Supporting Information

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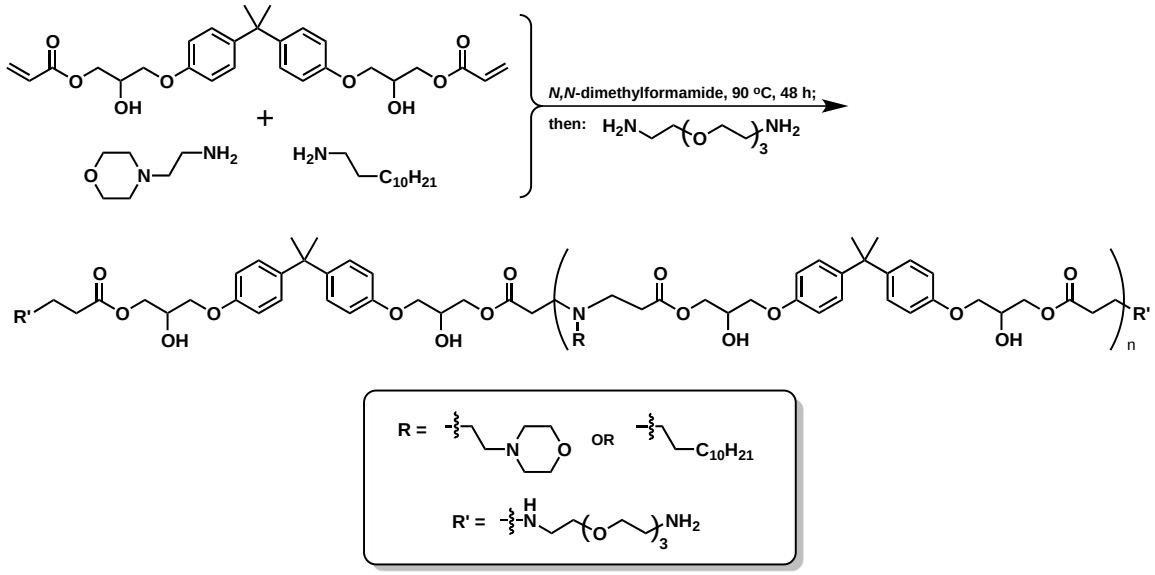
Chemical Synthesis and Characterization

Representative Synthesis of DD90-C12-122



To a 20 mL glass scintillation vial were added the diacrylate (396 mg, 0.82 mmol, 1.2 eqv.) and the hydrophilic amine (62 mg, 0.48 mmol, 0.7 eqv). The hydrophobic amine (38 mg, 0.20 mmol, 0.3 eqv.) was dissolved in anhydrous dimethyl formamide (5 mL) and added to the diacrylate and hydrophilic amine. The vial was then sealed, covered in aluminum foil, and heated to 90 ^{o}C. After 48 hours, the reaction was cooled to room temperature. The vial was opened to air and the end-capping amine was added in excess (192 mg, 1 mmol). The mixture was stirred until the end-capping amine was fully dissolved, and then was stirred at 500 rpm. After 24 hours, the reaction was diluted with diethyl ether (25 mL) followed by mixing. The heterogeneous mixture was then centrifuged for 2 minutes at 1250 G. The liquid was then decanted, leaving behind the polymeric solid. The ether wash/centrifugation/decanting process was repeated an additional time, and then the solid was dried under reduced pressure. ^{1}H Nuclear Magnetic Resonance (NMR) Spectra, Infrared Spectroscopy (IR), and Gel Permeation Chromatography (GPC) analyses were performed on these dried polymeric samples. The remainder of the material was diluted in anhydrous dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL for storage at -80 ^{o}C.

Instrumentation and Methods

Proton nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded with a Varian inverse probe INOVA-500 spectrometer (with a Magnex Scientific superconducting actively-shielded magnet), are reported in parts per million on the δ scale, and are referenced from the residual protium in the NMR solvent (DMSO-d_6: δ2.50)[1] displaying a window range of 9 to -0.5 ppm.

Infrared data (IR) were obtained with a Bruker Alpha FTIR spectrometer. Samples were collected neat on a ZnSe ATR crystal, and spectra are reported as percent absorbance as a function of frequency of absorption (cm^{-1}). Gel Permeation Chromatography (GPC) was carried out in tetrahydrofuran (THF) on Styragel columns utilizing a Malvern Viscotek^{TM} TDA 305 triple

detection system. Samples were filtered over 0.2 µm PTFE filters before injection using a 1.0 mL/min flow rate. Molecular weights and polydispersities were determined by comparing to a linear polystyrene standard.

