Contents

MATERIALS

a) Chemicals and reagents for synthesis. All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. 1,2-epoxydecane was purchased from TCI America. 1,2-epoxyoctadecane was purchased from Alfa Aesar. Hydrophobic acrylates octyl acrylate (Ac8), decyl acrylate (Ac10), tetradecyl acrylate (Ac14), and hexadecyl acrylate (Ac16) were synthesized as described below. Organic solvents were purchased from Fisher Scientific and purified with a solvent purification system (Innovative Technology). Lipid PEG2000 was chemically synthesized, as previously described.[1] $CDCl_3$, methanol-*d4*, and DMSO-*d6* were purchased from Cambridge Isotope Laboratories.

b) Nucleic acids and other reagents for biological assays. All siRNAs were purchased from Sigma-Aldrich. DNA oligonucleotides were purchased from Integrated DNA Technologies. Luciferase, mCherry, and Cas9 messenger RNA (mRNA) were purchased from Tri-Link Biotechnologies. Lipofectamine 3000 and OptiMEM were purchased from Invitrogen. Single guide RNA was prepared by *in vitro* transcription (IVT) using the MEGAshortscript T7 transcription kit (Life Technologies) followed by purification using the MEGAclear Transcription Clean-Up Kit (Life Technologies) according to the manufacturer's protocols. The Ribogreen reagent was purchased from Life Technologies. ONE-Glo + Tox and Cell Titer Glow were purchased from Promega. RIPA buffer and TRIzol reagent were purchased from Thermo Scientific. QuickExtract DNA Extraction Solution was purchased from Epicentre. Real-time qPCR was performed using iTaq Universal SYBR Green 2X Supermix (Bio-Rad). All antibodies were purchased from Cell Signaling.

c) Cell culture. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Hyclone containing high glucose, L-glutamine, and without pyruvate or phenol red. RPMI-1640 was purchased from Sigma Aldrich. Dulbecco's modified phosphate buffered saline (PBS), Trypsin-EDTA (0.25%) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. HeLa-Luc and A549-Luc cells were cultured in DMEM supplemented with 5% FBS. IGROV1 cells were cultured in RPMI-1640 supplemented with 5% FBS.

d) Animal studies. All experiments were approved by the Institutional Animal Care & Use Committee (IACUC) of The University of Texas Southwestern Medical Center and were consistent with local, state and federal regulations as applicable. C57BL/6 and athymic nude $Foxn1^{nu}$ mice were purchased from Envigo. NOD scid gamma (NSG) mice were purchased from the UT Southwestern animal breeding core. Rosa-CAG-LSL-tdTomato mice were purchased from The Jackson Laboratory (Stock number: 007909).

METHODS

Instrumentation

a) Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and ^{12}C NMR were performed on a Varian 400 MHz spectrometer or a Varian 500 MHz spectrometer.

b) Mass spectroscopy (MS). MS was performed on a Voyager DE-Pro MALDI-TOF. LCMS was performed on an Agilent LCMS system equipped with UV-vis and evaporative light scattering detectors (ELSD).

c) Flash chromatography. Flash chromatography was performed on a Teledyne Isco CombiFlash Rf- 200i chromatography system equipped with UV-vis and evaporative light scattering detectors (ELSD).

d) Nanoparticle size and zeta potential analysis. Particle sizes and zeta potentials were measured by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano ZS (He-Ne laser, $\lambda = 632$ nm).

e) Nanoparticle formulation for *in vivo* studies. Zwitterionic amino lipid (ZAL) nanoparticles (ZNPs) for *in vivo* studies were prepared using a two-channel microfluidic mixer with herringbone rapid mixing features (Precision Nanosystems NanoAssemblr). Ethanol solutions of lipid mixes (ZALs, cholesterol, and PEG-lipid) were rapidly combined with acidic aqueous solutions of nucleic acid at an aqueous: EtOH volumetric ratio of 3:1 and a flow rate of 12 mL/minute.

f) Real-time qPCR. RT qPCR was run on a Bio-Rad C1000 Touch Thermal Cycler (CFX384 Real-time System). Each reaction was made with iTaq Universal SYBR Green 2X Supermix (Bio-Rad).

g) Confocal laser scanning microscopy. Tissue sections were imaged using confocal laser scanning microscopy with a Zeiss LSM-700 and images were processed using ImageJ (NIH).

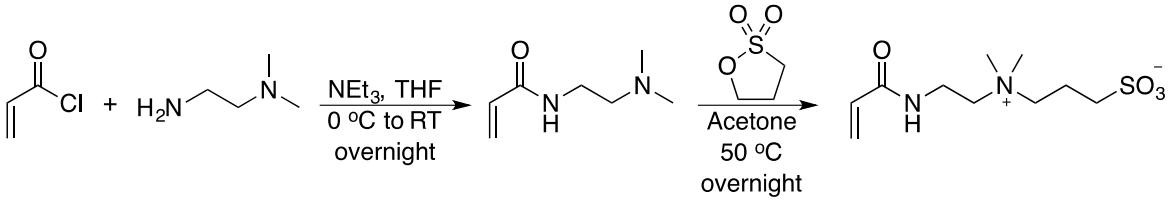
h) Flow cytometry. Flow cytometry was performed with BD FACSAria Fusion machine (BD Biosciences).

Chemical synthesis

Synthesis of 3-((2-acrylamidoethyl)dimethylammonio)propane-1-sulfonate (SBAm): A flame-dried 500 mL round-bottom flask equipped with a stir bar, and an addition funnel under a nitrogen atmosphere was charged with *N*,*N*-dimethyl ethenediamine (20 g, 226.9 mmol) and triethylamine (1 equiv, 227 mmol, 31.6 mL) in 250 mL dry THF, and cooled to 0 ^{o}C. Acryloyl chloride (0.9 equiv, 204.2 mmol, 16.6 mL) was dissolved separately in 50 mL dry THF and added dropwise via an addition funnel to the stirring amine solution. The reaction was allowed to warm to room temperature overnight which resulted in a yellow solution with white precipitate. The precipitate was filtered off and the filtrate was concentrated in vacuo. The crude product was purified by a silica gel column (20% MeOH in DCM). The product was dried with anhydrous sodium sulfate and concentrated under reduced pressure to yield the dimethylamino acrylamide intermediate as an orange liquid (9.36 g, 32.2% yield for step 1).

In a 250 mL round-bottom flask equipped with a stir bar, the dimethylamino acrylamide intermediate (9.36 g, 65.8 mmol) was dissolved in 100 mL acetone. In one portion, 1,3-propanesultone (1.1 equiv, 72.4 mmol, 8.85 g) was added. A rubber stopper with a needle vent was installed and the reaction mixture was heated to 50 ^{o}C overnight, yielding the formation of an off white solid precipitate. The precipitate was collected by vacuum filtration, washed with copious amounts of acetone, and dried under vacuum overnight yielding the SBAm product as a light yellow solid (14.77 g, 84.9% yield for step 2). Mass calculated *m/z* 264.11, observed M^{+1} (LCMS direct injection) *m/z* 265.1. ^{1}H NMR (400 MHz, D_2O) δ 6.33 – 6.17 (m, 2H), 5.80

(dd, J = 8.8, 2.7 Hz, 1H), 3.77 (t, J = 6.8 Hz, 2H), 3.52 (dd, J = 12.0, 5.5 Hz, 4H), 3.17 (s, 6H), 2.97 (t, J = 7.2 Hz, 2H), 2.30 – 2.17 (m, 2H).



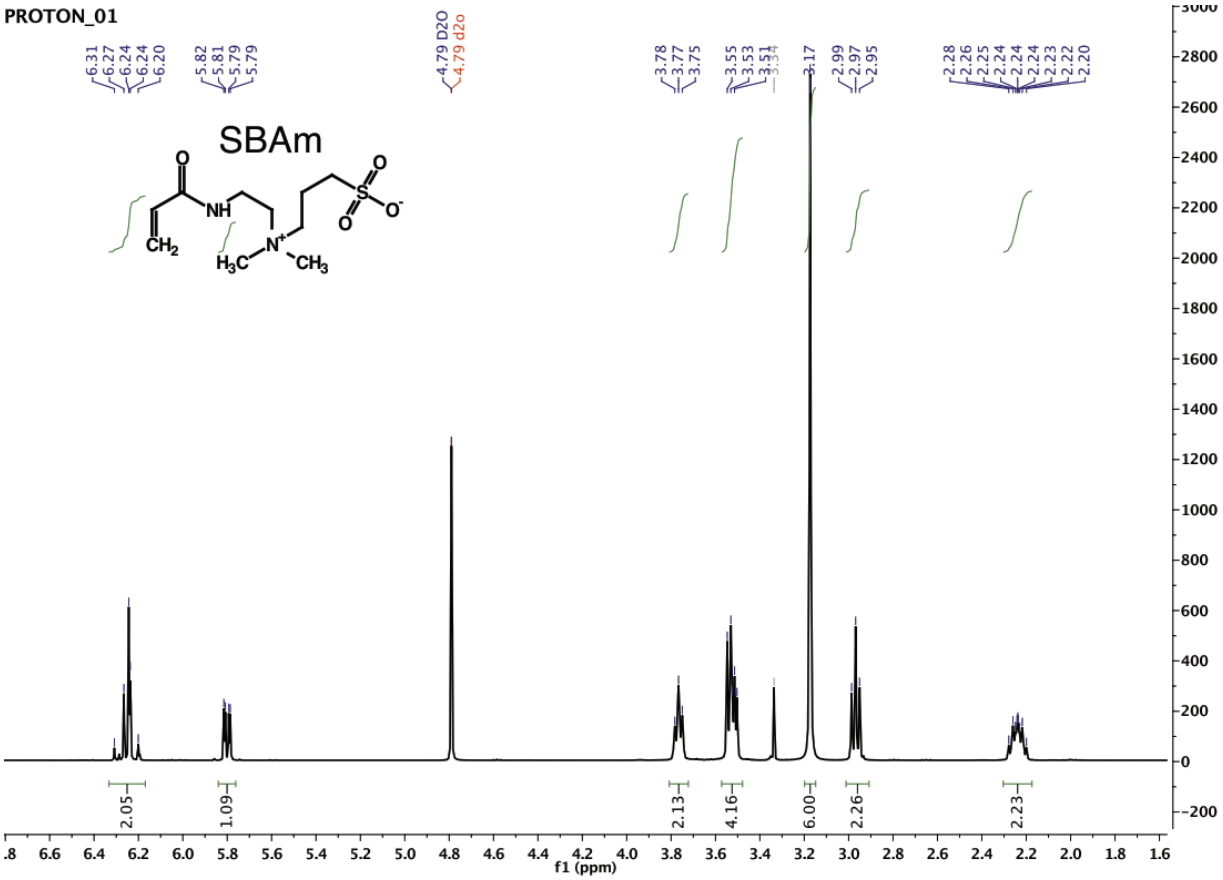


Figure S1. ^{1}H NMR of zwitterionic acrylamide precursor SBAm.

Amino sulfobetaine (ZA*x*) syntheses for library preparation:

General synthesis of propanesulfonate amide-bearing zwitterionic amines (ZA*x*). In a 20 mL vial equipped with a stir bar, 3-((2-acrylamidoethyl)dimethylammonio)propane-1-sulfonate (SBAm, 1.5 g, 5.67 mmol, 1 equiv) was dissolved in 5.67 mL deionized water to a concentration of 1M. The corresponding amine (28.35 mmol, 5 equiv) was added via pipette in one portion, the vial covered and stirred at room temperature overnight. After overnight reaction, the amino SBAm reaction mixture was transferred to several 50 mL polypropylene conical tubes was precipitated in >10 volumes acetone to remove the residual amine starting material, collected by centrifugation (4000 x g, 10 minutes). The supernatant was decanted, the pellet washed with acetone, and dried under vacuum to yield the amino SBAms, which were used without further purification.

ZA1: Light yellow sticky solid (2.40 g, 93.6% yield). Mass calculated *m/*z 452.31, observed M^{+1} (LCMS direct injection) *m/*z 453.3. ^{1}H NMR (400 MHz, D_2O) δ 3.65 (t, J = 6.8 Hz, 2H), 3.48 (ddd, J = 13.7, 9.5, 5.7 Hz, 4H), 3.14 (s, 6H), 2.95 (t, J = 7.2 Hz, 2H), 2.68 (s, 2H), 2.65 – 2.54 (m, 14H), 2.54 – 2.48 (m, 2H), 2.29 (d, J = 1.0 Hz, 9H), 2.22 – 2.16 (m, 4H).

