# Supporting Information

## Triphenylphosphonium-Modified Cationomers Enhance *in vivo* mRNA Delivery through Stabilized Polyion Complexation

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## 1. Materials and methods

### *Materials*

4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM) and Dextran sulfate (DS) with MW of 5 kDa were purchased from Wako Pure Chemical Industrial Ltd. (Osaka, Japan). 4-(Carboxybutyl)triphenylphosphonium Bromide and Deuterium oxide ($D_2O$) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). EGFP mRNA and Fluc mRNA were purchased from TriLink Biotechnologies (San Diego, CA, USA). VEE-GFP mRNA was *in vitro* transcribed from T7-VEE-GFP plasmid (Addgene #58977, Watertown, MA).[1] *In vitro* transcription was performed using mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Invitrogen, Carlsbad, CA, USA), followed by RNA purification with RNeasy Mini Kit (Qiagen, Hilden, Germany). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, Dulbecco's phosphate-buffered saline (D-PBS), fetal bovine serum (FBS), penicillin-streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (1.0 M) were purchased from Sigma Aldrich (St. Louis, USA). Primers and probe for qRT-PCR were purchased from Integrated DNA Technologies (Coralville, Iowa, USA), and TaqMan™ Universal PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Cell lines were obtained from Riken BioResource Center (Tsukuba, Japan).

### *Synthesis and characterization of block copolymers*

PEG-poly (L-lysine) (PEG-PLys) was synthesized as previously reported.[2] Briefly, PEG-PLys(TFA) was synthesized via ring opening reaction of Lys(TFA)-NCA from α-methoxy-ω-aminopropyl-PEG (PEG–NH2). TFA groups were then deprotected by alkaline hydrolysis to obtain PEG-PLys. Polymerization degree of PLys was determined to be 68 by $^1$H NMR. Triphenylphosphonium (TPP) moieties were introduced into lysine residues of PEG-PLys through amide coupling reaction. PEG-PLys (20 mg, 1 μmol), DMT-MM (90 mg), and 4-(Carboxybutyl)triphenylphosphonium Bromide (8.7, 14.6, 29.1, or 43.7 mg, corresponding to 19.6, 32.9, 65.6, or 98.6 μmol, respectively) were dissolved in 50 mM $NaHCO_3$/MeOH (3 mL/9 mL). The mixture was stirred at 4 ℃ overnight, adding DMT-MM (90 mg) to the solution after 2 and 4 h of reaction. The resulting solution was dialyzed (molecular weight cut-off (MWCO) of the membrane: 6000–8000 Da) against methanol and distilled water alternately for 7 days to remove unreacted reagents. The solution was then lyophilized to obtain PEG-PLys(TPP) as a white powder. TPP introduction ratio into PEG-PLys was determined by $^1$H-NMR ($D_2O$ at 80 ℃, δ= 3.4–3.6 ppm (4H: -CH2-CH2-O-), δ= 7.6-8.0 ppm (15H: $Ph_3P$-)). Gel

permeation chromatography (GPC) analysis of PEG-PLys(TPP) polymers was performed using Extrema 4500 Model (JASCO, Tokyo, Japan) (column: Superdex 200 Increase 10/300 gl; eluent: 10 mM PB (500 mM NaCl, 0.5 M arginine), pH 7.4; temperature: 28 ℃; flow rate: 0.5 mL/min; detector: UV: 270 nm). GPC analysis of PEG-PLys polymers was performed using Extrema 4500 Model (JASCO, Tokyo, Japan) (column: Superdex 200 Increase 10/300 gl; eluent: 10 mM PB (150 mM NaCl), pH 7.4; flow rate: 0.75 mL/min; detector: UV: 220 nm).

***NaCl responsiveness of block copolymers***

Block copolymers (9.2 μM) were dissolved in 10 mM HEPES buffer and mixed with 10 mM HEPES containing various NaCl concentration (from 0 to 4 M) at the same volume ratio. The resulting polymer solution was measured by dynamic light scattering (DLS). DLS measurement was performed at a detection angle of 90° under controlled temperature at 25 ℃ using Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). Scattered light intensity (SLI) was monitored to detect multimolecular assemblies. When the SLI increased, the hydrodynamic diameter and polydispersity index (PDI) of the assemblies were also monitored.

For determining critical micelle concentration (CMC), we prepared PEG-PLys(TPP) polymer solutions of varying concentrations through serial dilution in 10 mM HEPES buffer supplemented with NaCl (0, 0.15, 0.5, 0.75, or 1.5 M). Subsequently, the SLI of each solution was monitored using DLS at a detection angle of 173° under controlled temperature at 25 ℃ using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The CMC was identified as the lowest polymer concentration at which the SLI maintained initial linearity throughout the dilution series, starting from their self-assembled state.

***Preparation and characterization of mRNA-loaded polymeric micelles (mRNA/PMs)***

Block copolymers and mRNA were separately dissolved in 10 mM HEPES buffer without NaCl. 1-unit-volume of block copolymer solution was added to 2-units-volume of mRNA solution (50 ng/μL) under brief vortex mixing. The final molar ratio of cationic moieties (Lys or TPP moieties) in block copolymers/anionic moieties (phosphate groups) in mRNA, described as C/A ratio, was set to be 3. The resulting mixture was kept on ice for 10 min before analysis. Size distribution of mRNA/PMs was measured by DLS at a detection angle of 90°under controlled temperature at 25 ℃ using Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential of mRNA/PMs in 10 mM HEPES buffer was measured by electrophoretic light scattering (ELS) using

Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

For transmission electron microscopy (TEM) observation, the concentration of mRNA/PMs was adjusted to 6.6 ng mRNA/μL in 10 mM HEPES buffer. The mRNA/PM solution was mixed with the same volume of EM Stainer (Nisshin EM Co., Ltd., Tokyo, Japan), and TEM observation was performed using H7650 (Hitachi High-Technologies, Tokyo, Japan).

The mRNA association number in each PM was evaluated by fluorescence correlation spectroscopy (FCS). Fluc mRNA was labeled with Cy5 using Label IT Cy5 Labeling Kit (Mirus Bio LLC., Madison, WI), followed by encapsulation in PMs. The mRNA/PMs (3.3 ng mRNA/μL of 10 mM HEPES buffer) were evaluated by FCS using LSM 780 (Carl Zeiss, Germany) and He–Ne laser with an excitation wavelength of 633 nm and emission band-pass filter of 685/50 nm. The mRNA encapsulation number in each PM was calculated by comparing the count per molecule (CPM) of mRNA/PMs with that of free mRNA.

mRNA encapsulation efficiency was evaluated using the highly RNA-selective Qubit RNA HS dye (Q32852, Invitrogen, Waltham, MA, USA), as previously described.[3] Briefly, mRNA/PMs were added to Qubit RNA working buffer and incubated for 10 min. The fluorescence of the solution was measured using SPARK (Tecan Group Ltd., Zürich, Switzerland). The excitation/emission wavelength was set at 630/680 nm, respectively.