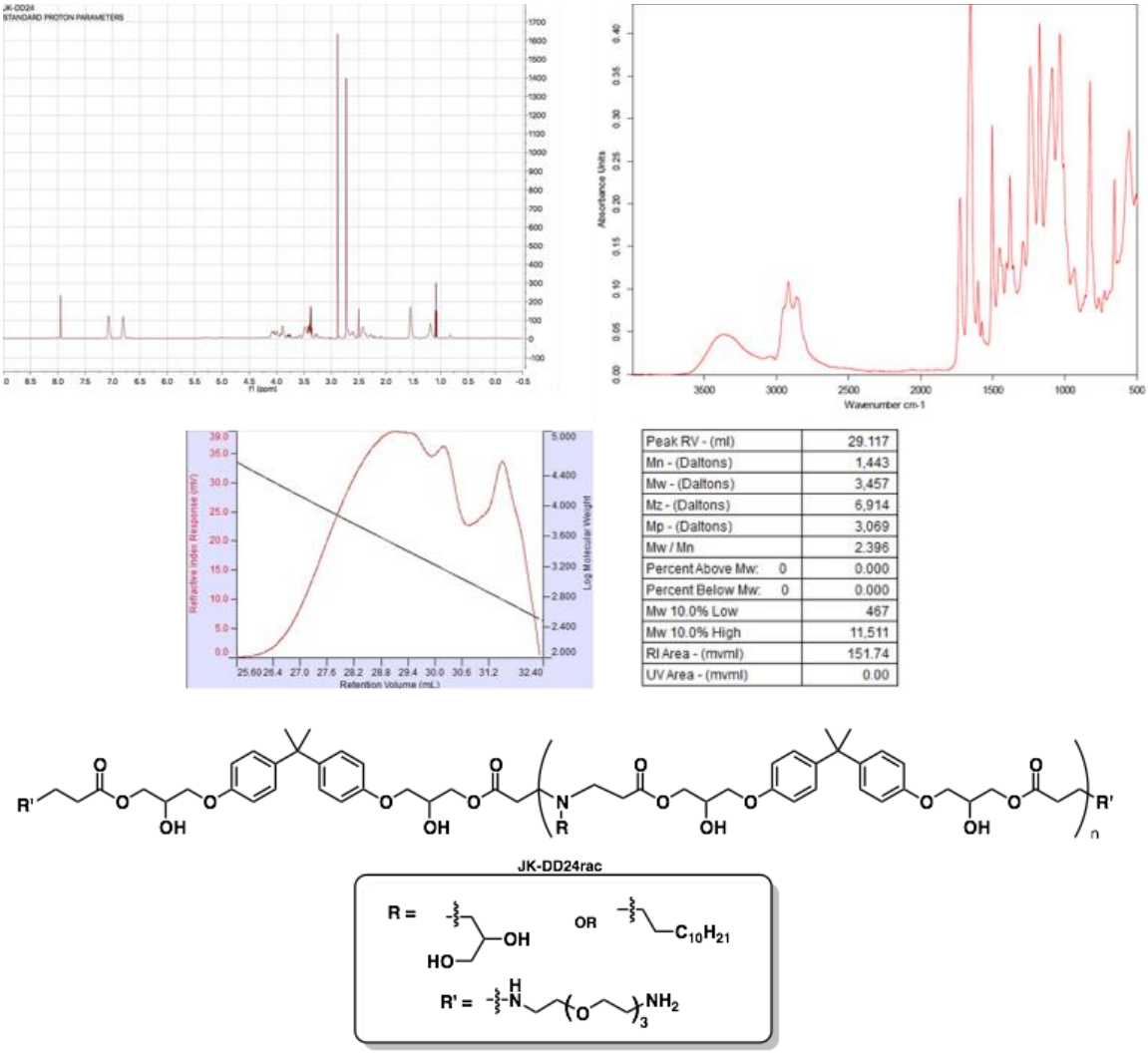


Figure S1: ^{1}H NMR, IR spectrum, GPC Trace, and Structure of DD24-C12-122

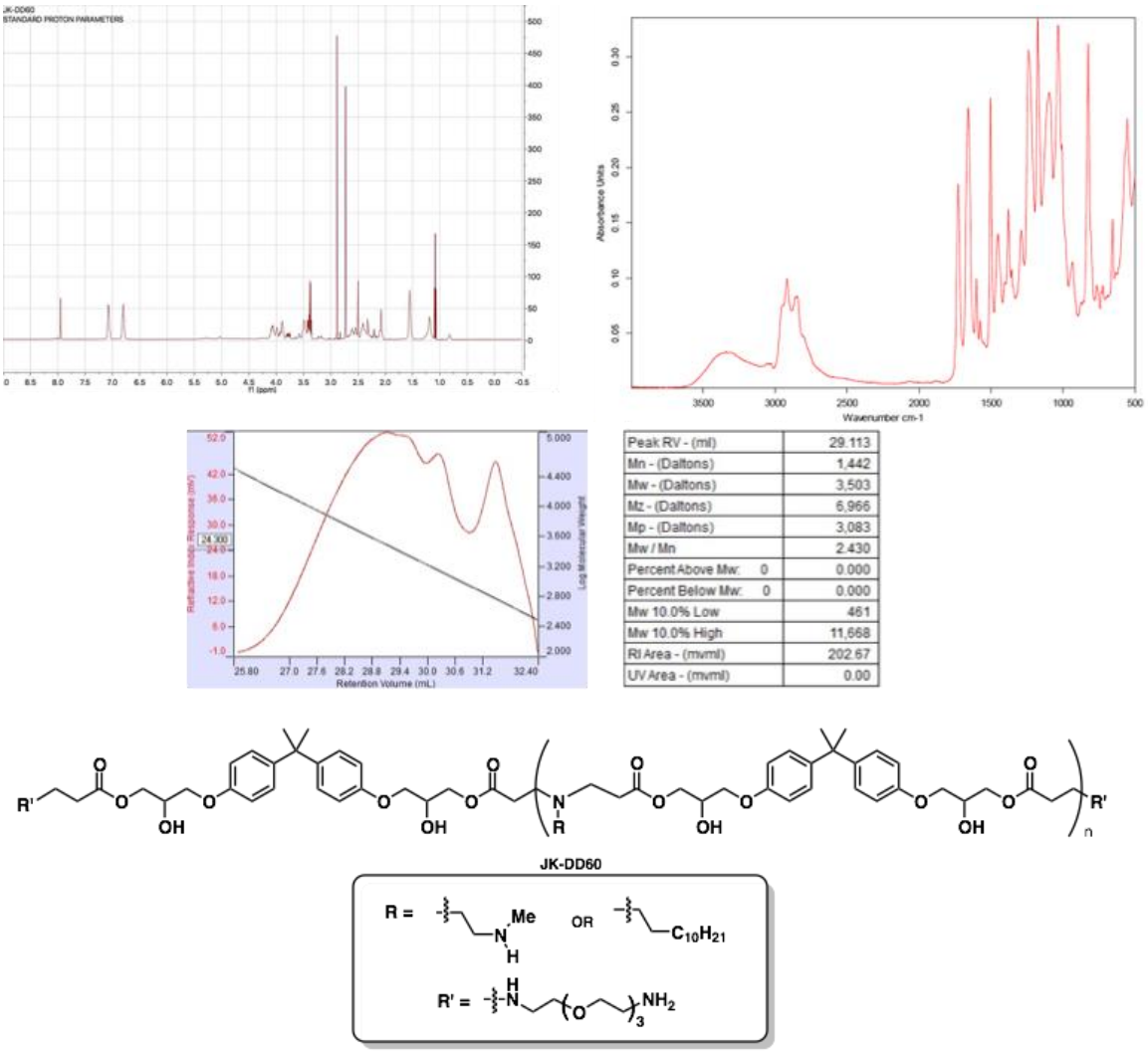


Figure S2: ^{1}H NMR, IR spectrum, GPC Trace, and Structure of DD60-C12-122

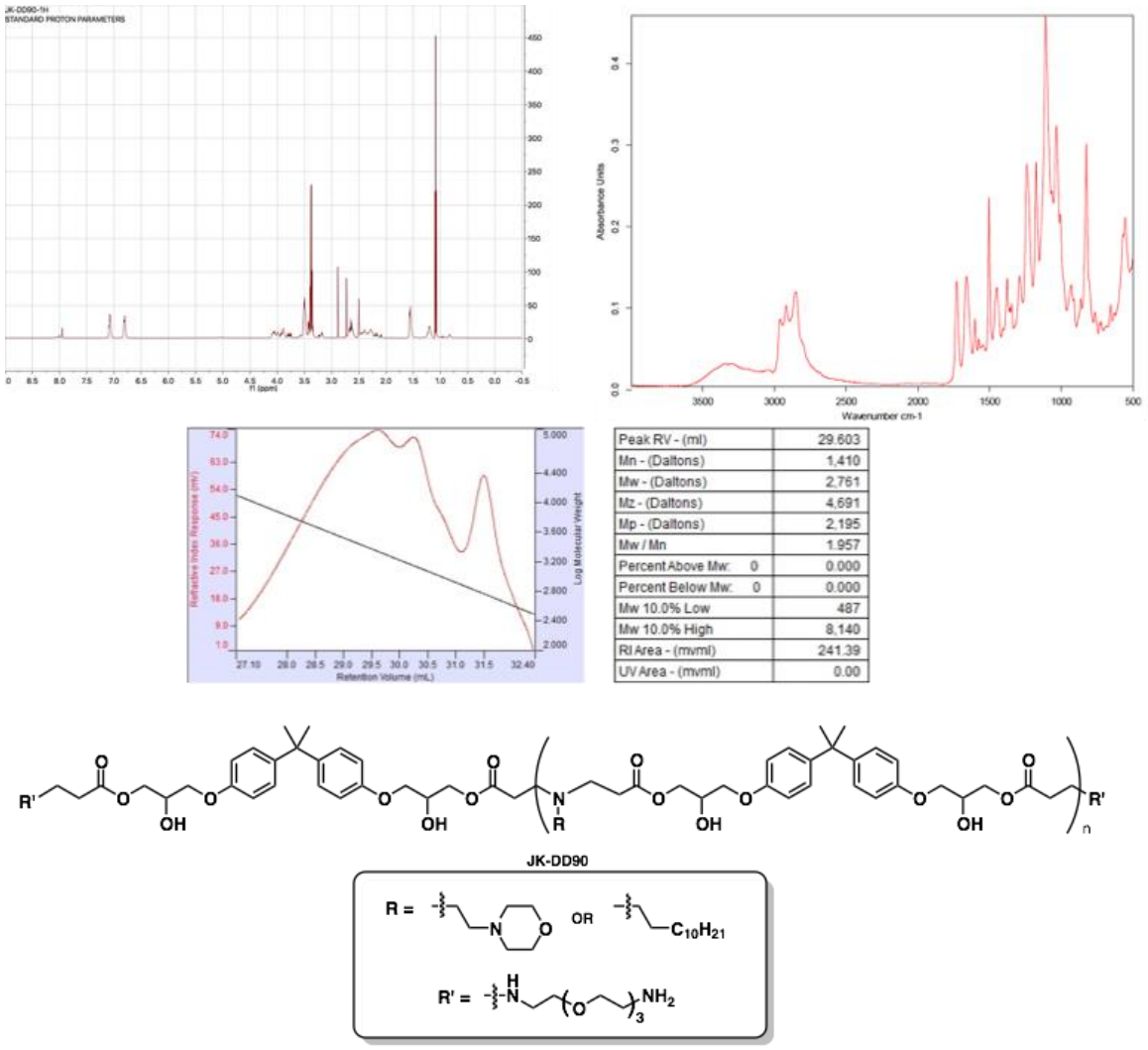


Figure S3: ^{1}H NMR, IR spectrum, GPC Trace, and Structure of DD90-C12-122

Extended Methods

Materials

Bisphenol A glycerolate (DD), bisphenol F ethoxylate (2 EO/phonol) diacrylate (DF), 4-(2-amino methyl) morpholine (90), N-N'-dimethyl ethylene diamine (60), (+/-)-3-amino-1,2-propanediol (24), dodecyl amine (C12), and 2-methyl-1,5-diaminopentane (118) were purchased from Aldrich (St. Louis, MO, USA). (Poly-ethylene oxide)$_4$-bis-amine (122) was purchased from Molecular Biosciences (Boulder, CO, USA). 1,4-butanediol diacrylate (C), 5-amino-1-pentanol (32), and heparin sodium salt from porcine intestinal mucosa were obtained from Alfa Aesar (Haverhill, MA, USA). 14:0 PEG2000 PE (PEG-lipid) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). jetPEI and *in vivo* jetPEI were obtained from VWR (Radnor, PA). Cy-5 labeled luciferase-encoding mRNA was purchased from TriLink Biotechnologies (San Diego, CA). Firefly luciferase-encoding mRNA was generously provided by Shire Pharmaceuticals (Lexington, MA, USA). All chemical reagents were used as received with no further purification.

mRNA Synthesis

mRNA was synthesized by an *in vitro* transcription from a plasmid DNA template encoding for the firefly luciferase gene. The *in vitro* transcription was followed by the addition of a 5' cap structure using a vaccinia virus-based guanylyl transferase system. A poly(A) tail of ~300 nt was incorporated via enzymatic addition using poly-A polymerase. Fixed 5' and 3' untranslated regions were constructed to flank the coding sequences of the mRNA.