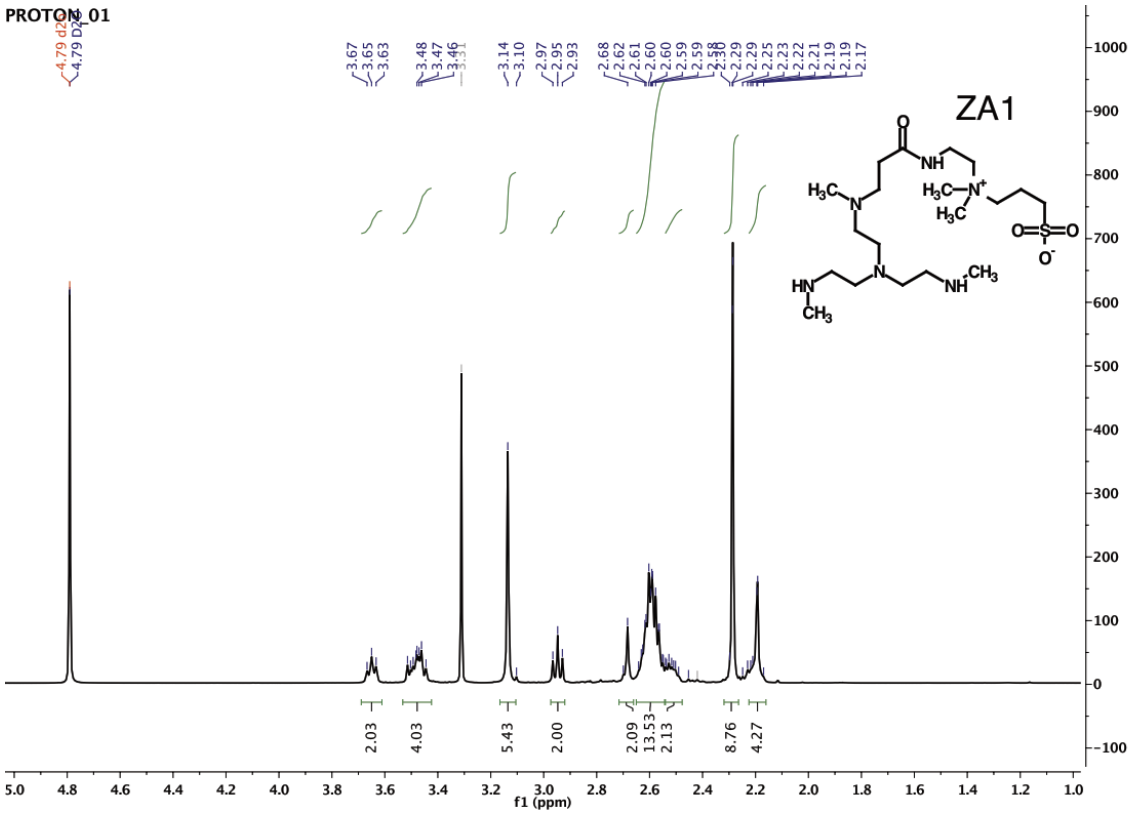
ZA2: Reaction done on a 0.776 g SBAm scale. Viscous yellow oil (0.36 g, 24.8% yield). Mass calculated *m/*z 536.41, observed M^{+1} (LCMS direct injection) *m/z* 537.4. ^{1}H NMR (500 MHz, Deuterium Oxide) δ 3.50 (t, J = 7.0 Hz, 2H), 3.33 (ddd, J = 22.0, 11.2, 5.7 Hz, 4H), 2.98 (s, 6H), 2.83 – 2.62 (m, 4H), 2.57 (dt, J = 21.3, 7.3 Hz, 4H), 2.44 (p, J = 7.1 Hz, 6H), 2.30 – 2.23 (m, 1H), 2.11 – 2.01 (m, 2H), 0.92 – 0.86 (m, 9H), 0.84 (d, J = 6.5 Hz, 4H).

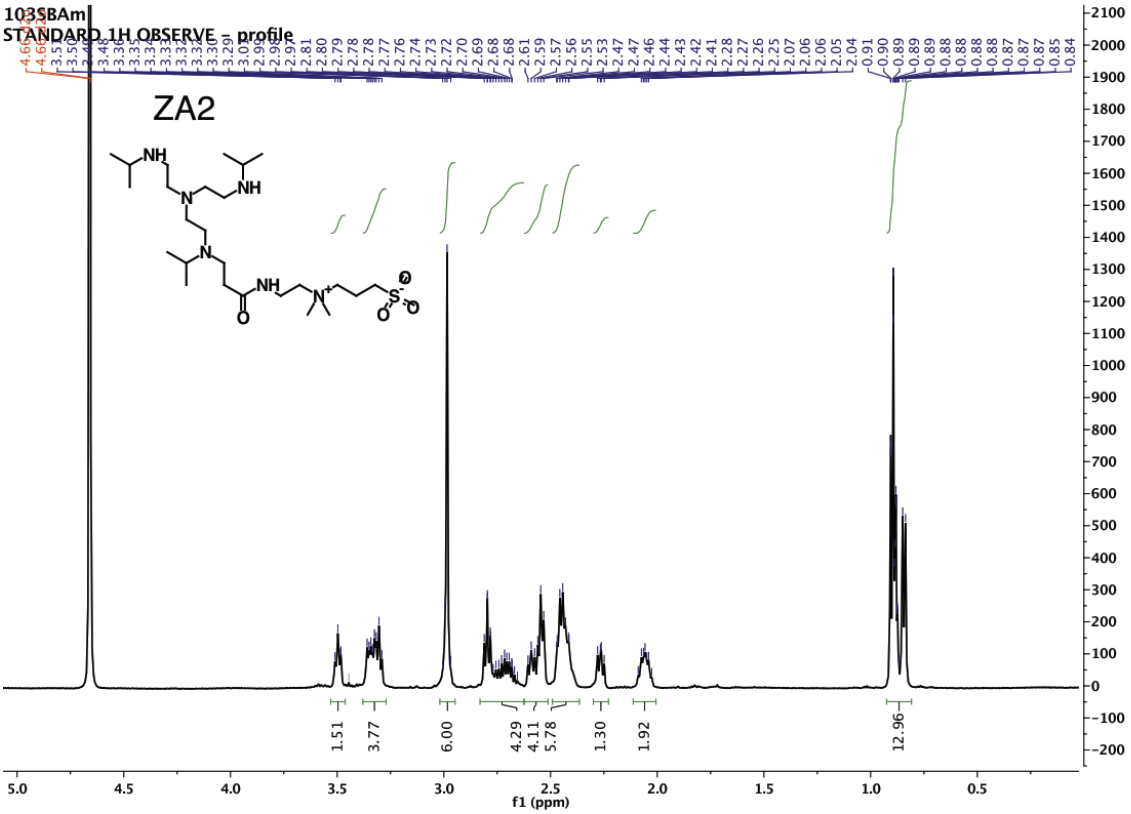
ZA3: Brown sticky solid (2.61 g, quantitative yield). Mass calculated *m/*z 410.58, observed M^{+1} (LCMS direct injection) *m/z* 411.3. ^{1}H NMR (500 MHz, D_2O) δ 3.62 (t, J = 6.7 Hz, 2H), 3.50 – 3.40 (m, 4H), 3.11 (d, J = 1.4 Hz, 6H), 2.92 (td, J = 7.2, 1.3 Hz, 2H), 2.82 – 2.68 (m, 5H), 2.66 – 2.49 (m, 8H), 2.41 (ddd, J = 8.2, 5.9, 1.3 Hz, 2H), 2.23 – 2.14 (m, 2H).

ZA4: Light yellow sticky solid (2.01 g, 92.9% yield) Mass calculated *m/*z 381.24, observed M^{+1} (LCMS direct injection) *m/z* 382.2. ^{1}H NMR (400 MHz, D_2O) δ 3.66 (t, J = 6.8 Hz, 2H), 3.49 (ddd, J = 13.7, 8.7, 5.8 Hz, 4H), 3.14 (s, 6H), 2.96 (t, J = 7.2 Hz, 2H), 2.86 – 2.64 (m, 6H), 2.57 – 2.40 (m, 5H), 2.28 – 2.14 (m, 6H).

ZA5: Sticky yellow solid (2.32 g, 84.1% yield). Mass calculated *m/*z 409.27, observed M^{+1} (LCMS direct injection) *m/z* 410.2. ^{1}H NMR (400 MHz, D_2O) δ 3.52 (t, J = 6.8 Hz, 3H), 3.35 (ddd, J = 13.8, 9.0, 5.6 Hz, 5H), 3.00 (s, 7H), 2.82 (t, J = 7.2 Hz, 3H), 2.65 (t, J = 7.1 Hz, 3H), 2.49 (q, J = 6.4, 5.5 Hz, 1H), 2.39 (t, J = 7.4 Hz, 2H), 2.26 (dq, J = 15.4, 5.4, 3.7 Hz, 7H), 2.14 – 1.99 (m, 7H), 1.55 – 1.41 (m, 4H).

ZA6: Sticky yellow solid (2.71 g, quantitative yield). Mass calculated *m/*z 464.31, observed M^{+1} (LCMS direct injection) *m/z* 465.3. ^{1}H NMR (500 MHz, D_2O) δ 3.64 (t, J = 6.9 Hz, 2H), 3.52 – 3.42 (m, 4H), 3.12 (s, 7H), 2.94 (t, J = 7.2 Hz, 3H), 2.82 – 2.68 (m, 5H), 2.53 (t, J = 7.4 Hz, 2H), 2.45 – 2.30 (m, 7H), 2.26 – 2.15 (m, 4H), 1.64 (tdd, J = 15.5, 12.1, 7.6 Hz, 4H).

