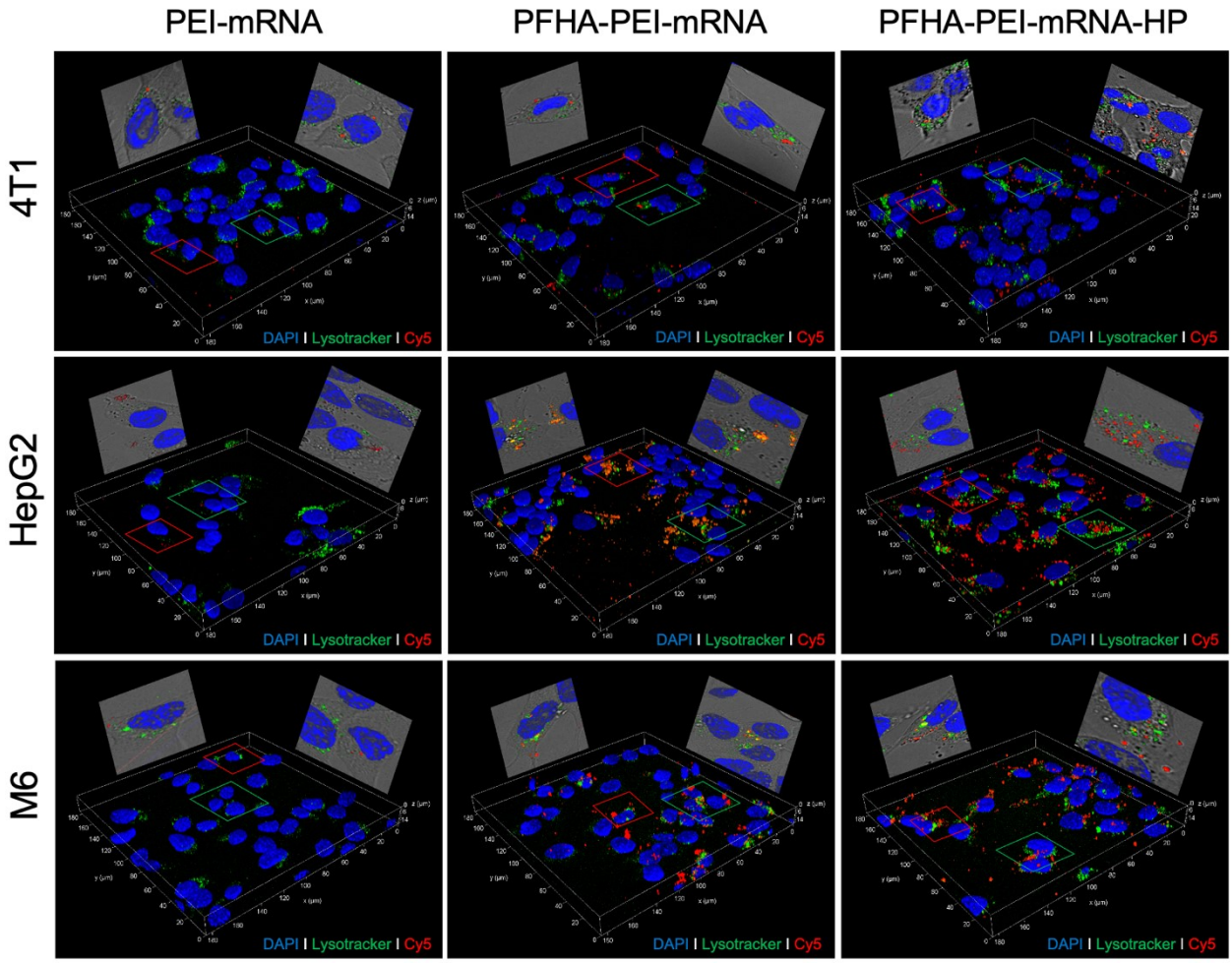


Figure S10. Cellular uptake and endosomal escape studies of PEI-mRNA, PFHA-PEI-mRNA and PFHA-PEI-mRNA-HP on 3 different cell types. All treatments were applied to cells at 37 °C for 12 hours at an mRNA concentration of 2 μg/mL. 3D Z stacked confocal images were taken with a z-resolution of 0.5 μm. In the images, blue represents cell nuclei; green represents lysotracker, and red represents mRNA. The red square in each image highlights the area further magnified and presented in the upper left inset. The green square in each image highlights the area further magnified and presented in the upper right inset. The insets include bright field background to show the cell membrane boundary.