Nanoparticle Synthesis

For particles prepared in the absence of PEG-lipid, mRNA was dissolved in sodium acetate buffer (NaOAc), pH 5.2, such that the mRNA concentration was between 25 and 200 ng μL^{-1}. The appropriate amount of polymer in DMSO (determined by the N/P ratio) was dissolved in an equivalent volume of 25 mM NaOAc. The polymer phase was then added to the mRNA phase and the solution was mixed vigorously in order to form the nanocomplexes, making the final mRNA concentration 12.5-100 ng μL^{-1}. For particles prepared with PEG-lipid, mRNA was dissolved in NaOAc as before while the appropriate amounts of polymer and PEG-lipid were co-dissolved in 200 proof ethanol. Nanoparticles were then dialyzed against PBS in a 20000 MWCO cassette at 4°C for 2-3 hours. Terpolymer nanoparticles used for high concentration *in vivo* dosing (>0.5 mg/kg) were synthesized at a 3:1 v/v ratio of mRNA phase to the polymer phase in order to limit the concentration decrease upon dialysis. jetPEI nanoparticles were made according to supplier protocol. Briefly, jetPEI and RNA were diluted in equal volumes of the provided buffer in order to yield the desired N/P. The jetPEI phase was added to the RNA phase and was mixed by vortexing, and the resulting nanoparticles were incubated at room temperature for 15 minutes prior to use. All particles were used no earlier than 15 minutes and no later than 4 hours following synthesis.

Nanoparticle Characterization

The mRNA concentration in dialyzed particles was determined via a modified Quant-iT RiboGreen RNA assay (Thermo Fisher). A nanoparticle dilution of ~1 ng μL^{-1} mRNA was made in TE buffer (pH 8.5) and mRNA standards were made ranging from 2 ng μL^{-1} to 0.125 ng μL^{-1}. 50 µL of each solution was added to separate wells in a 96-well black polystyrene plate. To each well was added 50 µL of 10 mg/mL heparin in TE buffer, which disrupted the electrostatic

forces binding the polymer and mRNA to allow for accurate quantification of nanoparticle mRNA content. The plate was incubated at 37ºC for 15 minutes with shaking at 350 rpm. Following the incubation, the diluted RiboGreen reagent was added (100 µL per well), and the plate was incubated as before for 3 minutes. RiboGreen fluorescence was measured according to the supplied protocol using a Tecan plate reader, and the mRNA standard was used to determine nanoparticle mRNA concentration. It should be noted that two separate standards were made: one with and one without 10 mg/mL heparin. Nanoparticle size was measured via dynamic light scattering via a standard (ZetaPALS, Brookhaven Instruments) or high-throughput (Dyna Pro Plate Reader, Wyatt) system. For size measurement, particles were diluted in PBS at a 1:16 v/v ratio and an intensity average measurement was reported for particle size. Zeta potential was measured using the ZetaPALS instrument. For zeta potential measurements, non-dialyzed (i.e. in NaOAc buffer) particles were diluted in reverse osmosis H_2O at a ratio of 1:5 v/v. To prepare particles for CryoTEM, nanoparticles were dialyzed against 0.1x PBS and imaged with a JEOL 2100F transmission electron microscope.

Serum Stability Turbidity Assay

Nanoparticle suspensions were diluted to 50 ng μL^{-1} mRNA in the appropriate buffer (PBS for dialyzed nanoparticles, NaOAc for non-dialyzed particles). An initial (*t*=0) absorbance measurement at 660 nm (Tecan plate reader) was taken after pipetting 90 µL per well of the diluted suspensions into a clear 96-well polystyrene plate. The wavelength of 660 nm was chosen to be sufficiently high to avoid absorbance by serum proteins, and is similar to that used in other nanoparticle agglomeration assays.[2] Following this, half of the particles were mixed with 10 µL per well of fetal bovine serum, while the other half were mixed with 10 µL of PBS per well as controls using a multi-channel pipette. The plate was immediately placed at 37ºC, and the absorbance of each well (660 nm) was measured at 15, 30, 45, 60, 90, and 120 minutes following incubation. All readings were normalized to the corresponding absorbance at *t*=0. The concentration of mRNA remaining in suspension was assayed immediately following incubation to confirm that decreases in optical transmittance corresponded to nanoparticle precipitation.

In vitro *transfections*

HeLa cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% v/v heat inactivated fetal bovine serum (Invitrogen) and 0.1% v/v Penicillin Streptomycin (Invitrogen). 24 hours before transfection, cells were seeded onto a 96-well polystyrene tissue culture plate (20,000 cells per well, 100 µL media containing serum and antibiotic per well). In a typical example, mRNA-loaded nanoparticles were diluted to 5 ng μL^{-1} in buffer (PBS for dialyzed nanoparticles, NaOAc for non-dialyzed particles) and mixed with media such that the volume ratio of nanoparticle solution to media was 1:9. The media in the plate was aspirated, and the nanoparticle-containing media was added to the wells, in this case at a final concentration of 50 ng mRNA per well. 24 hours following transfection, cell viability was assayed using a MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) and cellular luminescence was quantified using Bright-Glo Assay kits (Promega), both of which were measured using a Tecan plate reader. Cellular luminescence was normalized to live cell count, determined via a standard curve made using the viability assay. Relative cellular viability was calculated by taking the ratio of live cell fluorescent signal in treated cells (a readout in the MultiTox-Fluor assay) and normalizing to the live cell signal of untreated cells 24 hours following transfection. No wash step was used following particle transfection.