***Evaluation of mRNA condensation status in polymeric micelles***

Cy3/Cy5 double-labeled Fluc mRNA was prepared using Label IT Cy3 Labeling Kit and Label IT Cy5 Labeling Kit (Mirus Bio LLC., Madison, WI). The labeled mRNA was mixed with block copolymers to obtain mRNA/PMs, as described above. To evaluate mRNA condensation status, excitation wavelength was set at 520 nm and fluorescence intensities of Cy3 and Cy5 at 570 nm and 680 nm, respectively, were measured using SPARK (Tecan Group Ltd., Zürich, Switzerland). Fluorescence resonance energy transfer (FRET) ratio was calculated as follows:

FRET ratio = [Cy5 intensity (680 nm)] / [Cy3 intensity (570 nm) + Cy5 intensity (680 nm)]

***Thermodynamic analysis of polymer-mRNA complexation***

Isothermal titration calorimetry (ITC) was conducted to analyze the thermodynamics of polymer-mRNA interactions using an iTC200 instrument (GE Healthcare). Fluc mRNA was dissolved in 10 mM HEPES buffer and titrated with polymers dissolved in the same buffer at 25 ℃, with measurements taken every 120 s at 750 rpm. The concentration of

phosphate groups in the mRNA was adjusted to 0.19 mM for PEG-PLys, PEG-PLys(75% TPP), and PEG-PLys(97% TPP) polymers, and to 0.095 mM for PEG-PLys(26% TPP) and PEG-PLys(45% TPP) polymers. The concentration of cationic residues in the polymers was set to 1.9 mM for all measurements.

***Molecular dynamics (MD) simulation***

In this study, we conducted three types of simulations: 1) a complex between a 10-mer RNA and three 10-mer polymers (Lys, BA, TPA, or TPP), 2) a system with only a 10-mer RNA, and 3) a system containing ten 10-mer polymers. For simulations involving RNA, five distinct RNA sequences were used, as detailed in the main text. Input structures for MD simulations were initially prepared using ChemSketch, UCSF Chimera,[4] and AmberTools22.[5] Subsequent MD simulations were executed using GROMACS 2022.4,[6] employing the Amber ff19SB/OL3 force field.[7,8] We selected the ff19SB force field because each polymer, with the exception of Lys, can be considered a modified amino acid. The partial charges for these modified side chains were derived from the AM1-BCC model.[9] Each system was then solvated with TIP3P water in a rectangular box,[10] ensuring a minimum distance of 10 Å from the molecule to the edge of the box, applying periodic boundary conditions. To neutralize the systems, additional $NA^+$ or $Cl^-$ ions were introduced. Following this, each system underwent an energy minimization for 5000 steps using the steepest descent algorithm. The system was then equilibrated in the NVT ensemble, where the temperature was gradually ramped from 50 to 277.15 K over 200 ps and then continued for an additional 100 ps. Subsequent unrestrained simulations were carried out in the NPT ensemble at 277.15 K for 1 μs (for both the RNA-polymer complexes and the RNA-only systems) or 200 ns (for the ten 10-mer polymer-only systems). Throughout these simulations, the time step was maintained at 2 fs. Both Coulomb and van der Waals interactions used a cutoff distance of 10 Å. The particle mesh Ewald method handled long-range electrostatic interactions.[11] Covalent bonds that involved hydrogen atoms were constrained using the LINCS algorithm.[12] Snapshots were saved at intervals of 100 ps. We conducted three independent production runs with different initial velocities, accumulating 77.4 μs of aggregate simulation data. To account for relaxation from the initial structures, all trajectory analyses were based on the final 100 ns of each trajectory using the GROMACS package. Atomic contacts were defined when any pairs of atoms were within a 4.5 Å cutoff or closer. Results were either summed or averaged across the three independent trajectories for each system.

***Quantum mechanics (QM) calculations***

The monomeric forms of TPA and TPP were geometry-optimized using the B3LYP/6-31G(d,p) level in the Gaussian software.[13] Dipole moments for each molecule were then computed based on these optimized structures using the same method and basis set.

***Stability against polyanion***

To test the stability against polyanion exchange reactions, Cy3/Cy5 double-labeled Fluc mRNA was used to prepare mRNA/PMs. Dextran sulfate (DS) with MW of 5 kDa dissolved in D-PBS was added to the mRNA/PMs (final mRNA concentration was adjusted to 1.67 ng/μL). The final molar ratio of sulfate groups of DS / phosphate groups of mRNA/PMs (described as S/P ratio) varied from 0 to 3. The resulting mixture was incubated at 37 ℃ for 15 min, followed by fluorescence measurement of Cy3 and Cy5 to calculate FRET ratio as described above. The FRET ratio was utilized to evaluate mRNA/PM structural integrity.

***Stability in serum***

For assessing the stability against nucleases, Fluc mRNA/PMs were incubated in 50% FBS (final mRNA concentration was adjusted to 1.67 ng/μL) at 37 ℃ for 15 min, followed by RNA extraction using RNeasy Mini Preparation Kit. cDNA was synthesized by reverse transcription with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Lastly, qPCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primer/probe pair was used for qPCR:

・Forward: GTGGTGTGCAGCGAGAATAG
・Reverse: CGCTCGTTGTAGATGTCGTTAG
・Probe: TTGCAGTTCTTCATGCCCGTGTTG

***Polymer cytotoxicity***

CT26 cells (5,000 cells/well) were seeded on the 96-well plate and incubated overnight. Polymer solutions were incubated with the cells for 24 hours and their viability was analyzed using cell-counting kit-8 (Dojindo, Kumamoto, Japan)).

***Protein expression in cultured cells***

CT26 and B16-F10 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin under 5% CO2 at 37 ℃. HuH-7, HEK293, RAW264.7, and U87 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under 5% CO2 at 37 ℃. These cells (20000 cells/well) were

seeded onto 96-well plates. After 24 h of incubation, mRNA/PMs (400 ng Fluc mRNA/well) were added to the medium. Twenty-four hours later, the medium was removed, and cells were lysed using Cell Culture Lysis Reagent (Promega, Madison, WI, USA). Luminescence in the lysate was measured using Luciferase Assay System (Promega) and GloMax 96 Microplate Luminometer (Promega).

For positive control, in vivo-jetPEI® (jetPEI) (Polyplus-transfection S.A, Illkirch, France) was used to prepare mRNA complexes at the reagent nitrogen to RNA phosphorus (N/P) ratio of 6, according to the manufacturer's instructions. Branched polyethylenimine (bPEI) (MW: 2 kDa) (Sigma-Aldrich, St Louis, MO, USA) was similarly complexed with mRNA at N/P ratio of 6. Their protein expression efficiencies were evaluated as described above.