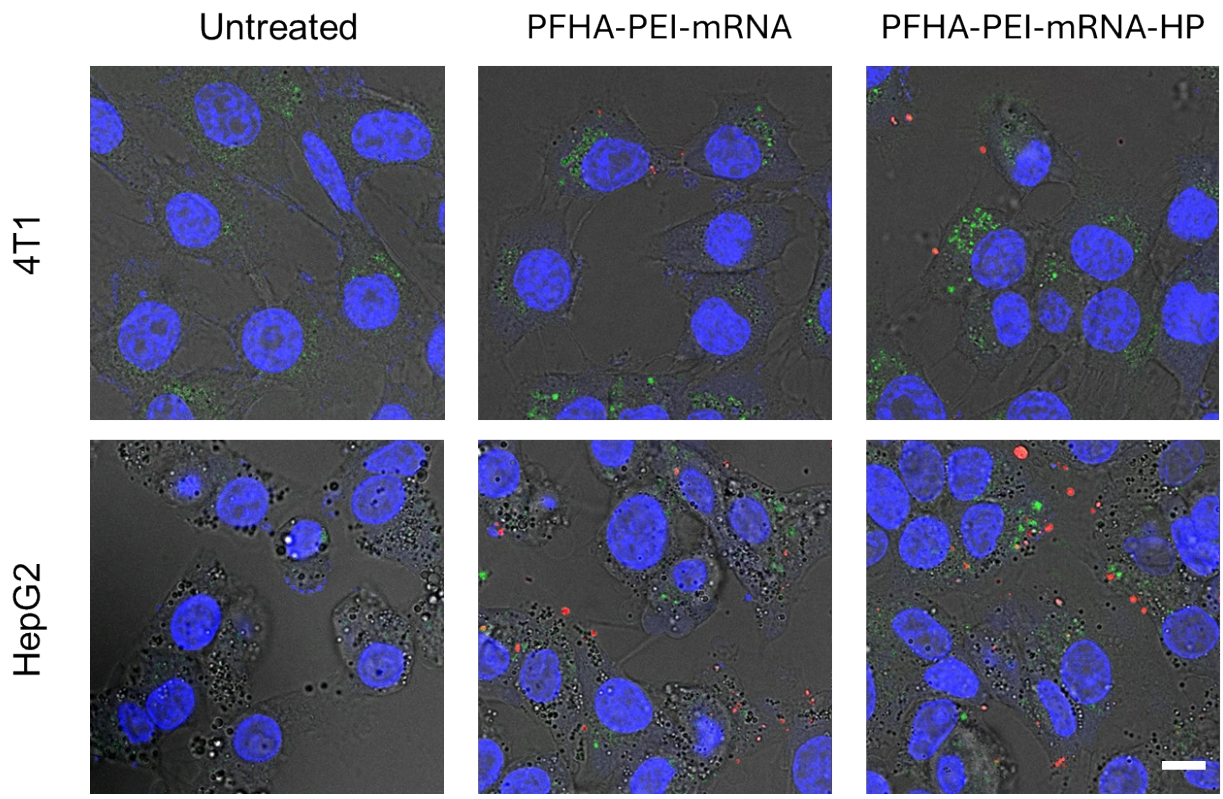


Figure S11. Cellular uptake and endosomal escape of PFHA-PEI-mRNA-HP in 4T1 and HepG2 cells. Cells were incubated at 37 °C for 3 hours with nanoparticles containing 2 μg/mL mRNA. Blue indicates cell nuclei (DAPI), green indicates endosomes/lysosomes (LysoTracker), and red indicates mRNA. Scale bar: 50 μm. Bright-field images were merged to delineate cell boundaries. Enhanced cytoplasmic mRNA signal (red) in the PFHA-PEI-mRNA-HP group suggests improved endosomal escape relative to PFHA-PEI-mRNA.