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1.30
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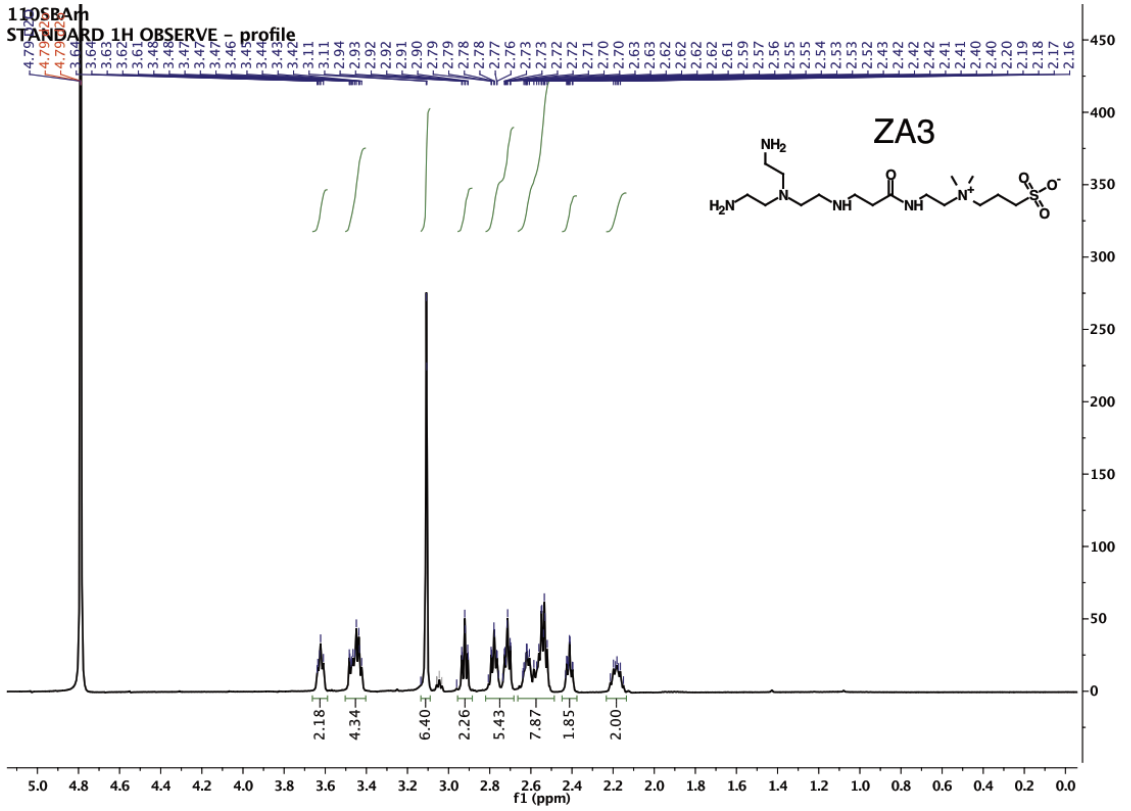
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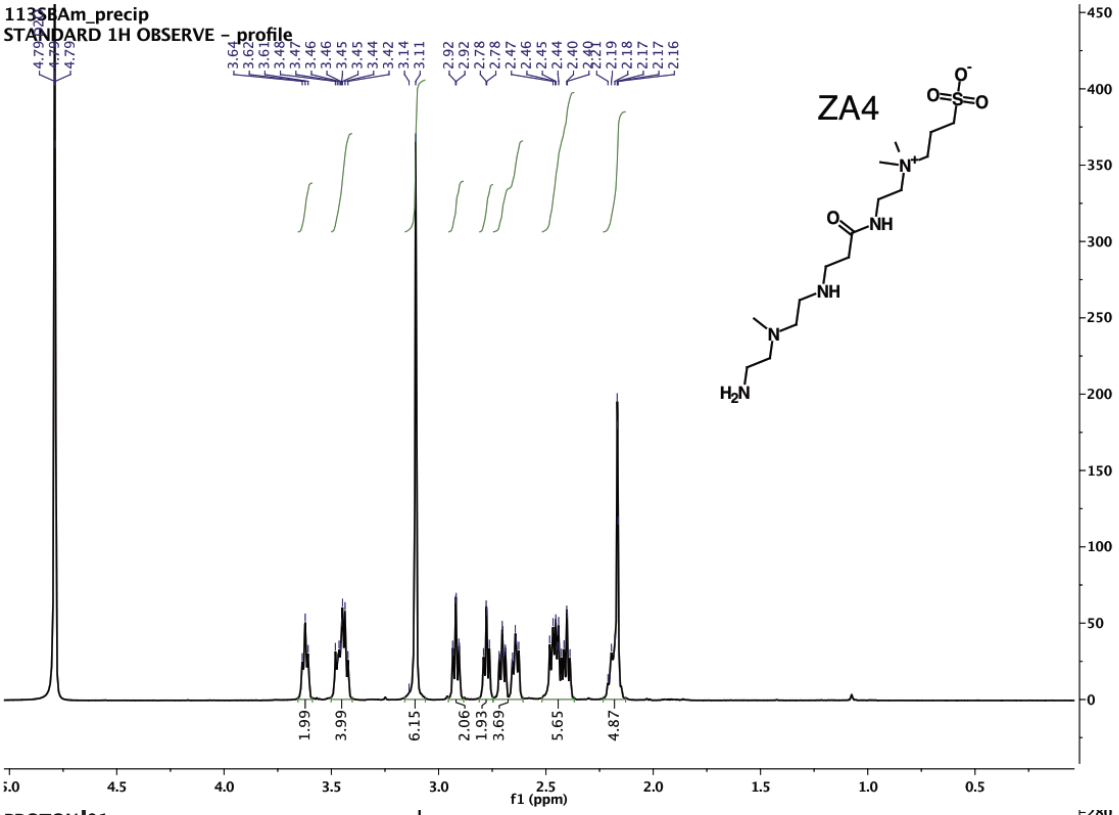
2.00
f1 (ppm)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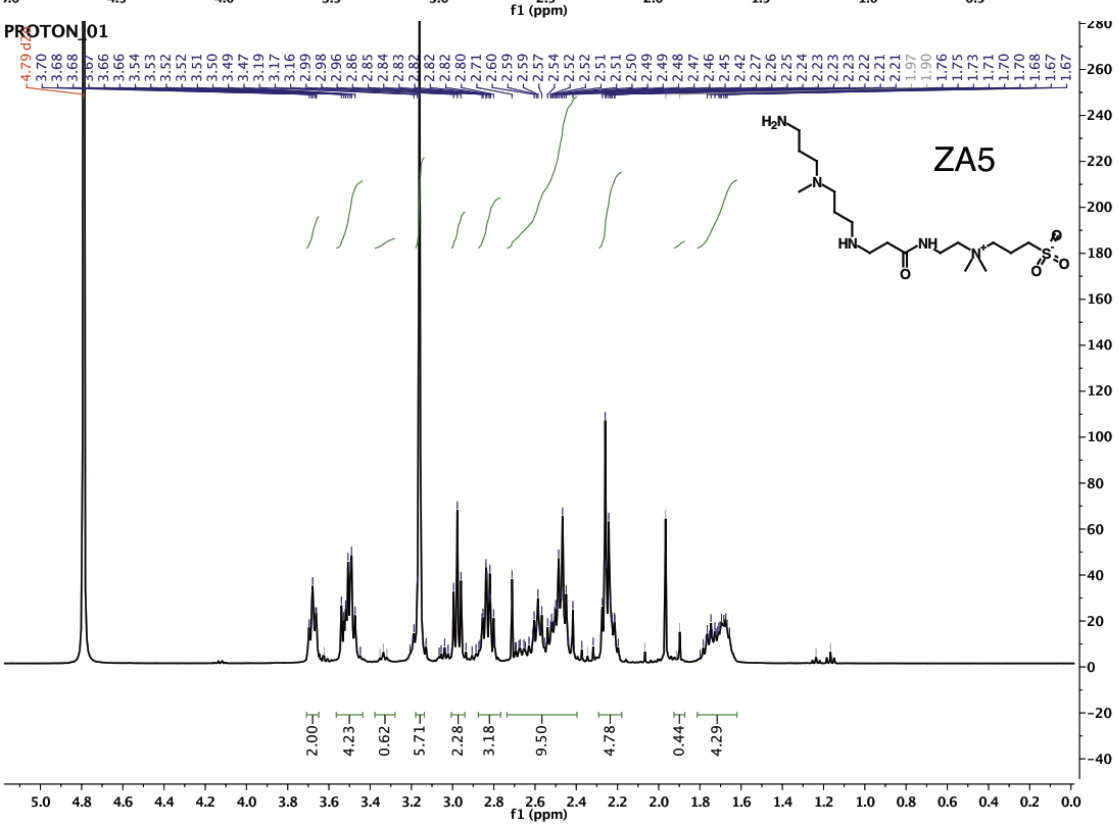
f1 (ppm)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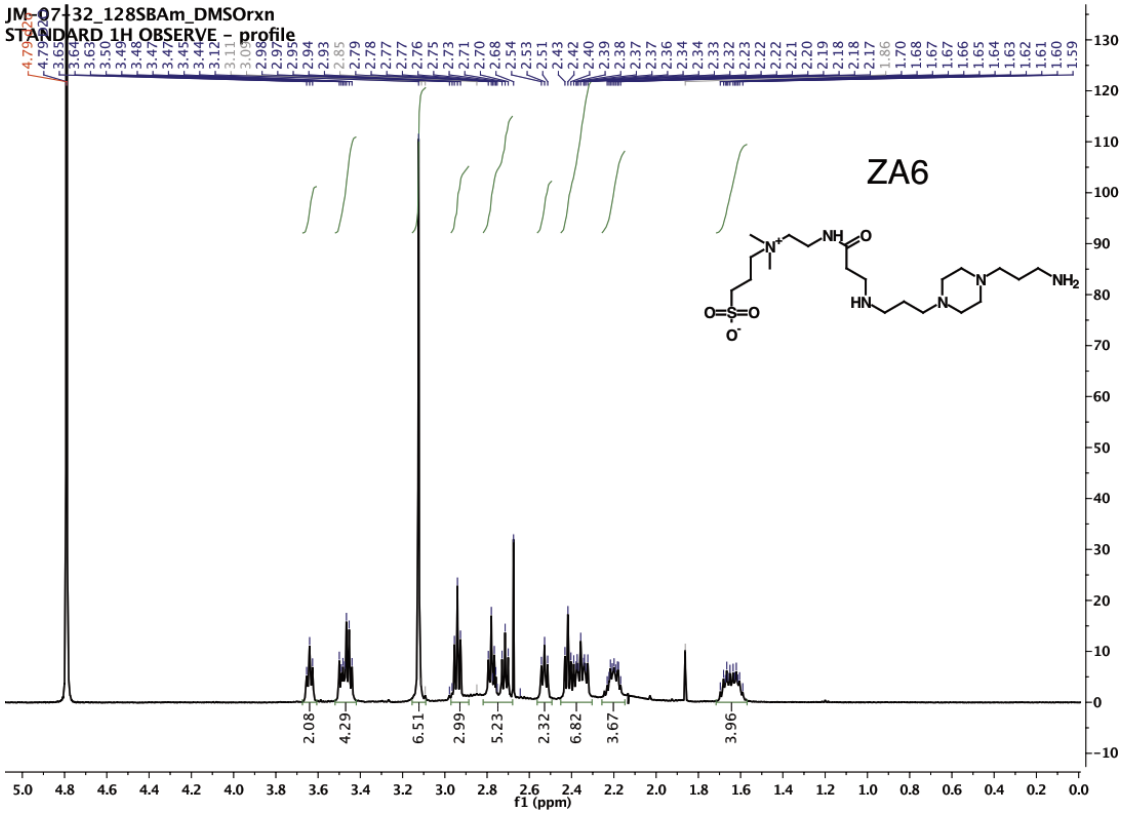
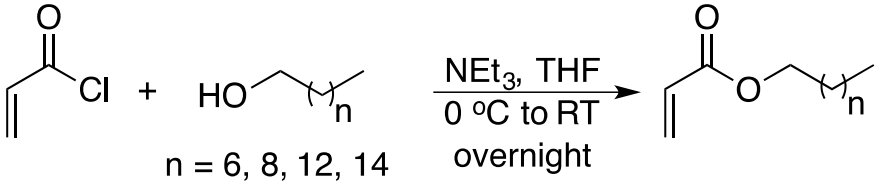


Figure S2. [1]H NMR spectra of zwitterionic amino SBAms (ZA*x*).

Hydrophobic acrylate (Ac*n*) synthesis. Hydrophobic acrylates were synthesized by the reaction of hydrophobic primary alcohols with acryloyl chloride at large scale. In a dry 250-mL round bottom flask under argon, the appropriate hydrophobic alcohol (10 or 15 g, 1 equiv) and triethylamine (1 equiv) were dissolved in 85 mL dry tetrahydrofuran and cooled to 0 °C on ice. Acryloyl chloride (0.9 equiv) was dissolved separately in 15 mL dry THF and added dropwise to the solution of alcohol and triethylamine, resulting in the formation of a white precipitate. The reaction was stirred and allowed to warm to room temperature. The precipitate was removed by fitration, the solvent removed under reduced pressure and the desired acrylate pruified on a column of silica gel with 5-10% ethyl acetate in hexanes to yield the products as pale yellow oils.

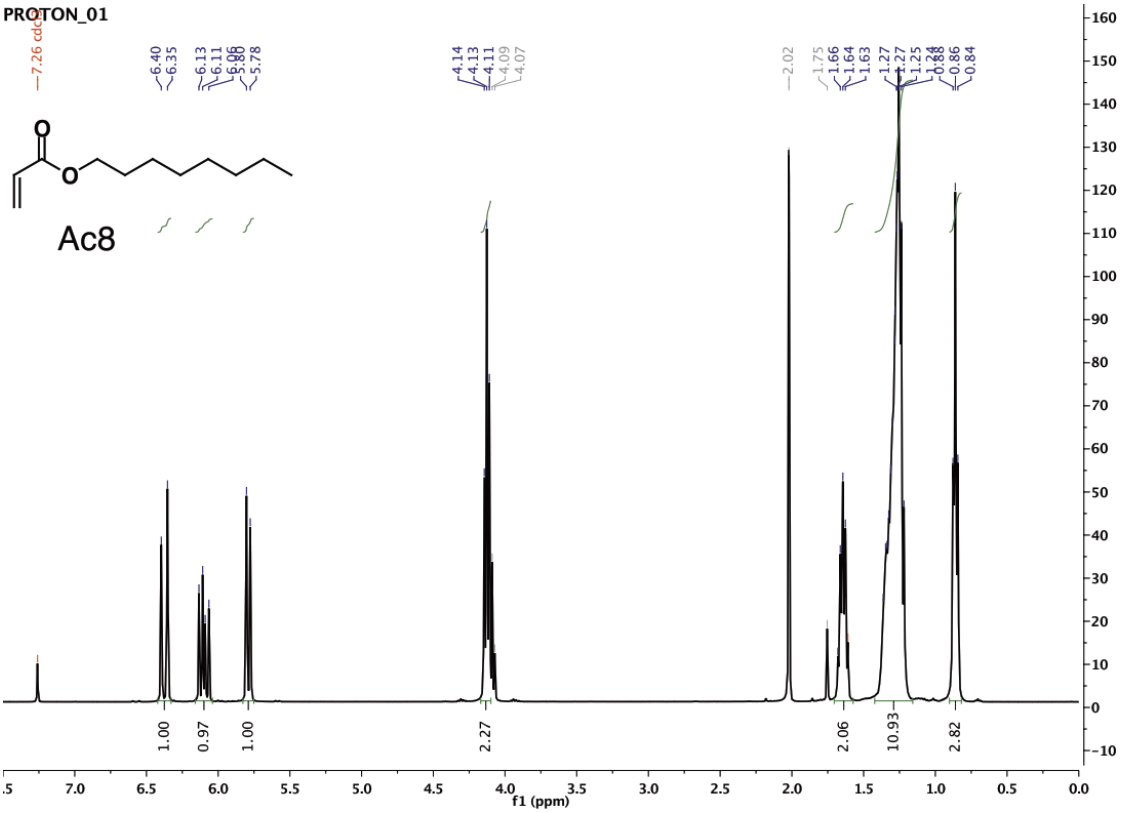


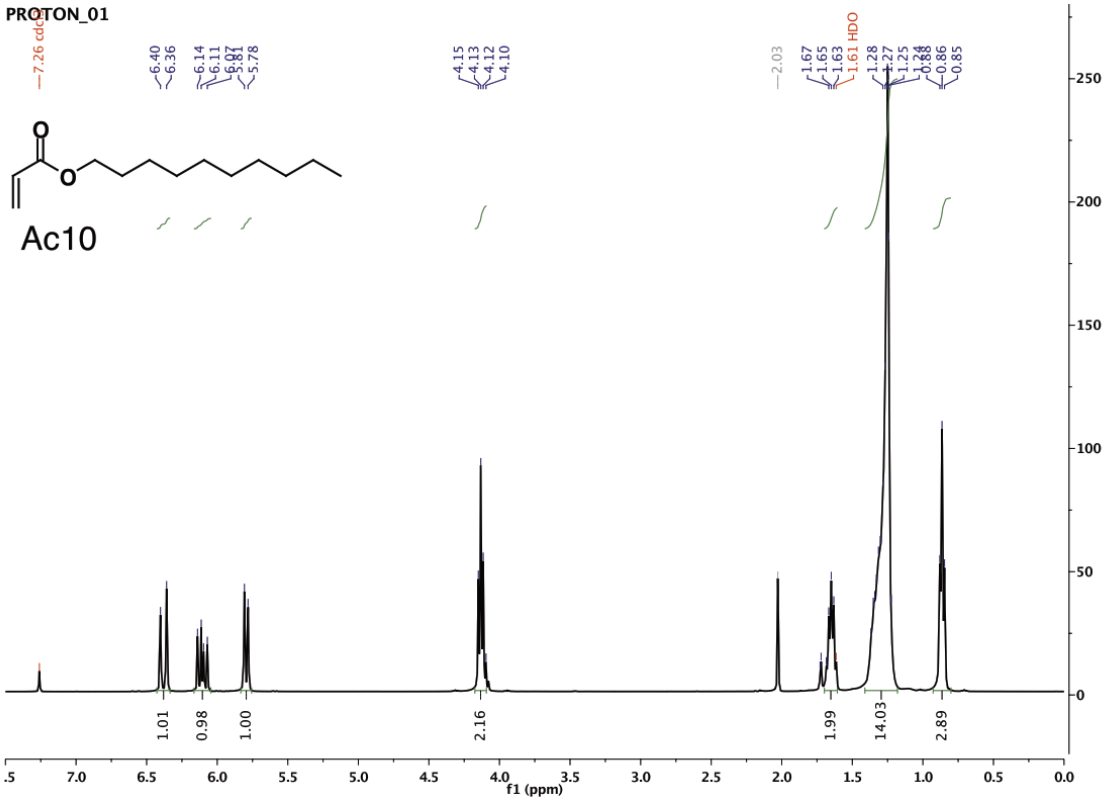
Ac8: 10 g alcohol scale, pale yellow oil, 9.75 g, 76.6% yield.
[1]H NMR (400 MHz, $CDCl_3$) δ 6.37 (d, J = 17.3 Hz, 1H), 6.10 (dd, J = 17.3, 10.4 Hz, 1H), 5.79 (d, J = 10.5 Hz, 1H), 4.13 (t, J = 7.0 Hz, 2H), 1.65 (q, J = 6.9 Hz, 2H), 1.42 – 1.16 (m, 10H), 0.86 (t, J = 6.7 Hz, 3H).