***mRNA uptake by cultured cells***

Cy5-labeled Fluc mRNA was prepared using Label IT Cy5 Labeling Kit (Mirus Bio LLC., Madison, WI). CT26 cells (12500 cells/well) were seeded onto 8-well chamber (IWAKI AGC Techno Glass Co. Ltd., Shizuoka, Japan). After 24 h incubation, PMs encapsulating Cy5-labeled mRNA were added to each well (300 ng Fluc mRNA/well) and incubated for 24 h. Cells were washed with D-PBS three times, followed by nuclear staining with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). The stained cells were observed by the Leica DMi8 Thunder fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

***Animal studies***

All animal studies described below were approved by the Animal Care and Use Committee of the University of Tokyo (Tokyo, Japan) (Approval number: A2023E005-01). All animals were maintained under standard conditions with ad libitum access to food and water. No animals were excluded from any experimental group, and all treated animals were included in the analysis. To ensure unbiased assessment, blinding was performed with the assistance of additional personnel during the administration or data measurement stages. All procedures were conducted in accordance with relevant guidelines and regulations to minimize animal suffering.

***Stability in blood after systemic injection***

mRNA/PMs in 10 mM HEPES buffer supplemented with 150 mM NaCl were intravenously injected into BALB/c mice (female, 7 weeks old; Charles River Laboratories Japan Inc., Kanagawa, Japan) at a dose of 20 μg Fluc mRNA/mouse through

the jugular vein. mRNA/PMs were formulated with either PEG-PLys or PEG-PLys(97% TPP), and injected into mice (N=4 per group). 2 μL of blood was collected from the tail vein at 5, 10, 15, 30, and 60-min post injection, and added to RLT buffer containing 1% 2-mercaptoethanol. To quantify the remaining Fluc mRNA, RNA extraction from this solution, reverse transcription, and qPCR were performed as described above. The remaining amounts of Fluc mRNA in blood were presented as the mean ± standard error of the mean, which were compared between the two treated groups using two-tailed Student's t-test.

***Protein expression in tumor after systemic injection***

U87 Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under 5% $CO_2$ at 37 ℃. To prepare the U87 tumor model, 5 x $10^6$ U87 cells were subcutaneously inoculated into BALB/c-nude mice (female, 7 weeks old; Charles River Laboratories Japan Inc., Kanagawa, Japan). Three to four weeks later, tumor size reached approximately 300-400 $mm^3$, and mice were randomly divided into two groups based on their tumor size (N=5 per group). The mice were intravenously injected with mRNA/PMs dissolved in 10 mM HEPES buffer supplemented with 150 mM NaCl (20 μg Fluc mRNA/mouse). The mice were sacrificed 24 h after injection and tumor tissues were collected. Four equivalent volume of cell culture lysis buffer was added to each tissue and homogenization was conducted using Multi Beads Shocker MBX (Yasui Kikai, Osaka, Japan). Fluc expression in each homogenate was measured using Luciferase Assay System (Promega) and GloMax 96 Microplate Luminometer (Promega). The protein expression levels in the tumor were compared between the two treated groups using two-tailed Student's t-test.

***Biodistribution of intact mRNA after systemic injection***

mRNA/PMs dissolved in 10 mM HEPES buffer supplemented with 150 mM NaCl were intravenously injected into tumor-bearing mice (20 μg Fluc mRNA/mouse). For administration, tumor-bearing mice, prepared as described above, were randomly divided into two groups (N=4 for each group). The mice were sacrificed 4 hours after injection and major organs were collected to be immediately frozen by liquid nitrogen. 10 equivalent volumes of RLT buffer with 1% 2-mercaptoethanol were added to each tissue. The tissues were homogenized using Multi Beads Shocker MBX (Yasui Kikai, Osaka, Japan) and RNA was extracted from 100 μL of the supernatant of the tissue lysates using the RNeasy Mini Kit (Qiagen). The extracted RNA (500 ng) was used for reverse transcription to synthesize cDNA using the High Capacity RNA-to-cDNA Kit (Applied

Biosystems, Foster City, CA, USA). Finally, qPCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) with the primer/probe pairs (Integrated DNA Technologies, Coralville, IA, USA) specified below. GAPDH mRNA was used as an internal control to normalize Fluc mRNA levels in each tissue. The normalized Fluc mRNA levels were presented as the mean ± standard error of the mean.

For Fluc:

・Forward: GTGGTGTGCAGCGAGAATAG

・Reverse: CGCTCGTTGTAGATGTCGTTAG

・Probe: TTGCAGTTCTTCATGCCCGTGTTG

For GAPDH:

・Forward: GTGGAGTCATACTGGAACATGTAG

・Reverse: AATGGTGAAGGTCGGTGTG

・Probe: TGCAAATGGCAGCCCTGGTG

***In vivo safety of mRNA/PMs after systemic injection***

BALB/c mice were randomly divided into three groups (N=3 per group), and were intravenously injected with mRNA/PMs formulated with either PEG-PLys or PEG-PLys(97% TPP), or with a control solution of 10 mM HEPES buffer supplemented with 150 mM NaCl. mRNA/PMs were formulated in a 10 mM HEPES buffer supplemented with 150 mM NaCl and injected at a dosage of 20 μg Fluc mRNA/mouse. After 24 hours, blood was collected and centrifuged at 1500 g for 10 min at 4 ℃ to obtain plasma. The levels of five biomarkers, including total protein (TP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to assess liver function, creatinine (CRE) and blood urea nitrogen (BUN) to evaluate kidney function, were measured using Fuji DRI-CHEM slide (Fujifilm, Tokyo, Japan) and DRI-CHEM NX700 (Fujifilm, Tokyo, Japan). The biomarker levels were presented as the mean ± S.D. and the statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test.