Animal Studies

All animal experiments were approved by the M.I.T. Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. Female C57BL/6 mice (Charles River Laboratories, 18-22g) were intravenously injected with nanoparticles via the tail vein. Nanoparticles formulated without PEG-lipid were not dialyzed, and were injected following a previously established protocol.[3] For luciferase imaging experiments, mice were injected intraperitoneally with 130 µL of 30 mg mL^{-1} D-luciferin (PerkinElmer) in PBS 24 hours after injection. 10 minutes following luciferin injection, mice were sacrificed via CO_2 asphyxiation. Six organs were collected (pancreas, spleen, kidneys, liver, lungs, and heart) and imaged for luminescence using an IVIS imaging apparatus (PerkinElmer) with the luminescence being quantified using Living Image Software (PerkinElmer). For fluorescent imaging experiments, mice were injected intravenously with nanoparticles loaded with Cy5-labeled mRNA, and mice were sacrificed 6 hours after injection. Biodistribution was determined by collecting organs as before (with the addition of the uterus) and imaging for fluorescence using an IVIS imaging apparatus.

Statistics

Data were expressed as mean ± SD for groups of at least three replicates, or as individual values with the mean indicated. Figure 2c was analyzed for statistical significance using an unpaired, two-tailed Student's *t* test. Figure 3b was analyzed for statistical significance using an unpaired, one-tailed Student's *t* test when comparing PEGylated DD90-C12-122/C32-C12-118 and *in vivo* jetPEI to controls (PBS and non-PEGylated DD90-C12-122) and both a one and two-tailed Student's *t* test (also unpaired) when comparing PEGylated DD90-C12-122/C32-C12-118 to *in vivo* jetPEI. Figure S9 was analyzed using an unpaired, two-tailed Student's *t* test. All statistical tests were done with 95% confidence and implemented in GraphPad Prism 6 (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Additional Nanoparticle Characterization

Table S1: Nanoparticle characterization (mean ± SD, *n*=3 for size)

	Particle Diameter, nm (No PEG-lipid)	Particle Diameter, nm (With PEG-lipid)	Zeta Potential, mV (No PEG-lipid)	Zeta Potential, mV (With PEG-lipid)	Zeta Potential, mV (With PEG-lipid, dialyzed)
DD90-C12-122	398.3±163.0	186.6±43.8	37.6	39.3	10.8
DD24-C12-122	388±99.3	101.2±14.0	35.7	37.1	12.3
C32-C12-118	552.6±31.7	233.0±5.4	31.4	23.2	14.2
DD60-C12-122	394.9±91.9	116.8±39.8	36.3	31.9	19.8
DF90-C12-122	393.3±164.1	159.5±36.3	36.5	38.5	16
DF60-C12-122	398.3±137.5	180.3±33.3	38.3	39.7	14.2
C32-118	315.6±70.3	295.0±140.5	26.7	24.2	16.6

Note: N/P=57 for all nanoparticles unless otherwise noted

Estimating N/P for PBAE Nanoparticles

Determining the number of nitrogen residues in a PBAE polymer is an approximation, since the polymers are rather polydisperse. Additionally, terpolymers require an additional approximation since the exact repeat unit structure is inherently random. A sample N/P calculation for the most potent compound, DD90-C12-122, is shown below in order to clarify the process used in the estimations.

Determining the Number of Phosphate Residues

Each µg of oligonucleotide (mRNA, DNA, siRNA, etc.) contains 3 nmol of negatively charged phosphate. Thus, 20 µg of mRNA would have 60 nmol of negatively charged phosphate, as would 20 µg of DNA.

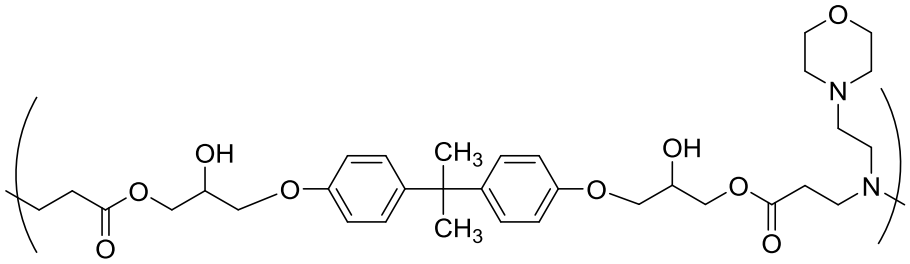
Determining the Number of Nitrogen Residues

The weight average molecular weight of the polymer (M_w) is obtained via GPC compared to a linear polystyrene standard. It is necessary to subtract the mass of the end caps to get the molecular weight of the polymer with only the repeat unit. Using DD90-C12-122 as an example:

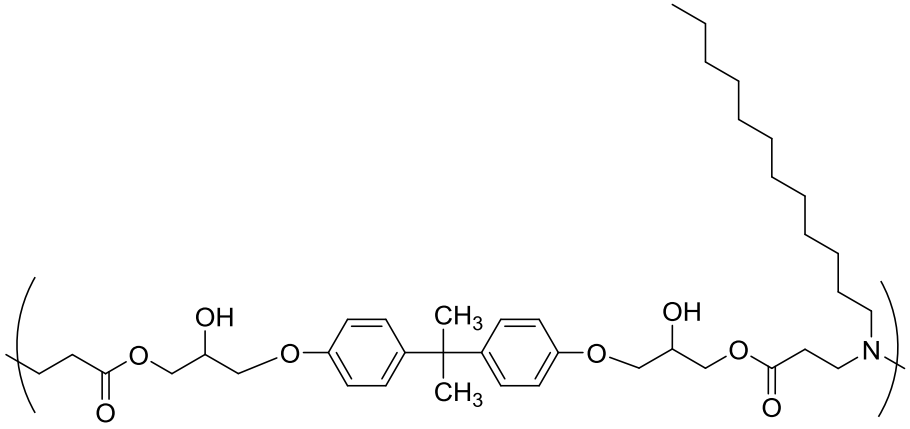
M_w: 2761
122 (end-cap) molecular weight: 192.25
$M_{w,adjusted}$=2376