Ac10: 10 g alcohol scale, pale yellow oil, 10.98 g, 90.9% yield.
[1]H NMR (400 MHz, $CDCl_3$) δ 6.38 (d, J = 17.3 Hz, 1H), 6.10 (dd, J = 17.3, 10.4 Hz, 1H), 5.79 (d, J = 10.5 Hz, 1H), 4.13 (t, J = 6.8 Hz, 2H), 1.66 (q, J = 7.0 Hz, 2H), 1.41 – 1.18 (m, 14H), 0.86 (t, J = 6.6 Hz, 3H).

Ac14: 15 g alcohol scale, pale yellow oil, 15.28 g, 90.5% yield.
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.37 (dd, J = 17.3, 1.5 Hz, 1H), 6.09 (dd, J = 17.3, 10.4 Hz, 1H), 5.77 (dd, J = 10.4, 1.5 Hz, 1H), 4.12 (t, J = 6.8 Hz, 2H), 1.69 – 1.59 (m, 2H), 1.39 – 1.15 (m, 21H), 0.85 (d, J = 7.1 Hz, 3H).

Ac16: 15 g alcohol scale, pale yellow oil, 11.27 g, 68.3% yield.
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39 (dd, J = 17.4, 1.5 Hz, 1H), 6.11 (dd, J = 17.3, 10.4 Hz, 1H), 5.80 (dd, J = 10.4, 1.5 Hz, 1H), 4.14 (t, J = 6.7 Hz, 2H), 1.72 – 1.61 (m, 2H), 1.25 (s, 26H), 0.87 (t, J = 6.8 Hz, 3H).

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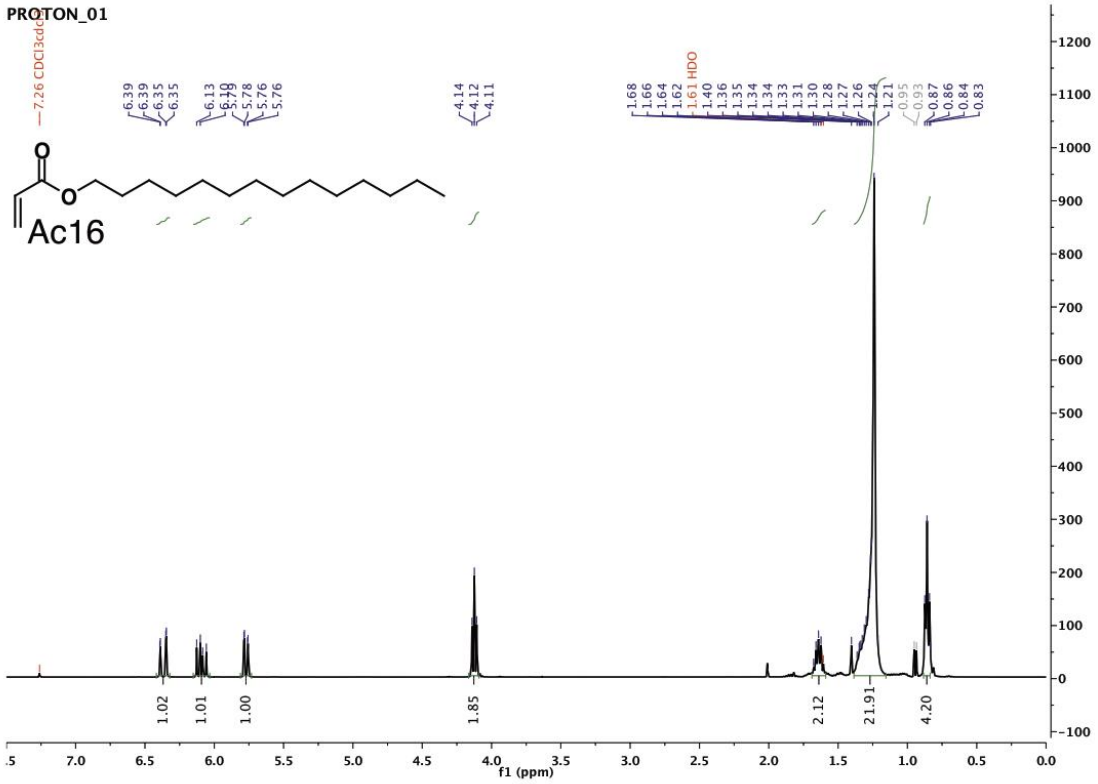
f1 (ppm)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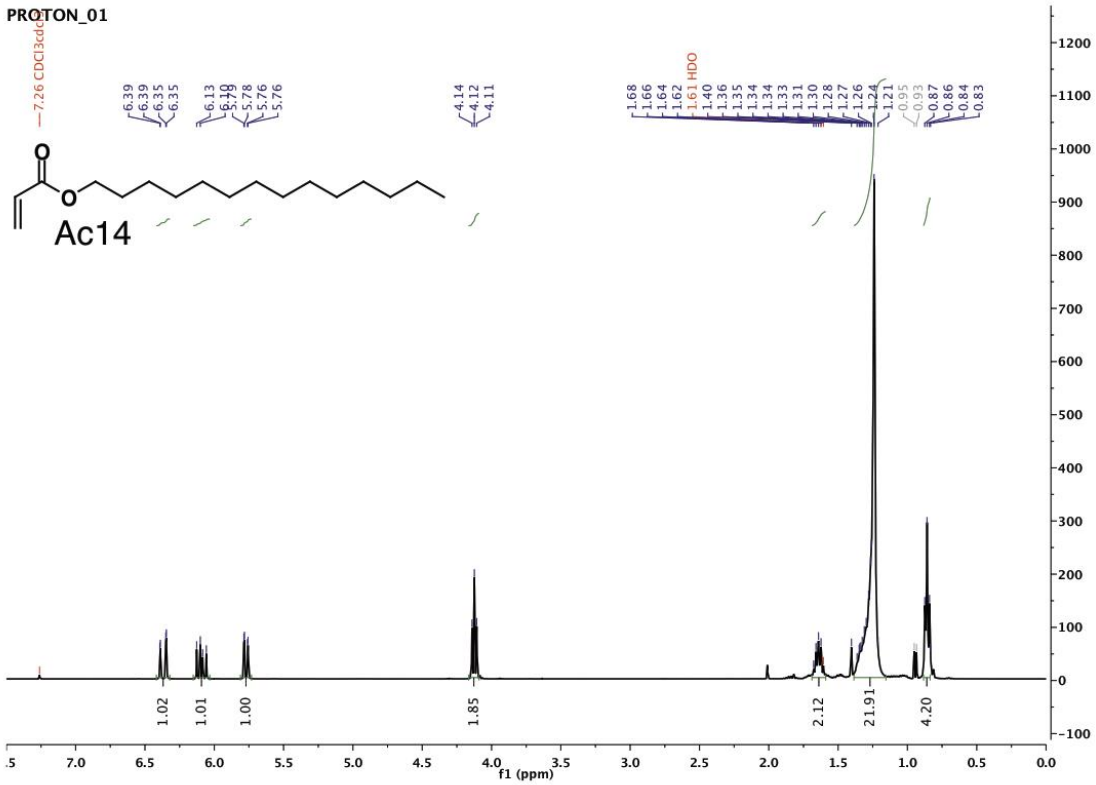
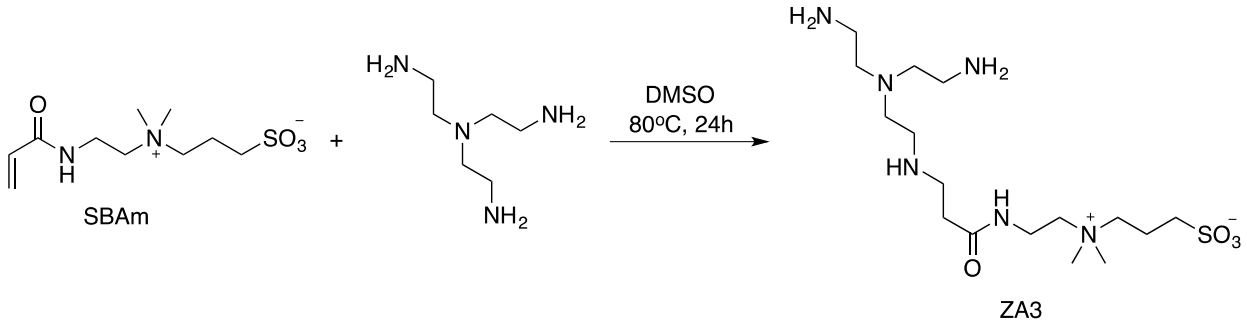


Figure S3. ^{1}H NMR spectra of hydrophobic acrylates Ac8, Ac10, Ac14, and Ac16.

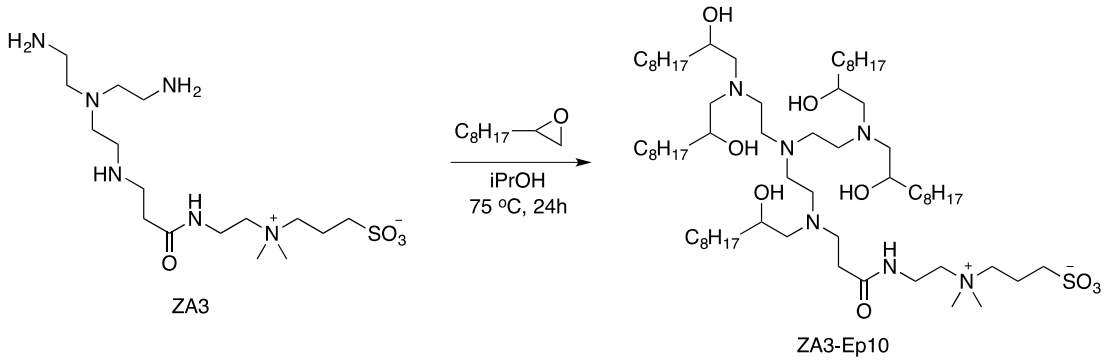
Synthesis of Amino SBAm epoxide and acrylate libraries of zwitterionic amino lipids (ZALs): A zwitterionic amino lipid (ZAL) library of all previously described amino SBAms was prepared by introduction of hydrophobic tails through reaction with 1,2-epoxy alkanes and hydrophobic acrylates. The epoxides (1,2-epoxyoctane, 1,2-epoxydecane, 1,2-epoxydodecane, 1,2-epoxytetradecane, 1,2-epoxyhexadecane, and 1,2-epoxyoctadecane) were purchased commercially and encoded to include the total number of carbon atoms in the molecule (Ep*n*, 8-18). The hydrophobic acrylates were either purchased commercially (Ac12, Ac18) or synthesized by the reaction of the appropriate primary alcohol with acryloyl chloride (Ac8, Ac10, Ac14, Ac16), and encoded to include the number of carbon atoms in the hydrophobic tail, but not including the acrylate moiety (Ac*n*, 8-18). To prepare the library, in a 4 mL vial equipped with a stir bar, the zwitterionic amines (0.1 mmol or 0.05 mmol) were weighed out by balance, and dissolved to a concentration of 1 M in *i*PrOH for epoxide ZALs or in DMSO for acrylate ZALs. The appropriate hydrophobic electrophile was added with N equivalents, where N is the number of amine reactive sites that would yield complete conversion of primary and secondary amines to tertiary amines. The vials were sealed and the reactions stirred for several days at 75 °C for epoxides and 80 °C for acrylates. After reaction, the reactions were precipitated in acetone to yield the zwitterionic aminolipids. The crude products were used to screen the library for siRNA delivery efficacy without further purification.

Alternative Synthesis of amino SBAm ZA3: A 20 mL vial equipped with a stir bar was charged with 3-((2-acrylamidoethyl)dimethylammonio)propane-1-sulfonate (SBAm, 0.8111 g, 3.068 mmol) and dissolved in 3 mL DMSO. Via syringe, tris(2-aminoethyl) amine (5 equiv, 15.32 mmol, 2.24 g) was added yielding a cloudy yellow/brown suspension. The reaction mixture was sealed and stirred at 80 °C overnight, yielding an orange cloudy suspension. The reaction mixture was further diluted in DMSO, transferred to several 50 mL conical tubes and precipitated in 10 volumes ethyl acetate. The precipitate collected by centrifugation (4,000 x g, 10 minutes), and the supernatant decanted to yield a sticky yellow/brown. The product was re-precipitated in DMSO/EtOAc several times to remove any residual tris(2-aminoethyl) amine, and finally dissolved in MeOH transferred to round-bottom flask and concentrated under reduced pressure. The product was dried overnight under vacuum to remove residual solvent, re-dissolved in methanol and precipitated in

ethyl acetate, and dried under vacuum to yield 110SBAm as an orange/brown oil (1.4058 g, >100% but ^{1}H NMR shows residual DMSO) and used in subsequent reactions without further purification.



Synthesis of ZA3-Ep10: A 20 mL vial equipped with a stir bar was charged with 110SBAm (300 mg, 0.7307 mmol) and iPrOH (730 μL, 1M SBAm) and stirred briefly at RT to yield a yellow/brown suspension. 1,2-epoxydecane (4.384 mmol, 685 mg, 6 equiv) was added, the vial was sealed and stirred overnight at 75 °C for approximately 24h resulting in a clear yellow/brown solution. The iPrOH was removed under reduced pressure to yield a yellow/brown oil. The crude product was dissolved in minimal 5% MeOH in DCM and purification was carried out on a silica gel column (24g) using the Combiflash system (Teledyne Isco). The product was eluted and fractionated with a solvent gradient of 5% MeOH in DCM to 20% MeOH, 2% saturated ammonium hydroxide in DCM and the product elution tracked by ELSD. The product containing fractions were concentrated under reduced pressure, and dried under vacuum overnight to yield the product as a sticky yellow solid (192.5 mg, 22.1% yield). Mass calculated *m/*z 1191.0246, observed M^{+1} (LCMS direct injection) *m/z* 1192.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (d, J = 5.8 Hz, 1H), 4.56 – 3.92 (m, 2H), 3.76 – 3.54 (m, 12H), 3.20 (d, J = 3.3 Hz, 6H), 2.88 (dq, J = 36.8, 13.1 Hz, 10H), 2.69 – 2.60 (m, 4H), 2.54 (d, J = 14.2 Hz, 3H), 2.49 – 2.30 (m, 9H), 2.22 (dd, J = 11.0, 5.5 Hz, 2H), 1.44 – 1.23 (m, 66H), 0.87 (t, J = 6.7 Hz, 15H).