## 2. Supporting figures

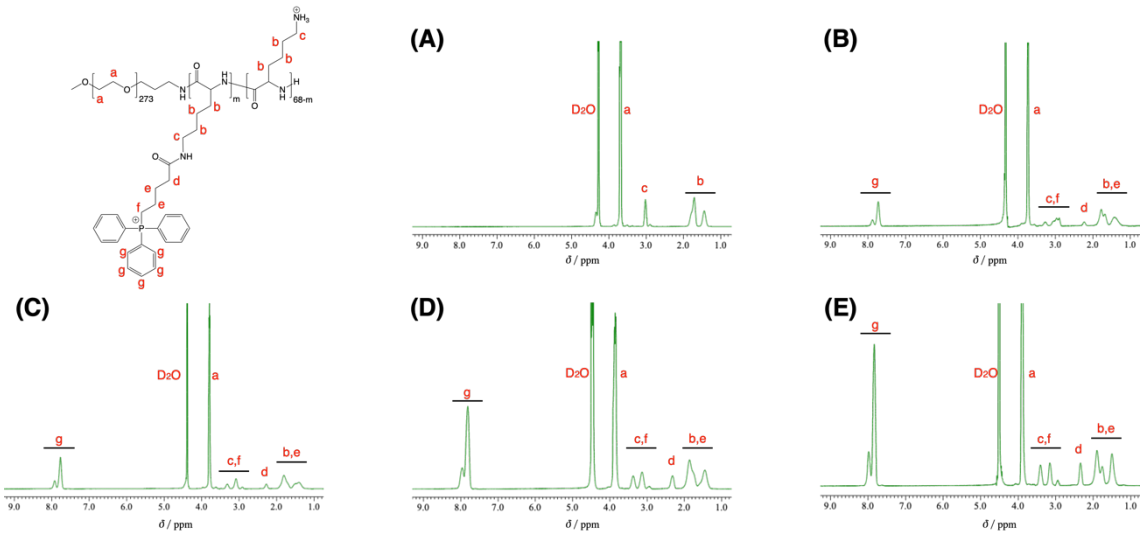


**Figure S1.** $^{1}$H NMR measurement of block copolymers. (A) PEG-PLys, (B) PEG-PLys(26% TPP), (C) PEG-PLys(45% TPP), (D) PEG-PLys(75% TPP), and (E) PEG-PLys(97% TPP). (solvent: $D_2O$, temperature: 80 ℃)

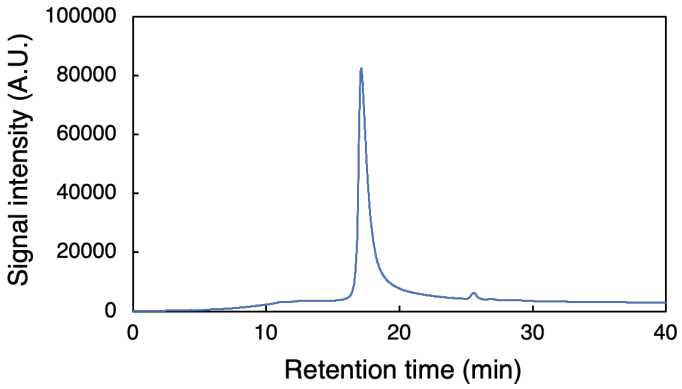


**Figure S2.** Size exclusion chromatography (SEC) analysis of PEG-PLys polymers. SEC was performed using Extrema 4500 Model (JASCO, Tokyo, Japan) (column: Superdex 200 Increase 10/300 gl; eluent: 10 mM PB (150 mM NaCl), pH 7.4; flow rate: 0.75 mL/min; detector: UV: 220 nm).

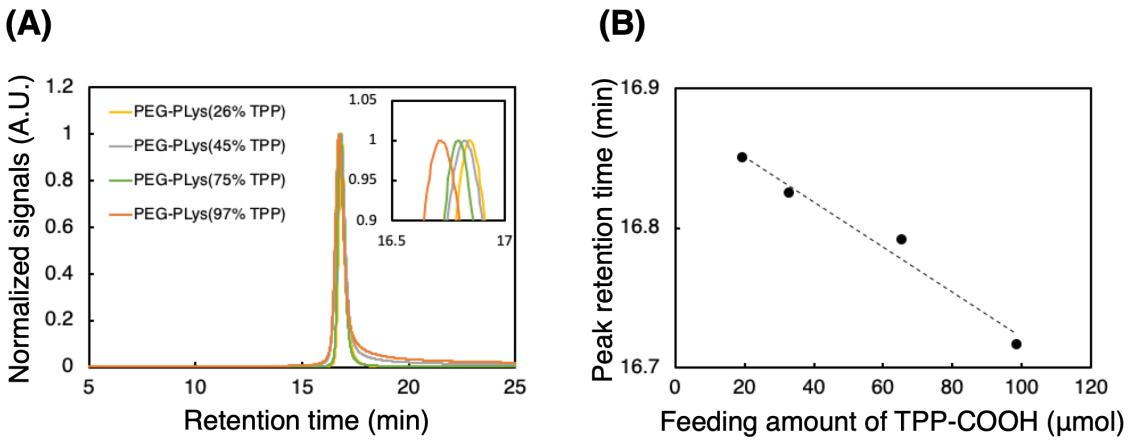


**Figure S3.** (A) SEC analysis of PEG-PLys(TPP) polymers. Each polymer exhibited a single peak and its retention time shifted corresponding to TPP introduction ratio (inset). SEC was performed using Extrema 4500 Model (JASCO, Tokyo, Japan) (column: Superdex 200 Increase 10/300 gl; eluent: 10 mM PB (500 mM NaCl, 0.5 M arginine), pH 7.4; temperature: 28 °C; flow rate: 0.5 mL/min; detector: UV: 270 nm). (B) Correlation between feeding amount of TPP-COOH during synthesis and peak retention time in SEC analysis.

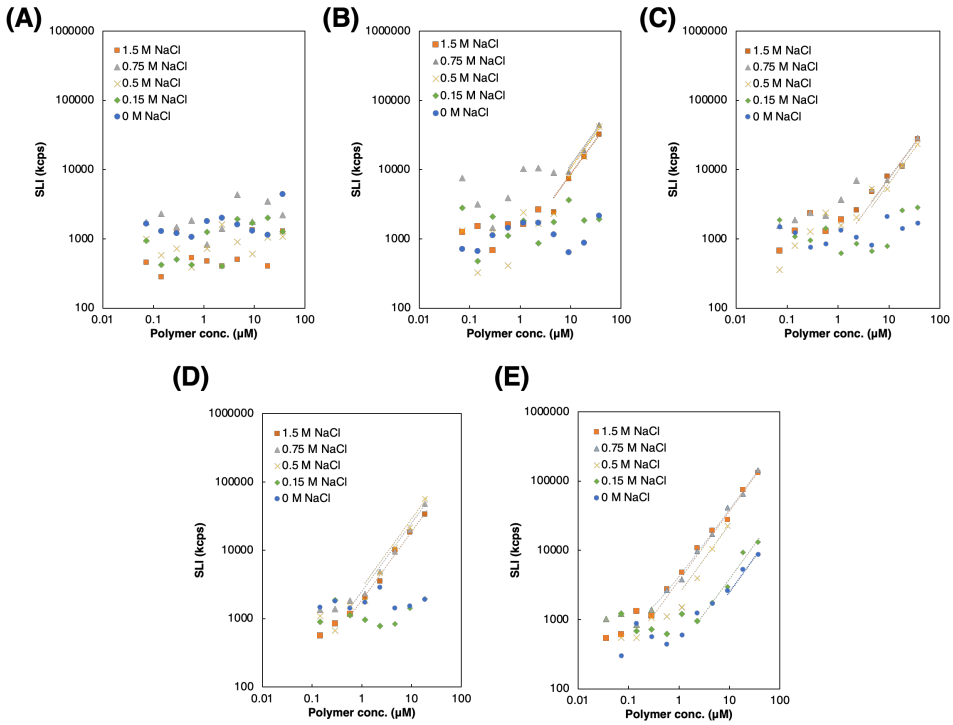


**Figure S4.** NaCl-responsive self-assembling properties of PEG-PLys(TPP) polymers of

varying concentrations. (A) PEG-PLys, (B) PEG-PLys(26% TPP), (C) PEG-PLys(45% TPP), (D) PEG-PLys(75% TPP), and (E) PEG-PLys(97% TPP). The scattered light intensity (SLI) of each polymer solution was measured using DLS.