Next, one can calculate the molecular weight of the polymer repeat unit. In the case of a terpolymer, there will be two possible repeat units. Thus, the molecular weight of the repeat unit will be the weighted average of the two. Continuing the example of DD90-C12-122:

Repeat unit 1 (hydrophilic amine): 614.73



Repeat unit 2 (hydrophobic amine): 669.39



Average repeat unit molecular weight: 643.22

Using the adjusted M_w and a weight average of the repeat unit molecular weights, the number of repeat units per polymer can be estimated. For the previous example, this value is 3.70. Thus, it is possible to determine the number of ionizable nitrogen residues per repeat unit. Again, this must be a weighted average of hydrophilic and hydrophobic repeat units. Using the weight percentages calculated in the previous example as the basis for the weighting, the number of nitrogen residues per repeat unit is 1.48. Combining all of this information, it is possible to obtain an estimate of the moles of nitrogen per mole of polymer, which in this example is 5.47. The end-capping amines are also included. For the “122” end cap, there are two end caps per mole of polymer. Thus, for the example above, the total number of amines in the molecule 9.47.

Calculating the N/P Ratio

TO calculate the N/P ratio, one only need divide the number of ionizable nitrogen residues in the PBAE/nucleic acid complex by the number of negatively charged phosphates to obtain the N/P ratio. For example, if 1000 nmol of DD90-C12-122 (9470 nmol of nitrogen residues) are complexed with 50 µg of mRNA (150 nmol of negatively charged phosphate), then the N/P ratio for the particles will be 63.

***In vitro* PEG-lipid optimization**

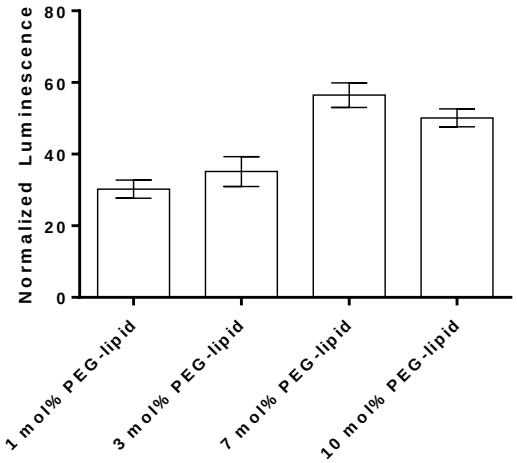


Figure S4. Optimization of PEG-lipid amount. DD90-C12-122 was formulated with luciferase mRNA (N/P = 57) and varying amounts of PEG-lipid. Nanoparticles were then used to transfect HeLa cells (50 ng mRNA/well in a 96 well plate). After 24 hours, cellular luminescence was measured and normalized to live cell number. Based on this experiment, all particles were formulated with 7 mol% PEG-lipid (mean ± SD, n = 4).