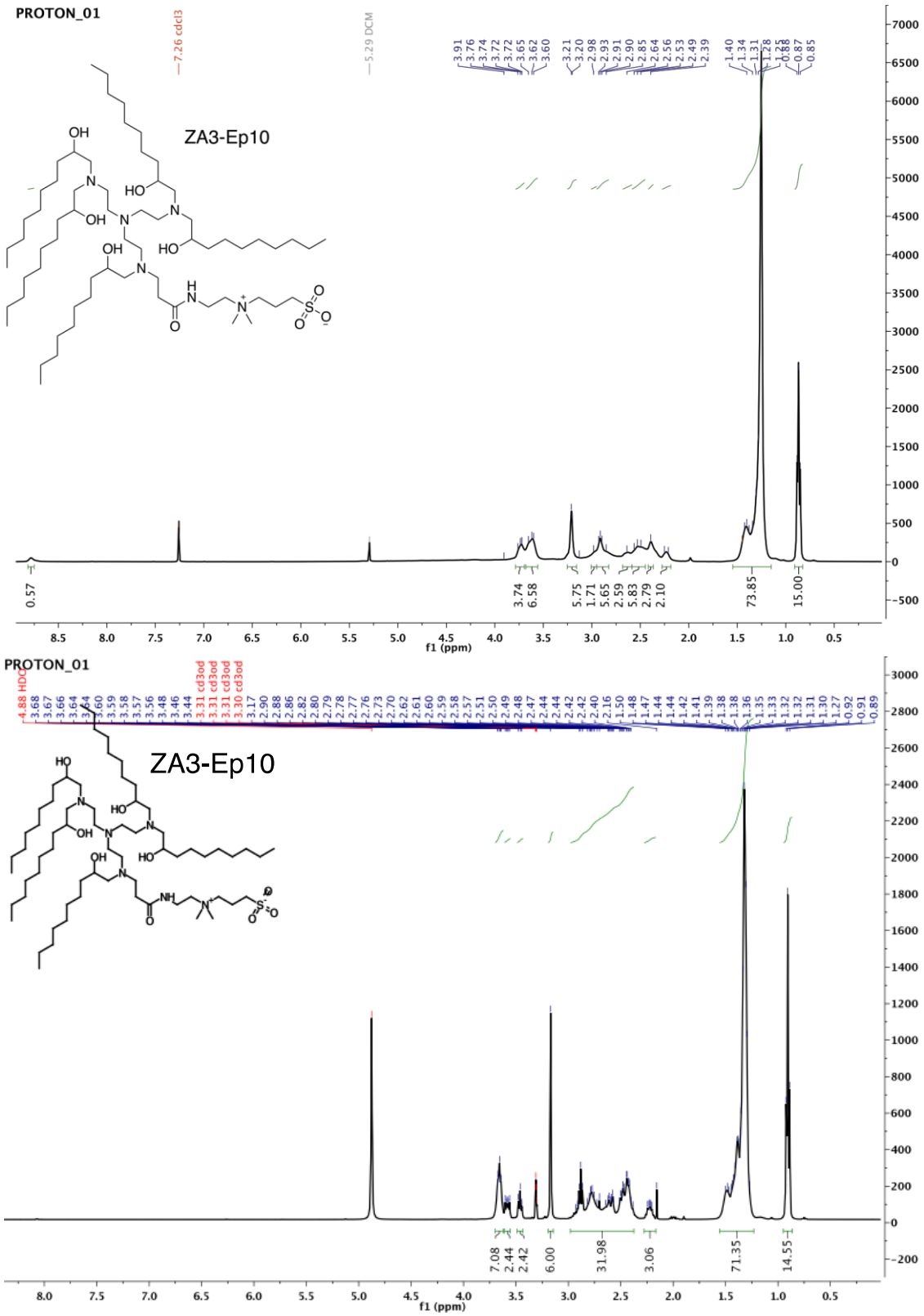


Figure S4. ^{1}H NMR of ZA3-Ep10 in $CDCl_3$ (top) and CD_3OD (bottom).

Nucleic Acid Sequences

Small interfering RNAs (siRNAs)
dT are DNA bases. All others are RNA bases.

siLuc (siRNA against Luciferase).
sense: 5'-GAUUAUGUCCGGUUAUGUA[dT][dT]-3'
antisense: 5'-UACAUAACCGGACAUAAUC[dT][dT]-3'

siCtrl (non-targeting siRNA)
sense: 5'-GCGCGAUAGCGCGAAUAUA[dT][dT]-3'
antisense: 5'- UAUAUUCGCGCUAUCGCGC[dT][dT]-3'

Single guide RNAs (sgRNAs). Guide RNAs were designed using the CRISPR.mit.edu platform and cloned into pSpCas9(BB)-2A-GFP (PX458) as previously reported.[2]

Table S1. sgRNA sequences

Guide name	Target	Guide sequence (5' to 3')	PAM	Strand
sgLuc1	Luciferase	CTTCGAAATGTCCGTTCGGT	TGG	Positive
sgLuc2	Luciferase	CCCGGCGCCATTCTATCCGC	TGG	Positive
sgLuc3	Luciferase	TCCAGCGGATAGAATGGCGC	CGG	Negative
sgLuc4	Luciferase	GGATTCTAAAACGGATTACC	AGG	Positive
sgLuc5	Luciferase	ATAAATAACGCGCCCAACAC	CGG	Negative
sgLoxP	LoxP	CGTATAGCATACATTATACG	AAG	Negative
sgCtrl	Mouse F7	GCTTCGATAATATCCGCTAC	TGG	Positive

Table S2. BbsI sgRNA cloning oligos

Probe	Sequence (5' to 3')*
sgLuc1_Top	CACCG**CTTCGAAATGTCCGTTCGGT**
sgLuc1_Bottom	AAACACCGAACGGACATTTCGAAGC
sgLuc2_Top	CACCG**CCCGGCGCCATTCTATCCGC**
sgLuc2_Bottom	AAACGCGGATAGAATGGCGCCGGGC
sgLuc3_Top	CACCG**TCCAGCGGATAGAATGGCGC**
sgLuc3_Bottom	AAACGCGCCATTCTATCCGCTGGAC
sgLuc4_Top	CACCG**GGATTCTAAAACGGATTACC**
sgLuc4_Bottom	AAACGGTAATCCGTTTTAGAATCCC
sgLuc5_Top	CACCG**ATAAATAACGCGCCCAACAC**
sgLuc5_Bottom	AAACGTGTTGGGCGCGTTATTTATC
sgLoxP_Top	CACCG**CGTATAGCATACATTATACG**
sgLoxP_Bottom	AAACCGTATAATGTATGCTATACGC
sgCtrl_Top	CACCG**GCTTCGATAATATCCGCTAC**
sgCtrl_Bottom	AAACGTAGCGGATATTATCGAAGCC

***Guide sequence shown in **bold.**

Table S3. T7 template PCR primers

Primer	Sequence (5' to 3')
IVT sgLuc-fwd	TAATACGACTCACTATAGGGATAAATAACGCGCCCAACAC
IVT sgLoxP-fwd	TAATACGACTCACTATAGGGCGTATAGCATACATTATACG
IVT sgCtrl-fwd	TAATACGACTCACTATAGGGGCTTCGATAATATCCGCTAC
IVT-rev (common)	AAAAGCACCGACTCGGTGCC

Table S4. Surveyor assay PCR primers

Primer	Sequence (5' to 3')	Amplicon	Expected cut bands
Luc 1_Forward	GGAACCGCTGGAGAGCAACT	510 bp	233 bp, 277 bp
Luc 1_Reverse	GTCCCTATCGAAGGACTCTGGCA		
Luc 2_Forward	GCTGGAGAGCAACTGCATAA	429 bp	202 bp, 227 bp
Luc 2_Reverse	CATCGACTGAAATCCCTGGTAATC		

Table S5. Real time qPCR primers

Primer	Sequence (5' to 3')
Cas9 forward	GGAACCGCTGGAGAGCAACT
Cas9 reverse	GTCCCTATCGAAGGACTCTGGCA
hActinB forward	AGAAGGATTCCTATGTGGGCG
hActinB reverse	CATGTCGTCCCAGTTGGTGAC

Biological assays

sgRNA preparation. Single guide RNAs were designed using the CRISPR.mit.edu platform and cloned into PX458 plasmid with standard BbsI cloning. T7 transcription templates were amplified by PCR and gel purified. sgRNAs were synthesized by *in vitro* transcription using the MEGAshortscript T7 transcription kit (Life Technologies) followed by purification using the MEGAclear Transcription Clean-Up Kit (Life Technologies) according to the manufacturer's protocols.

Screening of sgRNA using pDNA. sgRNA-cloned PX458 plasmids were used to evaluated efficacy of the sgRNAs against luciferase by transfection of the plasmid encoding both sgRNA and Cas9. Lipofectamine 3000 (LF3000, Invitrogen) was used to transfect the sgRNA-Cas9 plasmids according to manufacturer's protocols. HeLa-Luc cells were seed in a 96-well white-opaque tissue culture plate at a density of 10,000 cells per well. LF3000 pDNA particles were added to the cells at a dose of 100 ng pDNA per well. After 6 hours, the medium was removed and exchanged for 200 μL fresh growth medium. After 24, 48 and 72h, the relative expression of luciferase was determined using the One-Glo + Tox assay (Promega) and normalized to control. Non-targeting sgRNA (sgScr) and unguided Cas9 plasmids were used as a control. (N = 4 +/- standard deviation).

HeLa-Luc-Cas9 cell line preparation. HeLa-Luc-Cas9 stable cells were prepared by lentiviral transduction. Parental HeLa-Luc cells[1, 3] were seeded at a density of 70,000 cells per well in a 24-well plate in complete growth medium and allowed to attach in the incubator overnight. The medium was replaced with 1 mL pre-warmed pseudoparticle medium (DMEM, 3% FBS, 20 mM HEPES, 4 μg/mL polybrene). Cas9-Blast lentivirus supernatant was thawed on ice and 50-100 μL was added to the desired well. The cells were spinoculated at room temperature for 1 hour at 1,000 x g, and returned to the incubator overnight, after which the pseudoparticle medium was exchanged for complete growth medium. After 48h total time post spinoculation, selective pressure was applied (5 and 10 μg/mL Blasticidin S) and cells were maintained and expanded. Single cell clones were isolated by single cell sorting by flow cytometry. Cas9 protein expression was confirmed by western blot compared to parental HeLa-Luc cells by blotting for FLAG tag before single cell sorting and for Cas9 after single cell sorting.

***In vitro* ZAL nanoparticle (ZNP) formulations.** ZNPs were prepared by the ethanol dilution method. The RNA (whether an siRNA, sgRNA, or mRNA) was diluted in acidic aqueous buffer (unless otherwise indicated, 10 mM citric acid/sodium citrate buffer pH 3). The lipid mix was prepared in ethanol, with the appropriate molar ratios of ZAL, cholesterol and PEG-lipid from ethanol stock solutions of each component. Via pipette, the lipid dilution was added to the RNA dilution at a final volumetric ratio of 1:3, rapidly mixed by pipette, and incubated for 15-20 minutes. After this incubation period, the particles were either diluted 3-fold in, or dialyzed against 1X Dulbecco's Modified PBS without calcium and magnesium (Sigma-Aldrich). Dialyses were performed in Pur-A-Lyzer Midi dialysis chambers (Sigma-Aldrich) for 1 hour per 200 μL sample per chamber.

ZAL siRNA delivery library screen. The library of ZALs functionalized with epoxide and acrylate hydrophobic tails was screened for siRNA delivery efficacy in HeLa-Luc cells. In a white opaque 96-well plate tissue culture plate, HeLa cells were seeded at a density of 10 x 10^3 cells per well in 100 μL growth medium (DMEM without phenol red, 5% FBS), and allowed to attach overnight. The medium was exchanged for 200 μL fresh growth medium the day of the assay. Crude ZALs products were prepared using a formulation lipid mixture of 50:38.5 (ZAL: cholesterol), and a ZAL:siRNA ratio such that the number of hydrophobic tails in the ZAL times the ZAL:siRNA mole ratio in the formulation was ~1000, which resulted in a weight ratio range across the library of 16:1 to 45:1 ZAL:siRNA, with an average of 29.5 +/- 6.3 weight ratio across the library. ZAL NP formulations were performed in a 96-well plate by rapid mixing of ZAL lipid mix (20 μL) and siLuc dilution (60 μL, 13.33 ng/μL in 10 mM citric acid-sodium citrate buffer, pH 5) at 3:1 aqueous:EtOH *v:v* ratio with a multichannel pipette. After a 15-20 minute

incubation period, the formulations were diluted in 12 volumes (240 μL) PBS. The nanoparticles (40 μL) were added to the HeLa-Luc cells at a dose of 100 ng siRNA per well. The nanoparticles were incubated with the cells for 24 h after which time the cell viability and luciferase expression were evaluated with the ONE-Glo + Tox Assay cell viability and luciferase assay (Promega).

sgRNA delivery to HeLa-Luc-Cas9 cells. Select ZALs were evaluated in the delivery of single guide RNA (sgRNA) to HeLa-Luc-Cas9 cells. In a white opaque 96-well plate tissue culture plate, HeLa-Luc-Cas9 cells were seeded at a density of 5 x 10^3 cells per well in 100 μL growth medium (DMEM without phenol red, 5% FBS), and allowed to attach overnight and then supplemented with an additional 100 μL DMEM. ZNPs encapsulating sgRNA were formulated using the *in vitro* nanoparticle formulation protocol at the indicated lipid composition and weight ratio (maintaining 50:38.5 (ZAL:cholesterol mole ratio), tuning PEG-lipid additive from 5% to 0.5%, and tuning weight ratio from 20:1 ZAL:sgRNA to 5:1 ZAL:sgRNA). Non-targeting control sgRNA (sgCtrl) was used as a negative control. The nanoparticles were added to the cells at the appropriate dose of sgRNA and incubated with the cells for 48 h. The cell viability and luciferase expression were evaluated with the ONE-Glo + Tox Assay (Promega), normalized to untreated cells (N = 4 +/- standard deviation).