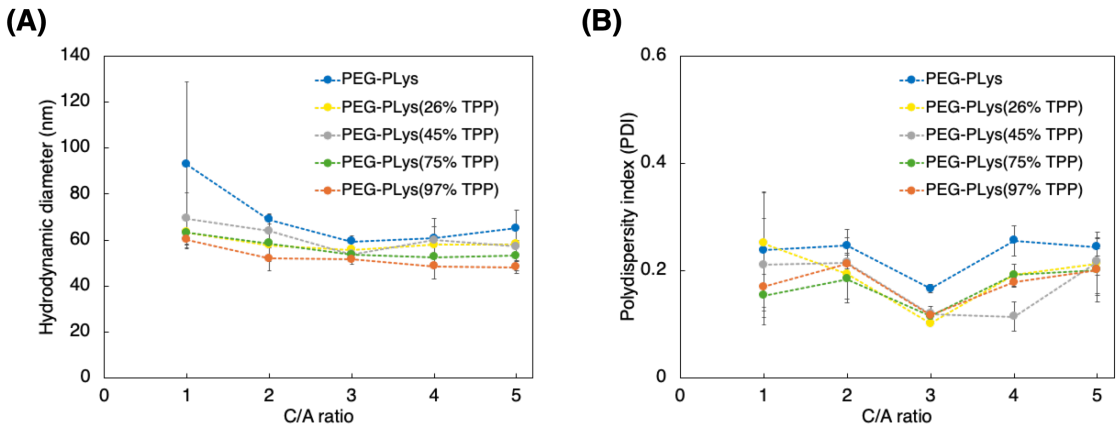


**Figure S5.** Size distribution of mRNA/PMs prepared at varying [Cationic moieties in polymers]/[Anionic moieties in mRNA] (C/A) ratios. (A) hydrodynamic diameter and (B) polydispersity index (PDI). Data shown as the mean ± S.D. (n = 3).

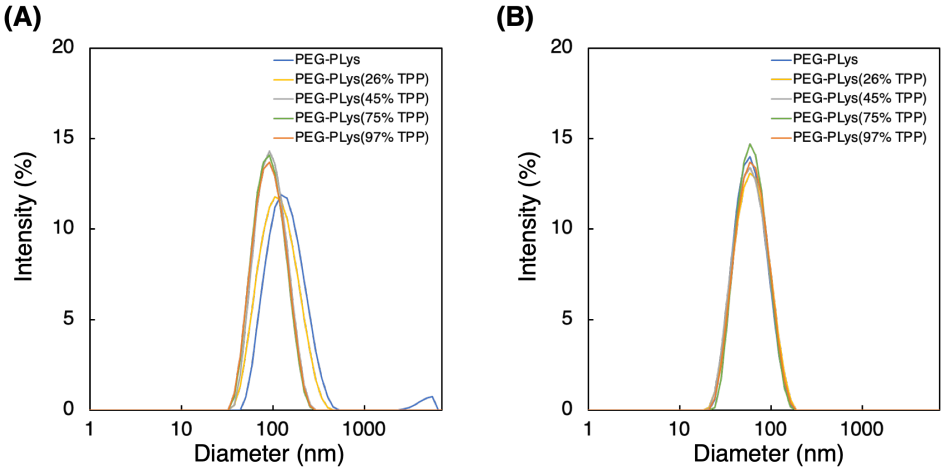


**Figure S6.** Size distribution of polymeric micelles (PMs) encapsulating various length of mRNA. (A) VEE-GFP mRNA (~7.6 kb) and (B) EGFP mRNA (~1 kb) were encapsulated into PMs, and their size distribution was determined by DLS.

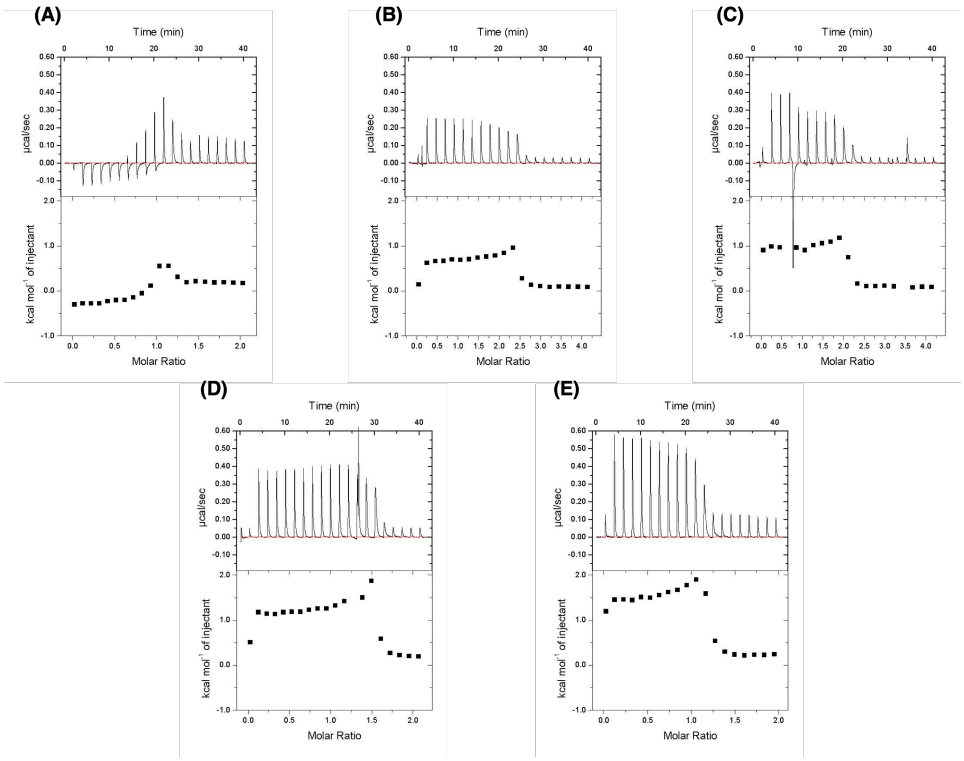


**Figure S7.** Isothermal titration calorimetry (ITC) thermograms of PEG-PLys(TPP) polymers titrated into Fluc mRNA in 10 mM HEPES buffer at 25 ℃. (A) PEG-PLys, (B) PEG-PLys(26% TPP), (C) PEG-PLys(45% TPP), (D) PEG-PLys(75% TPP), and (E) PEG-PLys(97% TPP). Raw heat per injection is shown on the top, with normalized data on the bottom.