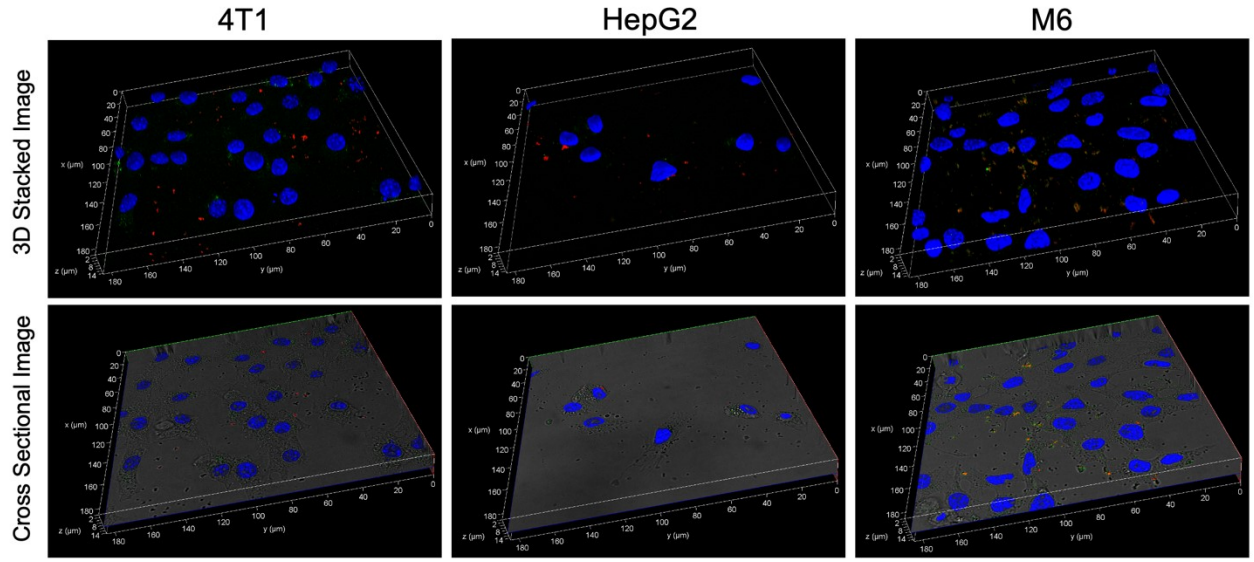


Figure S12. Cellular uptake and endosomal escape studies of PFHA-PEI-mRNA-HP on 3 different cell types conducted at 4 °C for 12 hours, using at an mRNA concentration of 2 μg/mL. 3D Z stacked confocal images were taken with a z-resolution of 0.5 μm. In the images, the blue represents cell nuclei, green represents lysotracker, and red represents mRNA. The cross-sectional image panels include a bright field background to show cell membrane boundary.