Kinetic assay of sgRNA and siRNA delivery. The kinetics of luciferase expression after silencing/editing by siRNA and sgRNA were determined in HeLa-Luc-Cas9 cells. For time points < 48h, ZNPs encapsulating sgRNA or siRNA were delivered to HeLa-Luc-Cas9 cells in 96-well plates at a density of 5K cells per well. After 0.5, 1, 2, 4, 11, 20, 30 and 44 h time point, the cell viability and luciferase expression were determined by the One-Glow + Tox assay. For longer time points, cells were treated in 6-well plates. Beginning at the 2 day time point, cells were aspirated, washed with 1X PBS, trypsinized in 200 μL trypsin and re-suspended in 1800 μL medium. 1 mL of each cell suspension was added to a fresh 6-well plate containing 1 mL DMEM (2 mL total) and returned to the incubator. Of the remaining cell suspension, 50 μL was transferred to a 96-well white-opaque plate (10 wells per sample). Cell viability was determined using the Cell-Titer Glo assay normalized to untreated cells, while relative luciferase expression was determined using the One-Glo assay and normalized against control (siCtrl or sgCtrl). Data was plotted as an average of 5 measurements +/- standard deviation.

Luciferase mRNA delivery *in vitro* assay. ZNPs with mRNA (Tri-Link Biotechnologies) were prepared using the *in vitro* nanoparticle formulation method outlined above. IGROV1 cells were seeded in white opaque 96-well tissue culture plates at a seeding density of 5 x 10^3 cells per well in 100 μL RPMI 1640 medium supplemented with 5% FBS, and allowed to attach overnight. After overnight incubation, an additional 100 μL medium was added to the wells. The ZAL:mRNA nanoparticles were prepared at ZAL:mRNA weight ratios of 20:1, 10:1, 7.5:1 and 5:1, and lipid mixture molar compositions of 50:38.5:n ZAL:cholesterol:PEG-lipid, where n = 5, 2, 1, and 0.5 at each weight ratio. The ZAL-mRNA nanoparticles were added to the cells at the appropriate mRNA dose and incubated for the indicated time (ranging from 6 h to 48 h), after which time cell viability and luciferase expression were evaluated with the ONE-Glo + Tox Assay (Promega) and normalized to untreated cells (N = 4 +/- standard deviation).

***In vitro* co-delivery of Cas9 mRNA and sgRNA.** ZNPs were evaluated in the co-delivery of Cas9 mRNA (Tri-Link biotechnologies) and single guide RNA (sgRNA) to luciferase expressing cancer cells. Cells were seeded at a density of 250,000 per well in 6-well plates and 2-mL DMEM. ZNPs were formulated using the *in vitro* formulation protocol. For co-delivery in a single particle, Cas9 mRNA and sgRNA were combined in acidic buffer together at pH 3 prior to the addition of ZAL lipid mix at the appropriate ZAL:total RNA weight ratio. Cells were incubated with ZNPs for 72 h prior to evaluation of editing by the surveyor assay. As a negative control, ZNPs with Cas9 only (unguided Cas9), sgLuc only, and Cas9 plus sgCtrl were added. sgRNA dose was fixed at 0.5 μg per well, while Cas9 mRNA dose was tuned from 0.5 μg (1:1) to 3 μg (6:1) per well. ZAL:total RNA ratio was fixed at 7.5:1. Staged co-delivery was carried out by the addition of Cas9 mRNA ZNPs followed by the addition of sgRNA ZNPs 24h later at a total ratio of

2:1 Cas9 mRNA to sgRNA. Following an additional 48h incubation time, cells were evaluated by gene editing by the surveyor assay.

Nucleic acid binding experiments. Nucleic acid binding was evaluated using the Ribogreen assay (Molecular Probes). In short, nanoparticles were prepared using the *in vitro* or *in vivo* formulation protocols. The nanoparticle formulations (5 μL) were added to a black 96-well opaque microplate (Corning). A standard curve of the appropriate nucleic acid was prepared in the same medium as the nanoparticles. Ribogreen reagent was diluted 1:1000 in 1 X PBS and 50 μL was added to each well via multichannel pipette. The mixture was stirred on an orbital mixer for 5 minutes, and the fluorescence of each well was read using a plate reader (λ_{Ex} 485 nm, λ_{Em} 535 nm). The amount of free nucleic acid was determined by fitting the signal from each nanoparticle sample to the nucleic acid standard curve, and the fraction bound determined by the following formula: Fraction nucleic acid bound = (total nucleic acid input-free nucleic acid)/ total nucleic acid input) (N = 3 or 4 +/- standard deviation).

***In vivo* nanoparticle formulations:** *In vivo* nanoparticle formulations were performed using the NanoAssemblr microfluidic mixing system (Precision Nanosystems). Lipids were dissolved in ethanol and nucleic acids were diluted in 10 mM citric acid-sodium citrate buffer pH 3. The lipid mixture and nucleic acid dilution were combined at a volumetric ratio of 3:1 nucleic acid:lipid mix at a total flow rate of 12 mL per minute, and a waste collection of 0.1 mL at the start and end of each formulation. The nanoparticles were dialyzed against 1 X PBS in Pur-A-Lyzer midi dialysis chambers (Sigma-Aldrich) for 1 hour per 200 μL volume in each chamber, and diluted in 1 X PBS to the appropriate nucleic acid concentration.

***In vivo* luciferase mRNA delivery:** All experiments were approved by the Institutional Animal Care & Use Committee (IACUC) of The University of Texas Southwestern Medical Center and were consistent with local, state and federal regulations as applicable. ZA3-Ep10 was formulated with *in vivo* formulation at 50 ZAL:38.5 cholesterol: 0.5, 1, or 2 PEG-lipid mole ratio in the lipid mix, and 7.5:1 ZAL:mRNA weight ratio. Mice were injected with ZAL-mRNA NPs at a dose of 1 mg/kg via tail vein injection or intraperitoneal injection. After 24 h and 48 h the luciferase expression was evaluated by live animal bioluminescence imaging Animals were anesthetized under isofluorane, and D-luciferin monosodium hydrate (GoldBio) substrate was injected subcutaneously in the neck scruff. After 10-12 minute incubation under anesthesia, the luciferase activity was imaged on an IVIS Lumina system (Perkin Elmer), and the images processed using Living Image analysis software (Perkin Elmer). *Ex vivo* imaging was performed on systemic organs after resection, and the tissue frozen on dry ice for *ex vivo* luciferase expression analysis.

Nanoparticle property characterization: Physical properties were measured using a Zetasizer Nano ZS (Malvern) with an He-Ne laser (λ = 632 nm). Particle sizes were measured by dynamic light scattering (DLS) (5 measurements, 3 runs x 10 seconds, automatic attenuator setting) by 173° back scattering. Zeta potential was measured in a folded capillary cell (Malvern) with samples diluted in PBS for ZAL NPs or citrate phosphate buffer pH 7.4 for CSAL NPs.

Surveyor Assay: Genomic DNA from transfected cells was isolated using QuickExtract DNA Extraction Solution (Thermo Fisher Scientific) according to the manufacturer's protocol. Then the target region was amplified by PCR, and the PCR products were gel purified on an agarose gel (QIAquick Gel Extraction Kit, QIAgen). Surveyor assay was performed using Surveyor Mutation Detection Kit (IDT): the PCR products were first hybridized, then half of the products were cut with Nuclease S; both the uncut and cut DNA were then run on the 4-20% polyacrylamide gel (Biorad). The gels were stained with SYBR Gold Nucleic Acid Gel Stain buffer (diluted 1:10000 in TBE buffer, Thermo Fisher Scientific) and imaged by UV light.

Western blot: The cells were lysed in cold RIPA buffer (Thermo Scientific), the lysate cleared by centrifugation and total protein in the supernatant quantified by the BCA assay (Pierce). 50 μg total protein was loaded on 4–20% precast polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad).

The membrane was blocked in 5% nonfat milk for 1 hour at RT, and then incubated with primary antibody at 4°C overnight (Cas9 antibody, 1:1000, Cell Signaling, 14697S; beta-actin antibody, 1:2000, Cell Signaling, 4970). Secondary antibodies were applied at RT for 1 hour (anti-rabbit IgG, HRP-linked antibody, Cell Signaling, 7074, anti-mouse IgG, HRP-linked antibody, Cell Signaling, 7076), and then the membrane was developed and detected on X-ray film.

Real-time RT-qPCR. Cells were transfected with Cas9 mRNA for the indicated time point in a 6-well plate and 0.5 μg/mL mRNA for the indicated time point. Total RNA was extracted using the TRIzol reagent according to the manufacturer's protocol. The RNA was reverse transcribed using the iScript Reverse Transcription kit (BioRad) and the real-time qPCR was run on a Bio-Rad C1000 Touch Thermal Cycler (CFX384 Real-time System). Each reaction was made with iTaq Universal SYBR Green 2X Supermix (Bio-Rad). The qPCR program is as follows:

1) 95 ℃ for 3min
2) 95 ℃ 10s and 55 ℃ 30s for 40 cycles
3) 95 ℃ 10s
4) 65 ℃ 5s
5) 95 ℃ 5s

Human β-actin was used as a control and mRNA levels were normalized to fold actin and plotted as an average of two independent experiments.

***In vivo* delivery of Cas9 mRNA and sgLoxP.** ZA3-Ep10 ZNPs encapsulating Cas9 mRNA and sgLoxp were prepared according to the *in vivo* nanoparticle formulation protocol using the Nanoassemblr microfluidic mixing device. The lipid mix contained 50 ZA3-Ep10: 38.5 cholesterol: 0.5 PEG-lipid molar ratios, and the particles were formulated at a 7.5:1 ZAL:total RNA weight ratio. The Cas9 mRNA: sgLoxP weight ratio was maintained at 4:1. Rosa 26-LSL-tdTomato mice were injected at 5 mg/kg total RNA (4 mg/kg mRNA, 1 mg/kg sgRNA) via tail vein injection and monitored for 1 week. After which they were sacrificed and the major organs imaged using the IVIS Lumina system for fluorescence expression (dsRed filter set) compared to an uninjected Rosa 26-LSL-tdTomato mouse. A liver specific Cre recombinase adeno-associated virus (Cre-AAV8) injected intravenously via tail vein injection (4 days) was used as a positive control.

Tissue sectioning. Tissue were fixed in 4% paraformaldehyde (PFA) at RT for 2 hours, then changed in 30% sucrose (in PBS) at 4 ℃ overnight. Then the tissues were embedded in Cryo-gel (Leica Biosystems), and frozen in dry ice. The blocks were sectioned using Cryostat machine (Leica Biosystems) at 8 μm thickness. The sections were air-dried and incubated in 0.25% Triton X-100 (Biorad) 5% FBS in PBS for 1h at RT. Then the slides were mounted with DAPI (Vector Laboratories) and covered.

Primary hepatocytes isolation. Primary hepatocytes were isolated by two-step collagenase perfusion. Liver perfusion medium (Thermo Fisher Scientific, 17701038), liver digest medium (Thermo Fisher Scientific, 17703034) and Hepatocytes wash medium (Thermo Fisher Scientific, 17704024) were used.

Flow Cytometry. For detection of Tomato positive populations, primary hepatocytes (2x106/mL) were isolated and stained with DAPI (Roche, 2ug/mL) for dead cell exclusion. Cells were analyzed with BD FACSAria Fusion machine (BD Biosciences). Tomato positive cells were counted in DAPI negative (live cell) populations.

Statistical analysis. Statistical analysis was performed using a Student's t-test in GraphPad Prism.

SUPPORTING INFORMATION FIGURES

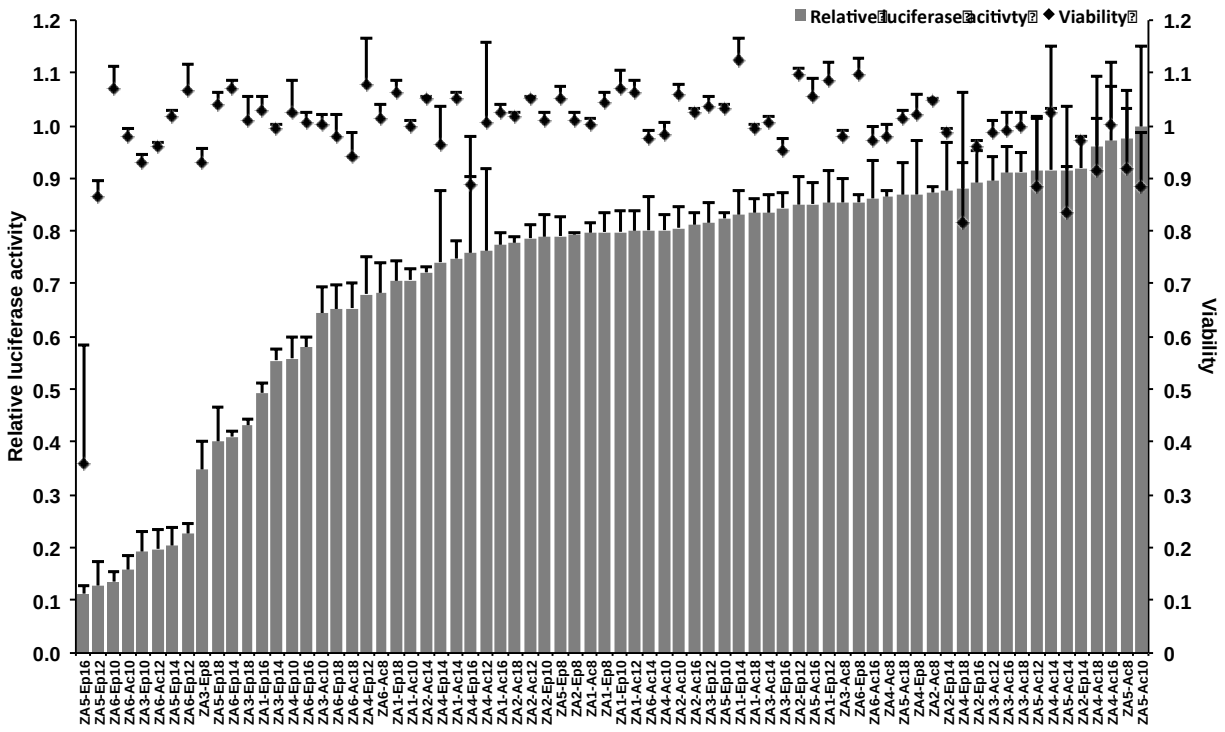


Figure S5. siRNA delivery to HeLa-Luc cells by the 72-member ZAL library. Cells were treated with 34 nM siRNA for 24 h and cell viability (dots) and relative luciferase activity (bars) were determined by normalizing to untreated cells.