Additional CryoTEM Images

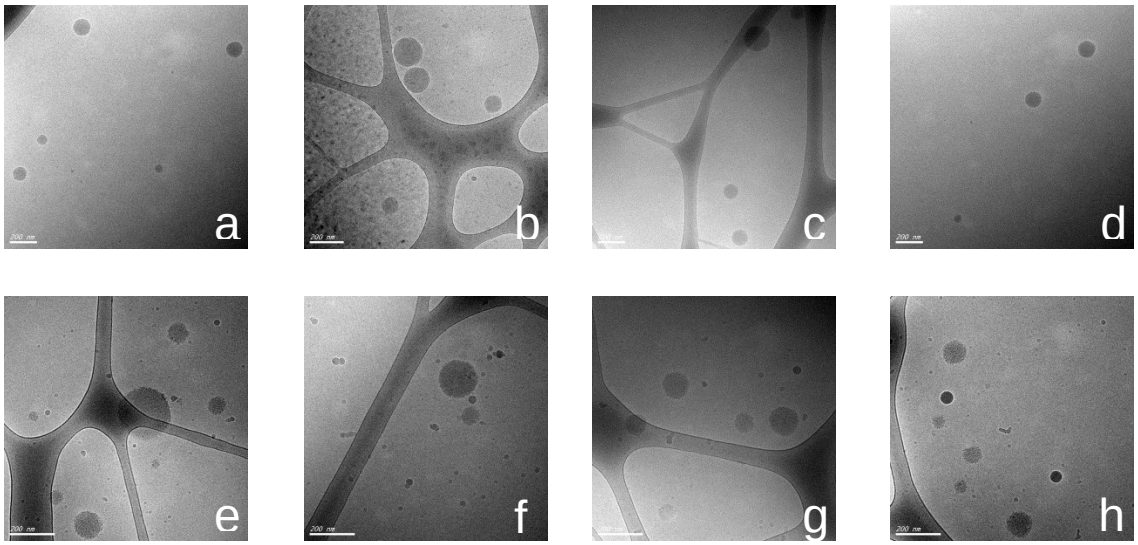


Figure S5. Additional CryoTEM images of DD90-C12-122 with 7 mol% PEG-lipid**.**

Additional Nanoparticle Stability Data

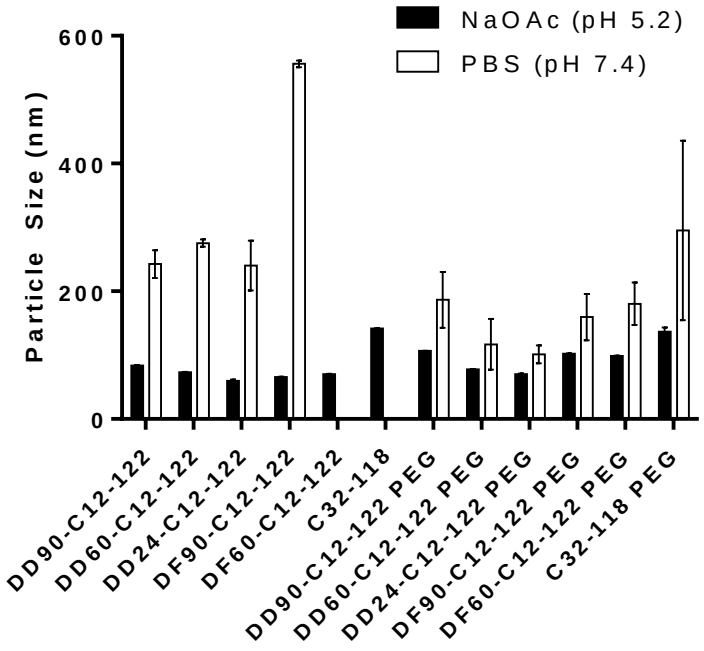


Figure S6. Dialysis at different pH. Particles that were dialyzed against NaOAc (pH 5.2) did not agglomerate, while non-PEGylated particles showed a large size increase, likely indicating particle aggregation, following dialysis against PBS (pH 7.4). Particles were dialyzed for 2 hours at 4°C.

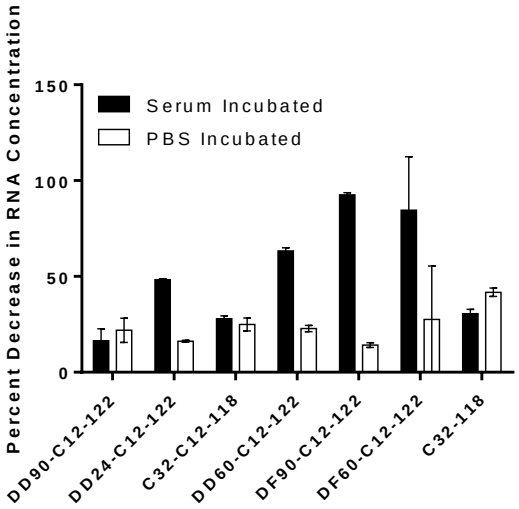


Figure S7. Changes in concentration following serum stability assay. Using the same modified RiboGreen assay described in the methods section, RNA concentration of the supernatant in the wells following the serum stability assay were measured. The largest decreases in RNA concentration correspond to the rapid decreases in absorbance seen in Figure 3, suggesting that the decrease is due to agglomerated particles crashing out of solution (mean ± SD, *n* = 3).

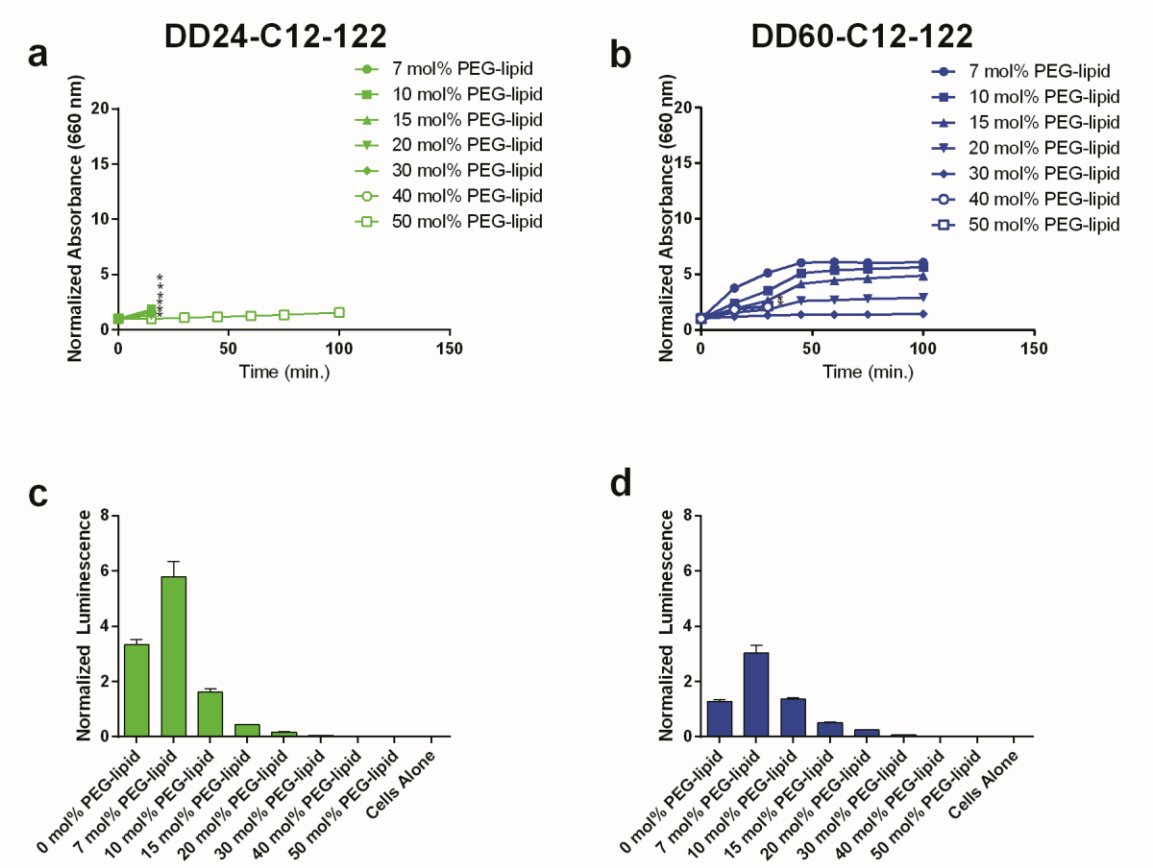


Figure S8. **(a)** Increasing the amount of PEG-lipid formulated with DD24-C12-122 nanoparticles rescues the particle serum stability, but it must be increased to 50 mol% (mean ± SD, *n* = 3). **(b)** Increasing the PEG-lipid content in DD60-C12-122 nanoparticles up to 30 mol% rescues serum stability, but at 40 and 50 mol% the particles are also unstable (mean ± SD, *n* = 3). This could be a result of the high PEG-lipid content interfering with mRNA binding. **(c)** Increased stability of DD24-C12-122 nanoparticles does not translate to increased potency in HeLa cells at a dose of 50 ng/well of mRNA (mean ± SD, *n* = 4). **(d)** As with DD24-C12-122, DD60-C12-122 transfection decreases with increasing PEG-lipid content past 7 mol% despite increases in serum stability. This trend is likely a result of decreased particle uptake and/or endosomal escape due to PEG-lipid interference with nanoparticle-membrane interactions [4] (mean ± SD, *n* = 4).