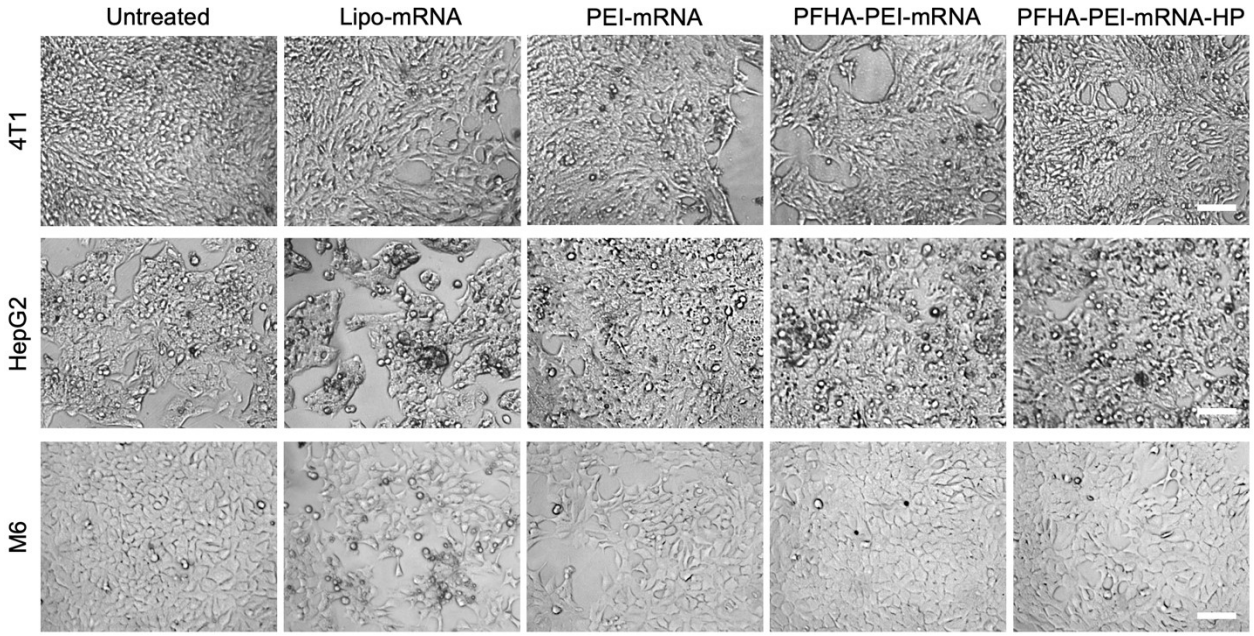


Figure S13. Bright field images of 4T1, HepG2, and M6 treated with Lipofectamine 2000-mRNA, PEI-mRNA, PFHA-PEI-mRNA and PFHA-PEI-mRNA-HP at a 2 μg/mL mRNA concentration. Untreated cells are included as a cell control.

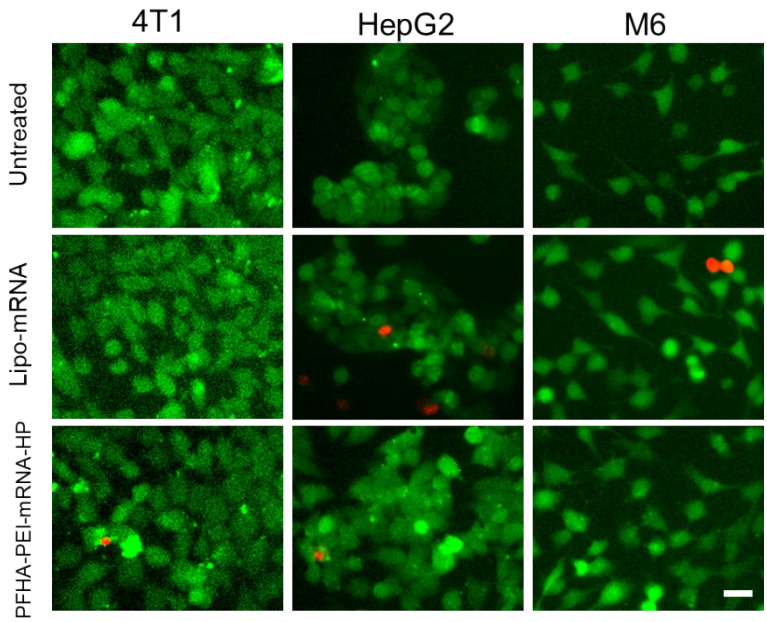


Figure S14. Live/Dead staining of 4T1, HepG2, and M6 cells after treatment with PFHA-PEI-mRNA-HP nanoparticles. Cells were stained with Calcein AM (green, live cells) and Propidium Iodide (red, dead cells) 24 hours post-treatment. Most cells exhibit strong green fluorescence and minimal red signal, indicating high viability and low cytotoxicity. Scale bar = 50 μm.