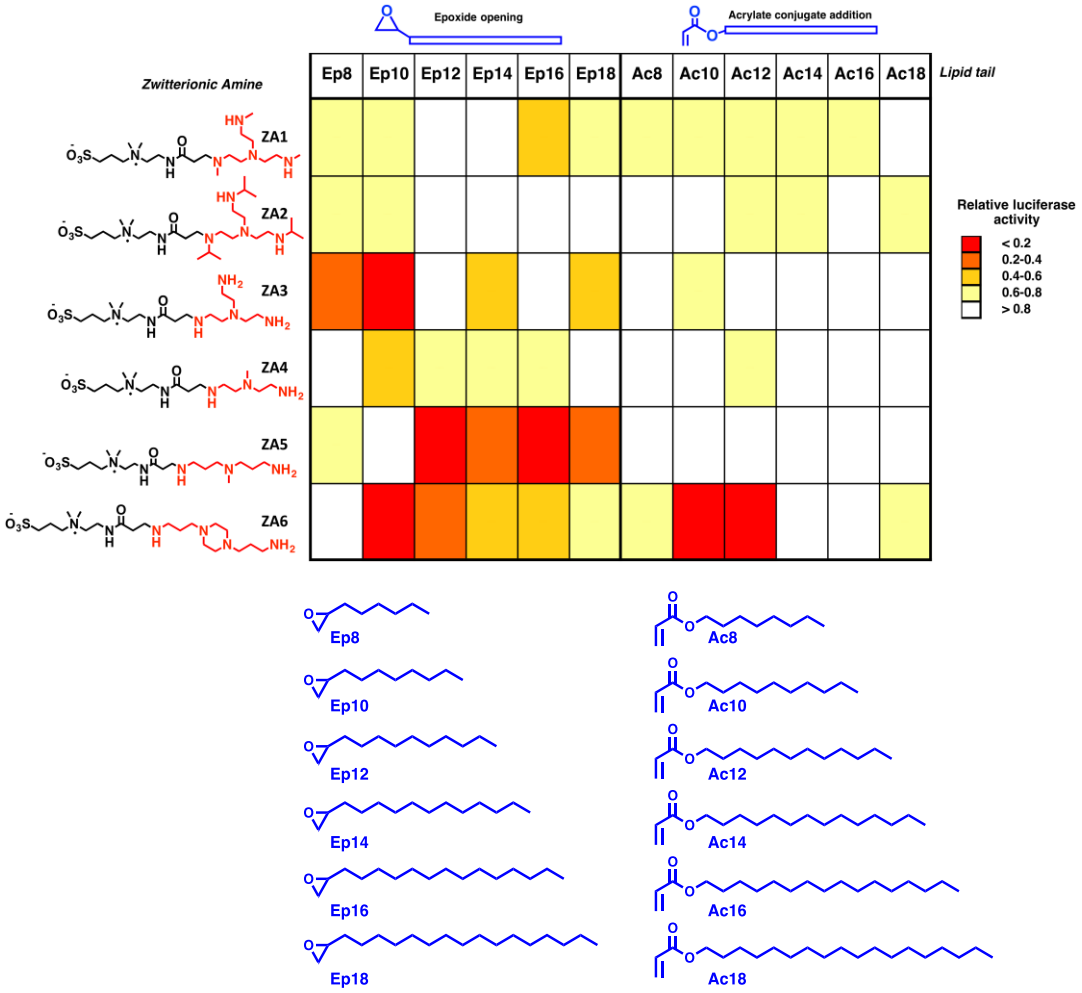


Figure S6. The heat map of siRNA delivery of the ZNPs to HeLa-Luc cells reveals structure-activity relationships within the library. Epoxide derived ZALs were generally much more potent than acrylate derived ZALs, while key core amines ZA3, ZA5, and ZA6 showed potent delivery.

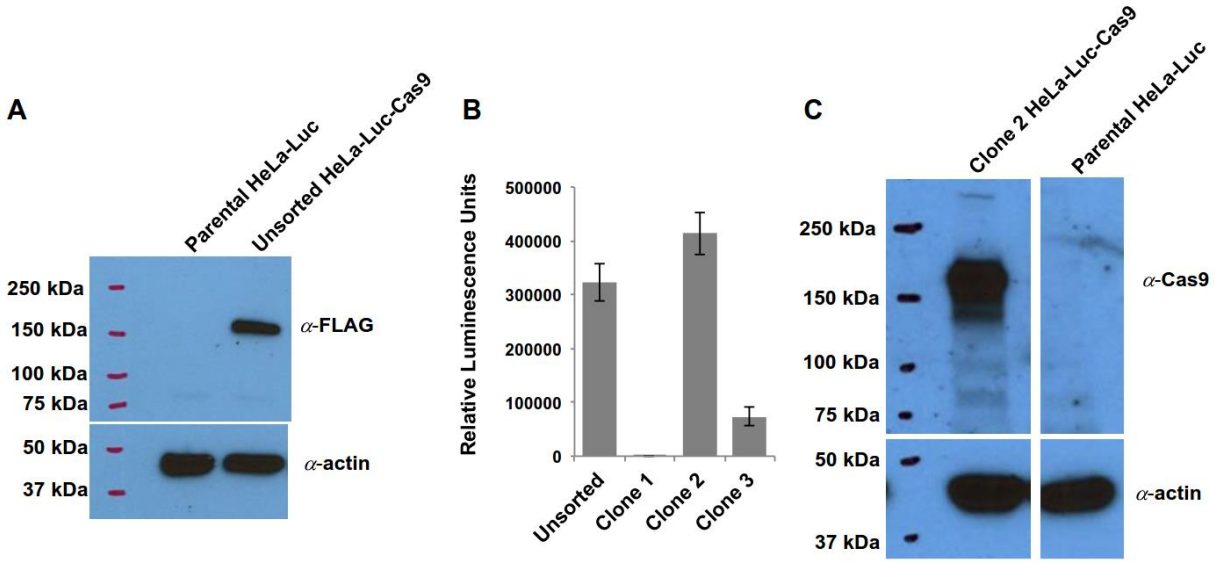


Figure S7. Cas9 expression was validated in HeLa-Luc-Cas9 cells by western blot. **(A)** Blotting with α-FLAG antibody in the pool of cells after Blasticidin S selection. **(B)** Luciferase expression of single cell clones as evaluated by the One-Glo assay (5,000 cells, 48h growth). **(C)** Cas9 expression of single cell clone 2 of HeLa-Luc-Cas9 blotted with α-Cas9.

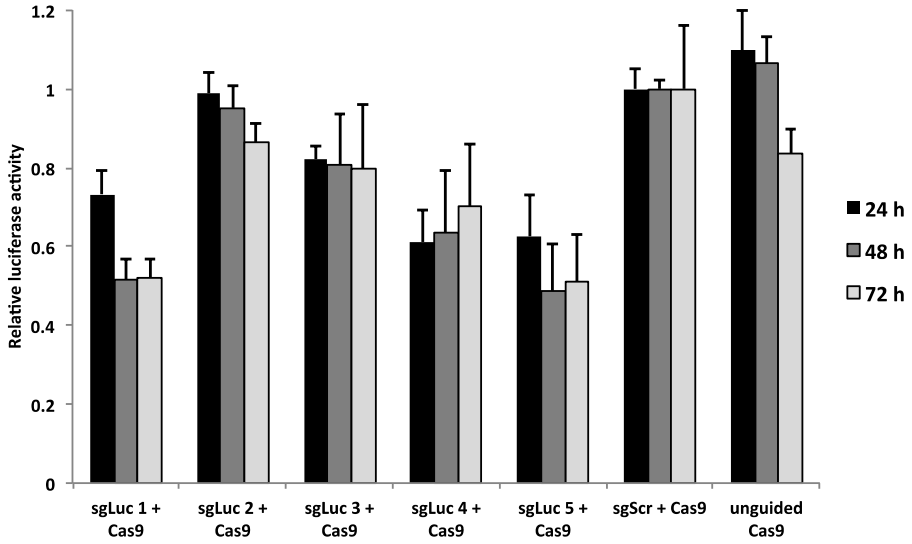


Figure S8. The evaluation of panel of single guide RNAs against luciferase using commercial reagent (LF3000) transfection of plasmid DNA encoding sgRNA and Cas9 protein reveals sgLuc5 as the most potent sgRNA sequence for silencing luciferase in unsorted HeLa-Luc cells. Values are normalized to non-targeting sgRNA control and plotted as mean +/- standard deviation (N = 4).

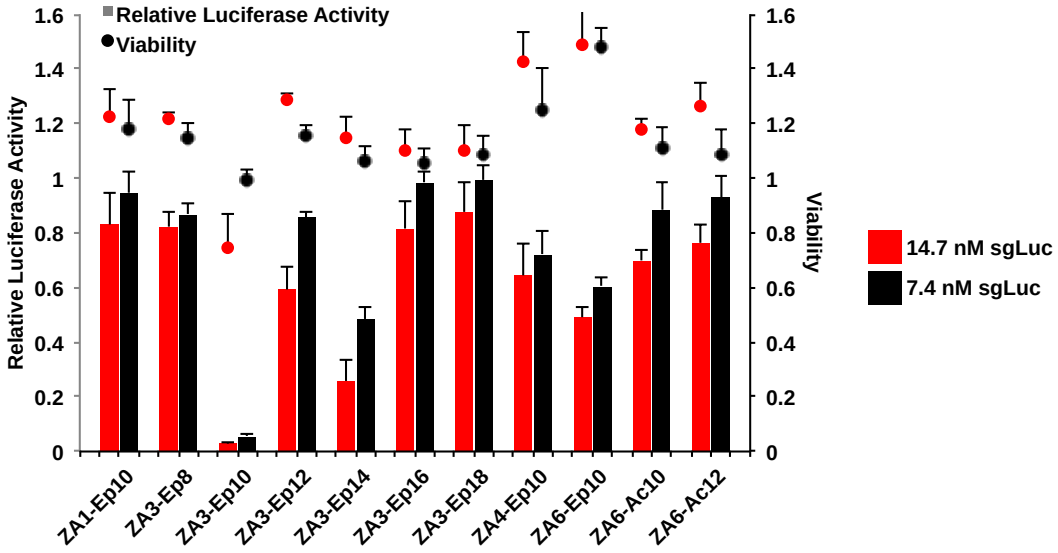


Figure S9. Lead ZALs identified from the siRNA screen were evaluated for sgRNA delivery to HeLa-Luc-Cas9 cells. ZNPs were formulated at 50:38.5:1 (ZAL:cholesterol:PEG-lipid molar ratios) in the lipid mix and 20:1 ZAL:sgRNA weight ratio. sgRNA was administered at both 14.7 nM and 7.4 nM for 48 h. ZA3-Ep10 emerged as the most highly potent (>95% luciferase silencing). Viability (dots) and relative luciferase activity (bars) were determined relative to untreated cells (N = 4 +/- standard deviation).

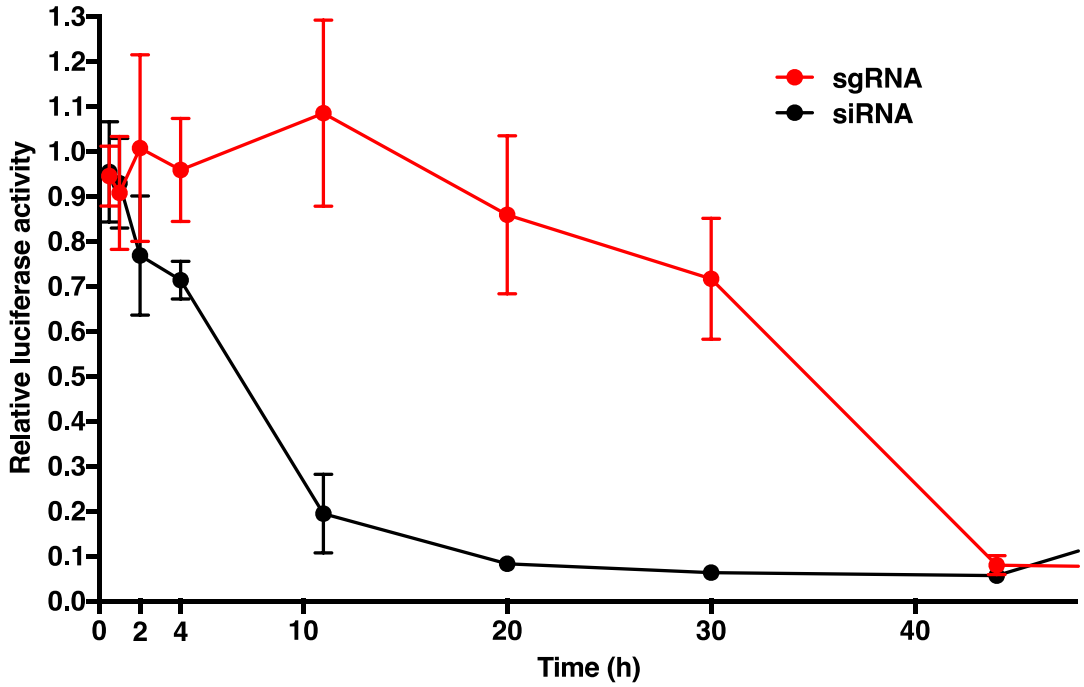


Figure S10. Magnification of the early time points of the kinetic curve of luciferase silencing comparing sgRNA versus siRNA by ZA3-Ep10 ZNPs shows that siRNA silencing is much faster than sgRNA editing.