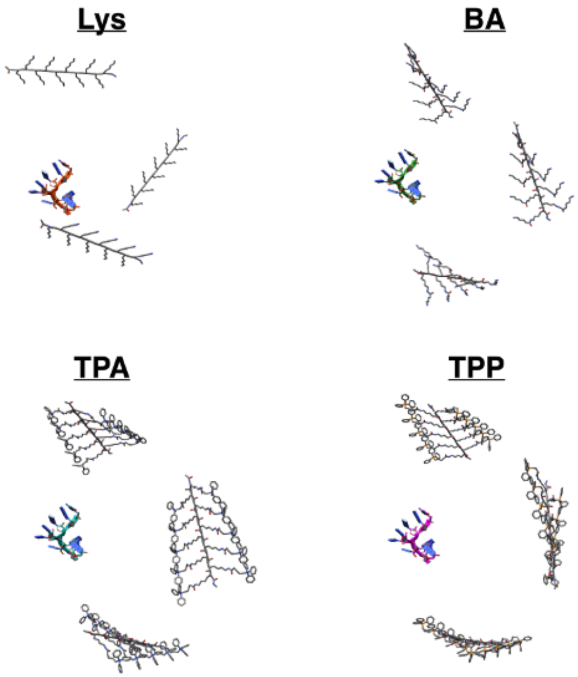


**Figure S8.** Initial coordinates for the molecular dynamics (MD) simulations of RNA-polymer systems, with RNA-seq01 shown as a representative example. Water molecules, neutralizing ions and hydrogen atoms are not shown for clarity.

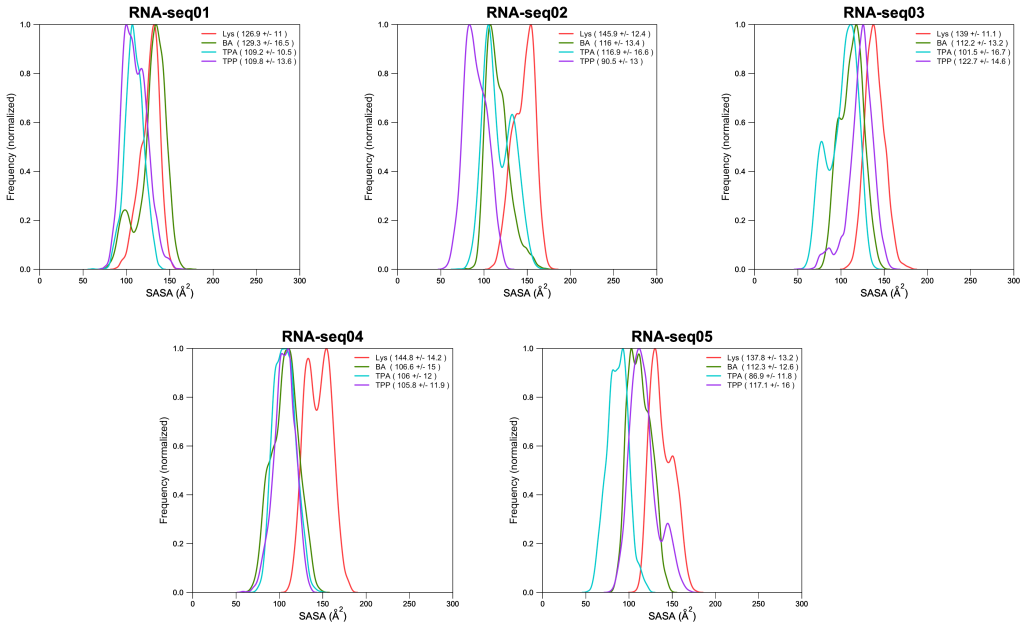


**Figure S9.** Solvent accessible surface area (in Å$^2$) of RNA during the final 100 ns of 1 μs MD simulations for the RNA-polymer systems.

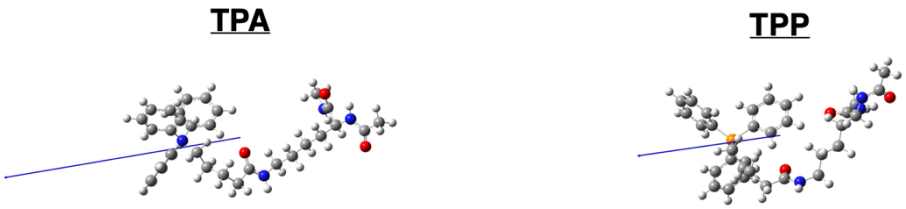


**Figure S10.** Dipole moments of the monomeric forms of TPA and TPP calculated by QM methods. Blue arrows represent the dipole moment of each molecule.