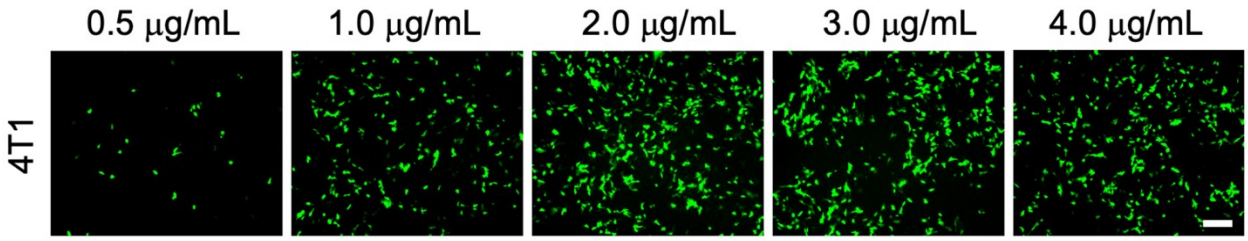


Figure S15. Fluorescence images of 4T1 transfected with PFHA-PEI-mRNA-HP for dose-dependent effect on transfection efficiency. PFHA-PEI-mRNA-HP was added to 4T1 at various mRNA doses and incubated for 16 hours before imaging to determine the optimal dose for transfection on cancer cell lines. The image results showed that transfection reached optimal efficiency at 2.0 μg mRNA/mL for cancer cell lines. The scale bar is 200 μm.