Highly PEGylated DD24-C12-122 *in vivo*

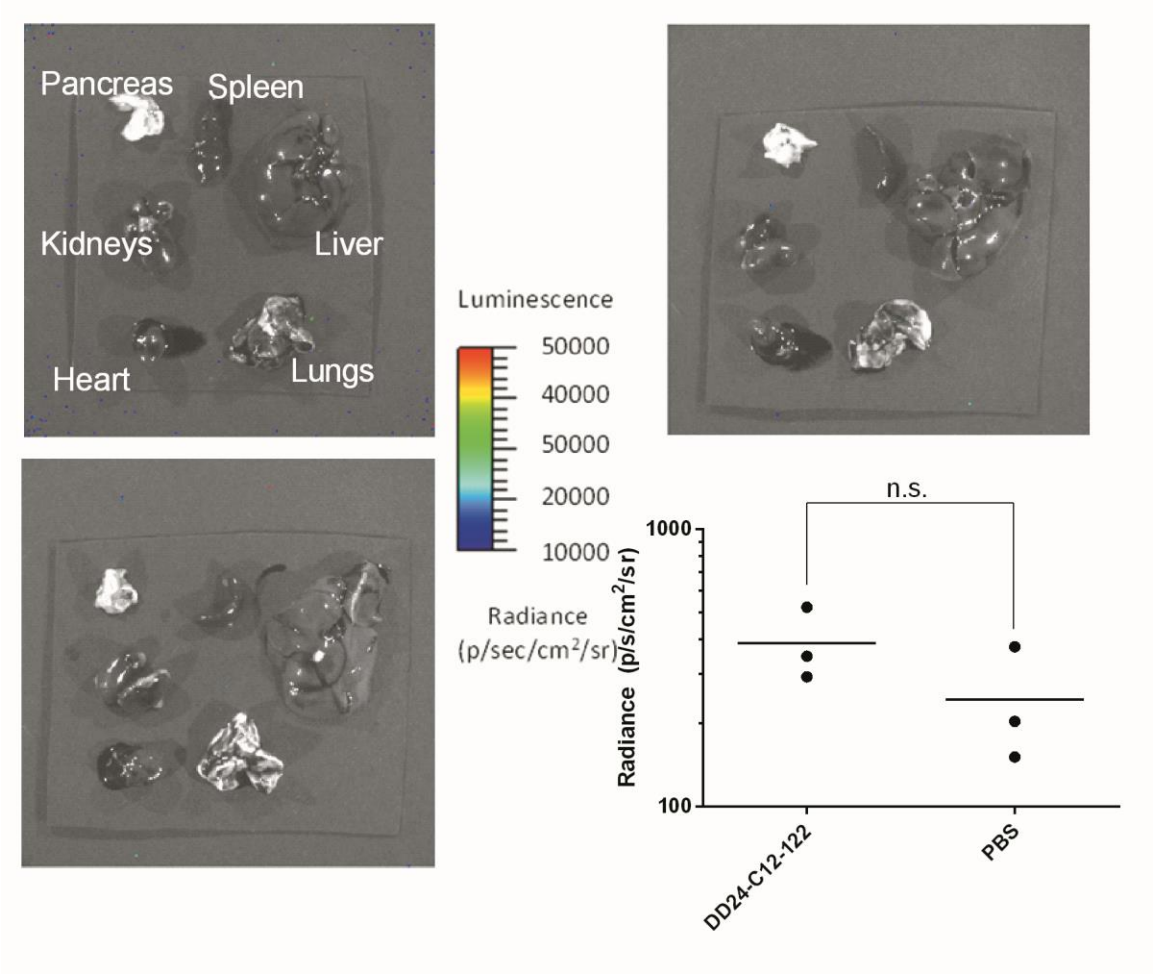


Figure S9. Intravenous injection of DD24-C12-122 with 50 mol% PEG-lipid in BL/6 mice (0.5 mg mRNA/kg mouse) is not lethal, but also does not result in any visible luminescence above background levels.

Nanoparticle Biodistribution Quantification

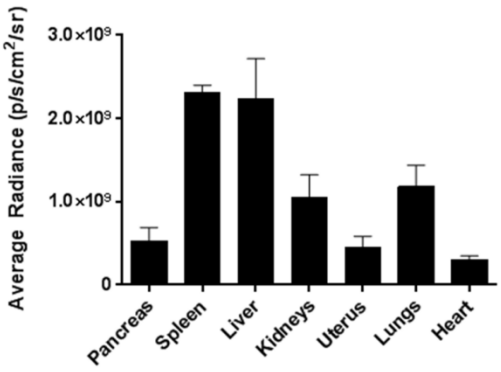


Figure S10. PEGylated DD90-C12-122 biodistribution. PEGylated DD90-C12-122 nanoparticles were loaded with Cy5-labeled mRNA and injected into mice via tail vein injection. Mice were sacrificed 6 hours after injection and their organs (pancreas, spleen, liver, kidneys, uterus, lungs, and heart from top left to bottom right) were imaged for fluorescence using an IVIS imaging apparatus. Fluorescent signal was quantified for each organ, demonstrating that similar amounts of mRNA localize to each organ, despite the fact that the most mRNA translation occurs in the lungs (mean ± SD, n = 4).

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