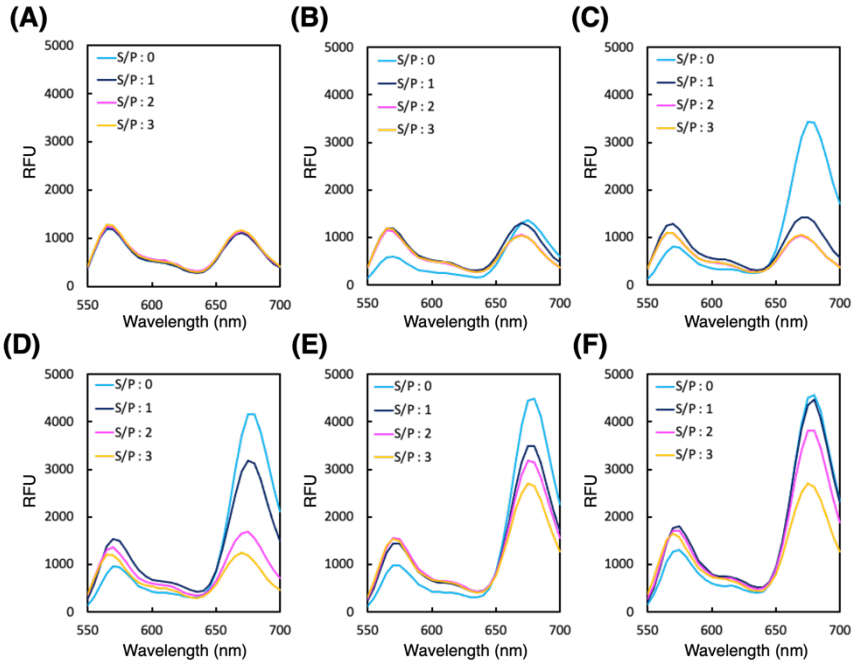


**Figure S11.** Fluorescence emission spectra of Cy3/Cy5-labeled mRNA-loaded polymeric micelles (mRNA/PMs). After 15-min incubation with dextran sulfate (DS) at various S/P (sulfate groups of DS / Phosphate groups of mRNA) ratio, fluorescence emission spectrum of each mRNA/PM (at 520 nm excitation) was measured. (A) free mRNA, (B) mRNA/PMs with PEG-PLys, (C) mRNA/PMs with PEG-PLys(26% TPP), (D) mRNA/PMs with PEG-PLys(45% TPP), (E) mRNA/PMs with PEG-PLys(75% TPP), and

(F) mRNA/PMs with PEG-PLys(97% TPP).

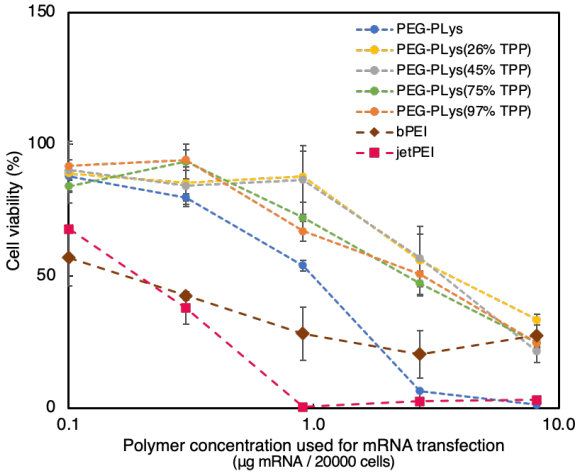


**Figure S12.** Cytotoxicity of PEG-PLys(TPP) polymers. The polymers were incubated with CT26 cells for 24 hours. The cell viability was evaluated with cell-counting kit-8. Transfection reagents, jetPEI and bPEI (for N/P ratio of 6), were included for comparison. Data shown as the mean ± S.D. (n = 3).

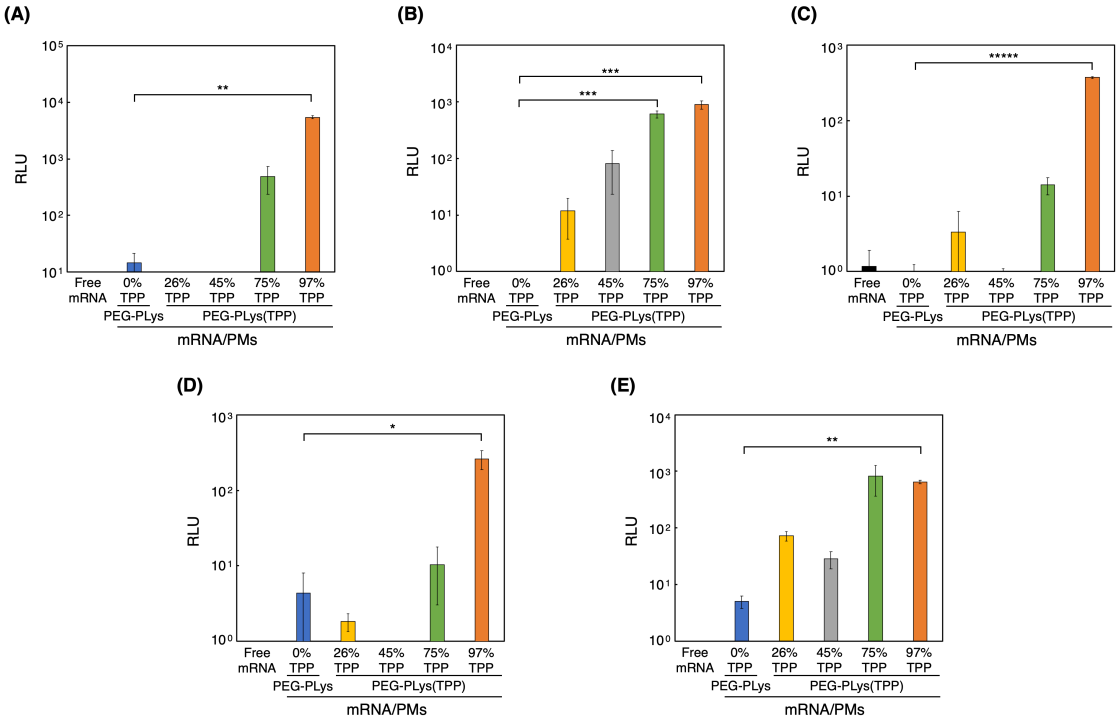


**Figure S13.** Fluc expression by mRNA/PMs in multiple cell types. After 24 h of

incubation, Fluc protein levels in cell lysate were quantified. (A) U87, (B) HEK293, (C) B16-F10, (D) HuH-7, and (E) RAW264.7 cells. Data are shown as the mean ± standard error of the mean (n=4 for (A)(E), n=6 for (B)(C)(D)). *p < 0.05, **p < 0.01, ***p < 0.001, and *****p < 0.00001, determined by one-way ANOVA with Bonferroni post hoc test.

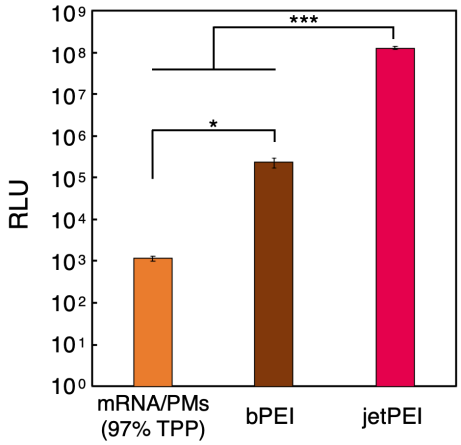


**Figure S14.** Comparison of protein expression efficiency between mRNA/PMs with PEG-PLys(97% TPP) and polyethylenimines (PEIs). After 24 hours of incubation with CT26 cells, Fluc protein levels in cell lysate were quantified. Two commercial PEI reagents (jetPEI and bPEI) were used as positive controls. Both PEIs were complexed with mRNA at the polymer nitrogen to RNA phosphorus (N/P) ratio of 6. Data are shown as the mean ± S.D. (n=4). *p < 0.05 and ***p < 0.001, determined by one-way ANOVA with Bonferroni post hoc test.