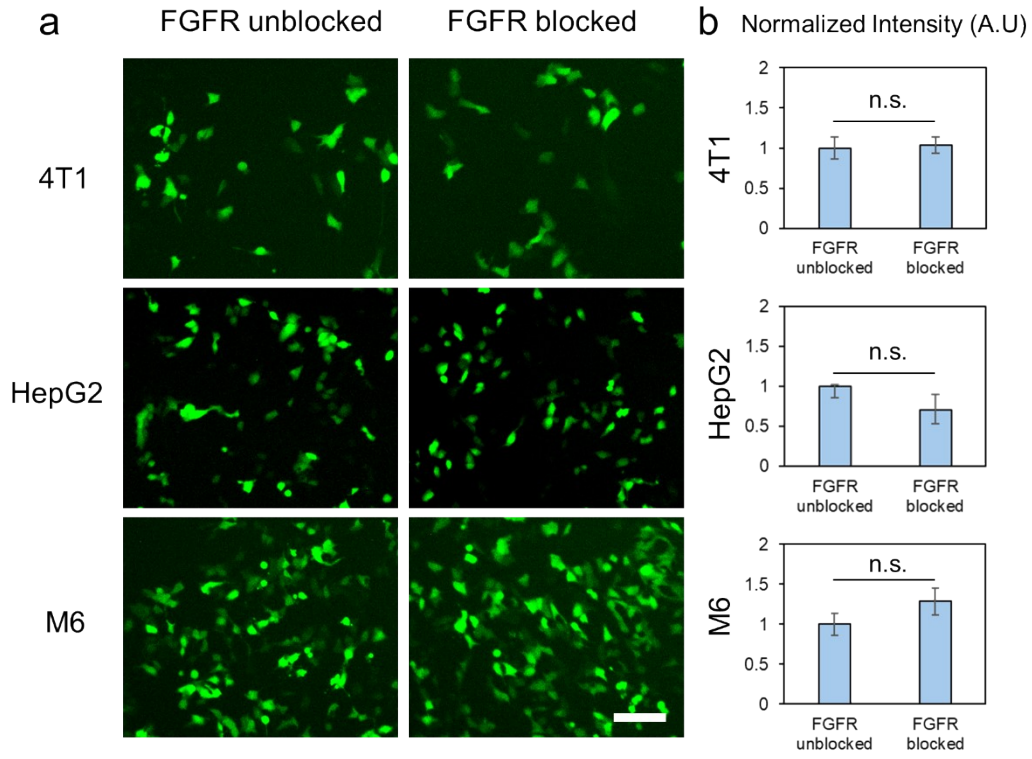


Figure S16. Assessment of FGFR involvement in PFHA-PEI-mRNA-HP nanoparticle uptake. (a) Representative fluorescence images of 4T1, HepG2, and M6 cells transfected with PFHA-PEI-mRNA-HP nanoparticles in the presence or absence of the FGFR inhibitor PD173074. Cells were pretreated with PD173074 (250 nM) for 1 hour prior to nanoparticle administration and maintained in the inhibitor-containing medium throughout transfection. Green fluorescence indicates EGFP expression. Scale bar = 100 μm. (b) Quantification of normalized EGFP fluorescence intensity from transfected cells. No significant differences (n.s.) were observed between FGFR-blocked and unblocked groups across all cell lines.

Supplementary Methods

Purity Analysis of PFHA-PEI by HPLC

The purity of PFHA-PEI copolymer was assessed using high-performance liquid chromatography (HPLC) with a Shimadzu LC-20 AD system equipped with a UV. The reversed phase column was a 4.6 mm × 250 mm Synergi Hydro-RP with an injection volume of 15 μL. The mobile phase consisted of two solvents: (A) 85% Acetonitrile and (B) 15% DI-H2O. The separation was achieved through a linear gradient of A–B (v/v). The flow rate was maintained at 0.2 mL/min. Absorbance was monitored at 220 nm.

Raman Spectroscopy Analysis

Raman spectroscopy analysis (inVia™ confocal Raman microscope, RENISHAW) was performed to study the structural information of PFHA-PEI. After ultrasonic dispersion of 10mg PFHA-PEI in 1mL ethanol, it was dropped on a silicon wafer and dried naturally in a fume hood overnight to obtain the test sample. The excitation source was a 514 nm laser. The Raman spectra of PFHA-PEI were recorded to determine their vibrational characteristics and to provide information on their structural features.

Quantitative ^{19}F NMR Analysis

Standard PFHA solutions (30 mg/mL and 60 mg/mL) were prepared in deuterated methanol (D-methanol) and mixed 1:1 (v/v) with trifluoroacetic acid (TFA, 10 mg/mL) as an internal standard. The PFHA-PEI sample (153 mg/mL) was similarly mixed 1:1 (v/v) with TFA (10 mg/mL) in D-methanol. 19F NMR spectra were acquired on a Bruker AVANCE NEO 500 MHz spectrometer with a nitrogen-cooled Prodigy BB-H&F CryoProbe. Spectra were processed using Topspin 4.2.0 with 5th-order polynomial baseline correction. The TFA peak (−76.15 ppm) and PFHA peak (−82.42 ppm) area integrals were measured to quantify relative signal intensities.

mRNA Encapsulation Efficiency Study

Free mRNA, PFHA-PEI-mRNA and PFHA-PEI-mRNA-HP, each containing equal amount of mRNA, along with PFHA-PEI-HP without mRNA, were incubated with 1 mg/mL Ethidium bromide solution for 10 minutes at room temperature before subjected to fluorescence analysis using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA, USA), conducted at an excitation wavelength of 260 nm excitation and an emission wavelength of 590 nm.

Live/Dead staining assay

For the Live/Dead staining assay, 4T1, HepG2, and M6 cells were treated with PFHA-PEI-mRNA-HP or Lipofectamine 2000-mRNA (2 μg/mL mRNA) or left untreated and incubated for 24 hours.

Cells were then washed twice with PBS, stained with Calcein AM (2 μM, green fluorescence for live cells) and propidium iodide (10 μg/mL, red fluorescence for dead cells) for 30 minutes at 37°C, and washed again with PBS. Imaging was performed using a Nikon TE300 inverted fluorescence microscope (Tokyo, Japan).