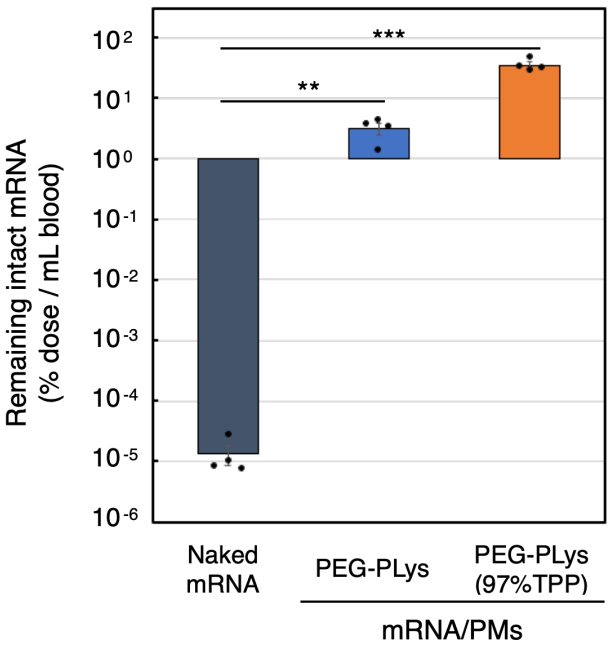


**Figure S15.** Stability of naked Fluc mRNA at 5 min post intravenous injection. Intact mRNA circulating in the bloodstream was quantified by qRT-PCR. Stability of mRNA encapsulated in PMs is also shown for comparison. Data are shown as the mean ± standard error of the mean (s.e.m.) (n=4). **p < 0.01 and ***p < 0.001, determined by one-way ANOVA with Bonferroni post hoc test.

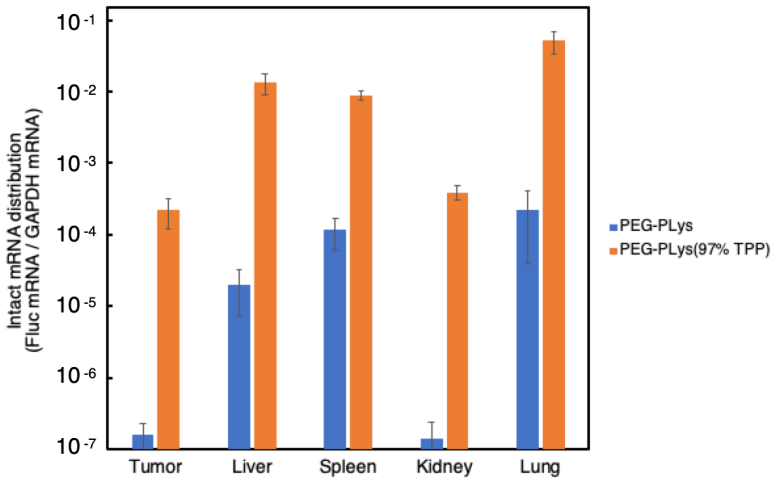


**Figure S16.** Biodistribution of intact Fluc mRNA after systemic injection. mRNA/PMs formulated with either PEG-PLys or PEG-PLys(97% TPP) were intravenously injected into tumor-bearing mice. Intact Fluc mRNA accumulation in each organ at 4 hours post-injection was evaluated using qRT-PCR. Fluc mRNA levels were normalized to GAPDH

mRNA. Data are shown as the mean ± standard error of the mean (n=4).

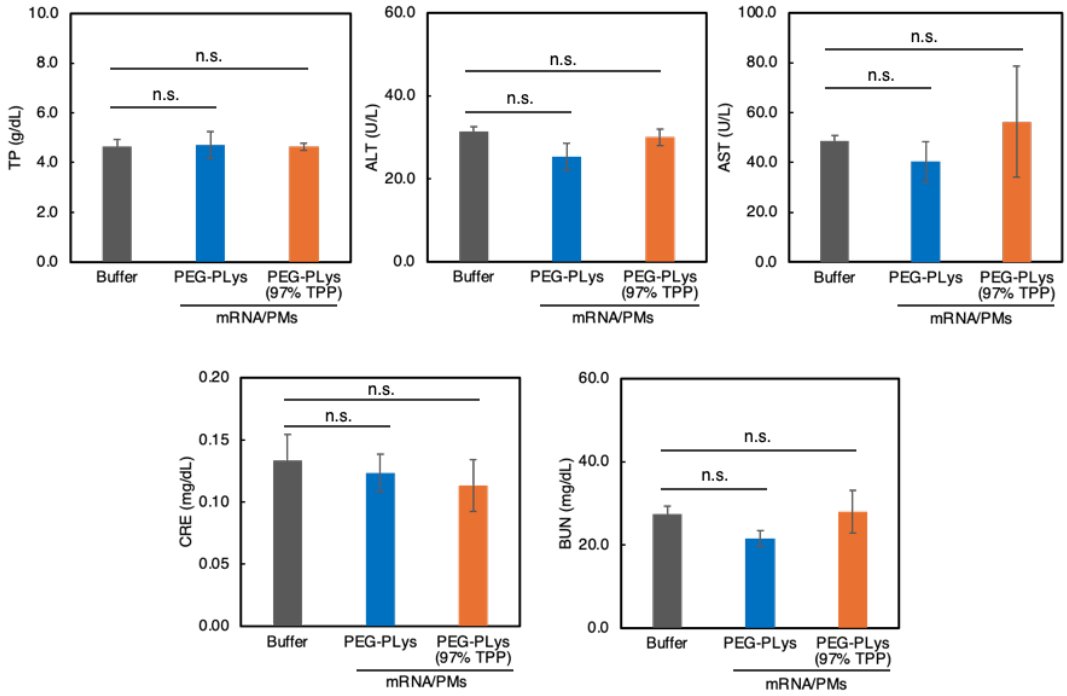


**Figure S17.** *In vivo* safety profiles of mRNA/PMs. mRNA/PMs formulated with either PEG-PLys or PEG-PLys(97% TPP) were intravenously injected into mice. After 24 hours, the levels of five plasma biomarkers - total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), and blood urea nitrogen (BUN) - were measured. Data are shown as the mean ± S.D. (n=3). (n.s.: not significant, determined by one-way ANOVA with Bonferroni post hoc test.)

## 3. Supporting tables

**Table S1.** RNA sequences used in MD simulations.

| RNA | Sequence |
|---|---|
| Seq01 | CAGGUUCAUA |
| Seq02 | GUUAGACCAU |
| Seq03 | GAAUUGCCAU |
| Seq04 | UGUCUAGCAA |
| Seq05 | UGCUAUCAGA |

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