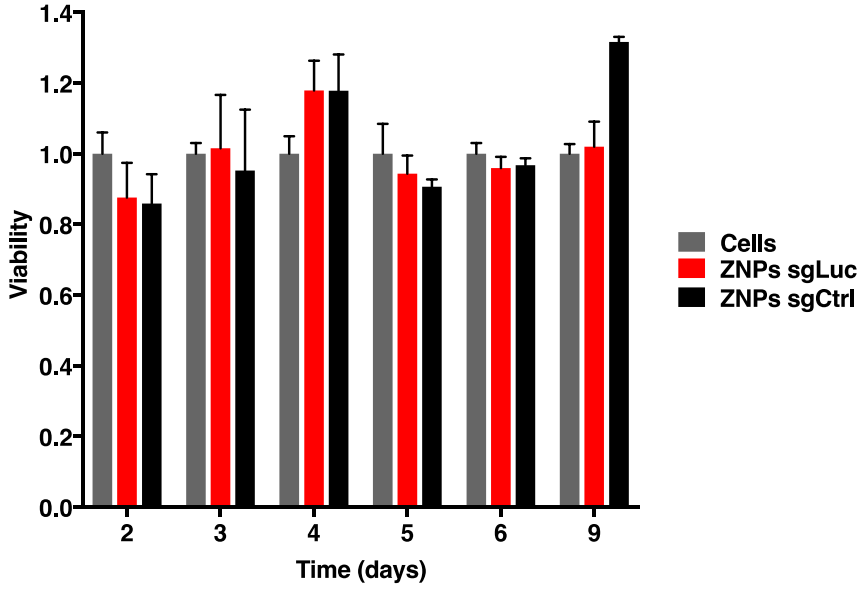


Figure S11. The relative viability of ZNP edited HeLa-Luc-Cas9 cells (sgLuc) versus unedited cells (sgCtrl) shows similar growth rates by the Cell-Titer Glo assay when normalized to untreated cells (N = 5 +/- S.E.M.)

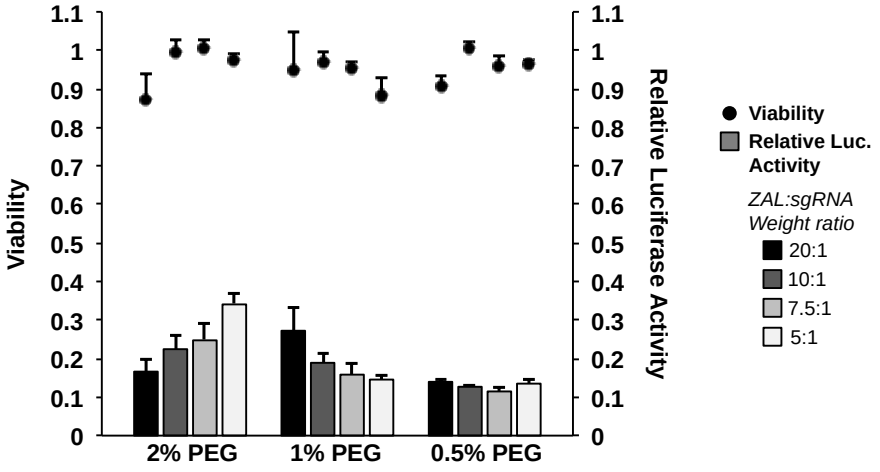


Figure S12. The optimization of ZA3-Ep10 ZNPs for sgRNA delivery was explored by tuning the PEG content of the formulation (2%, 1%, and 0.5%) and the ZAL:sgRNA weight ratio (20:1, 10:1, 7.5:1 5:1). All formulations were potent for sgLuc delivery at 7.4 nM, 48 h incubation, while 7.5:1 weight ratio and 0.5% PEG showed the best luciferase editing.

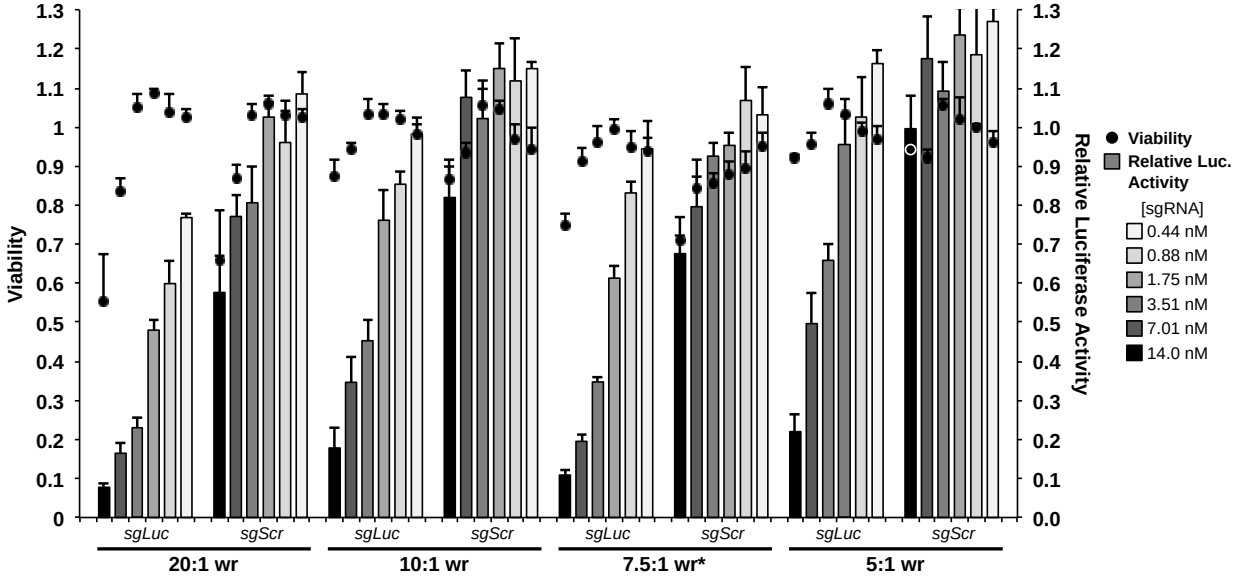


Figure S13. ZA3-Ep10 ZNPs show dose responsive, sequence-specific editing of luciferase. The effect of weight ratio on sgRNA delivery (50:38.5:2 ZAL:cholesterol:PEG lipid) for sgRNA delivery to HeLa-Luc Cas9 cells. 20:1, 10:1 and 5:1 weight ratio (wr) ZNPs were incubated for 48 h while 7.5:1 wr ZNPs were incubated for 66h. Cell viability and relative luciferase activity were normalized to untreated cells (N = 4 +/- standard deviation).

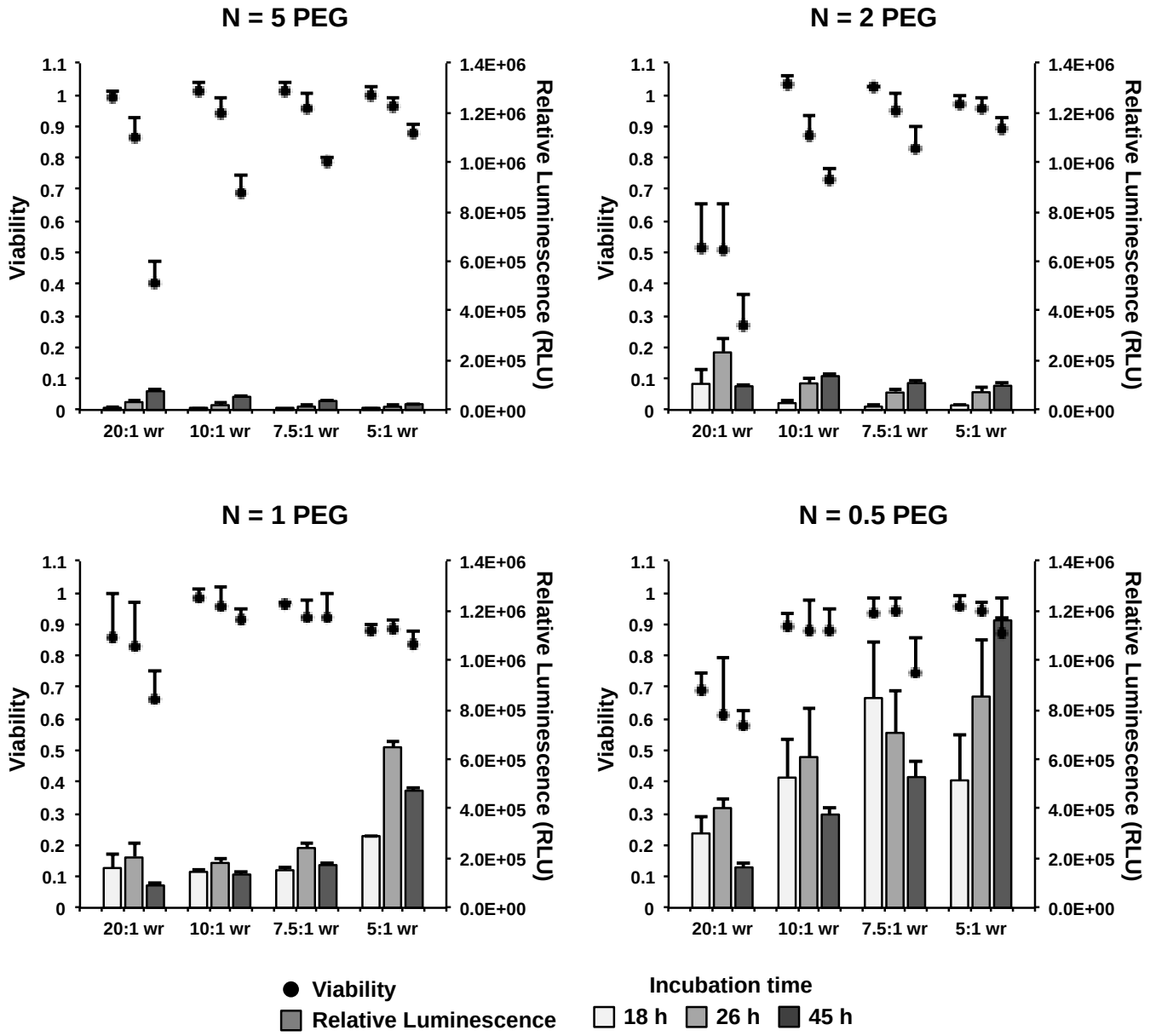


Figure S14. The optimization of the ZA3-Ep10 ZNPs for mRNA delivery was performed in IGROV1 cells. The weight ratio of the ZAL:mRNA was set at 20:1, 10:1, 7.5:1 and 5:1. The lipid mix was prepared with a relative molar ratio of 50:38.5:n, ZAL:cholesterol:PEG-lipid, where n = 5, 2, 1 or 0.5. Cells were treated in 96-well plates with 100 ng mRNA and incubated for the indicted time (18 h light gray, 26 h gray, 45 h dark gray) prior to evaluation of cell viability (dots) and luciferase expression (bars) using the One-Glo + Tox assay. Cell viability was determined compared to untreated cells and luminescence was normalized to viability to determine relative luminescence. Values are plotted as a mean +/- standard deviation, N = 4.

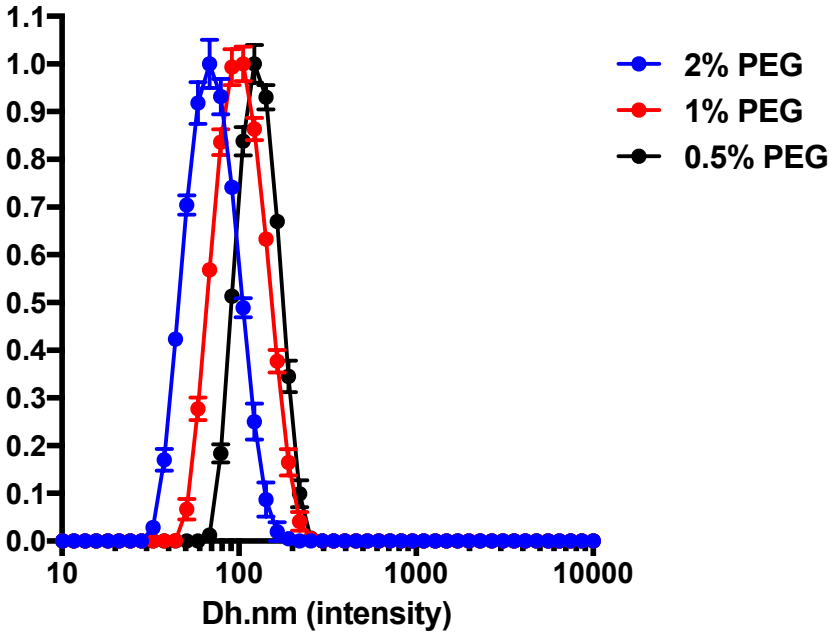


Figure S15. The effect of PEG lipid composition of ZA3-Ep10 Luc mRNA NPs formulated for *in vivo* assays. The ZAL:cholesterol ratio was fixed at 50:38.5 molar ratio while PEG-lipid was included at the indicated percentage. As expected increased PEG leads to smaller particle size, but poorer expression of mRNA.

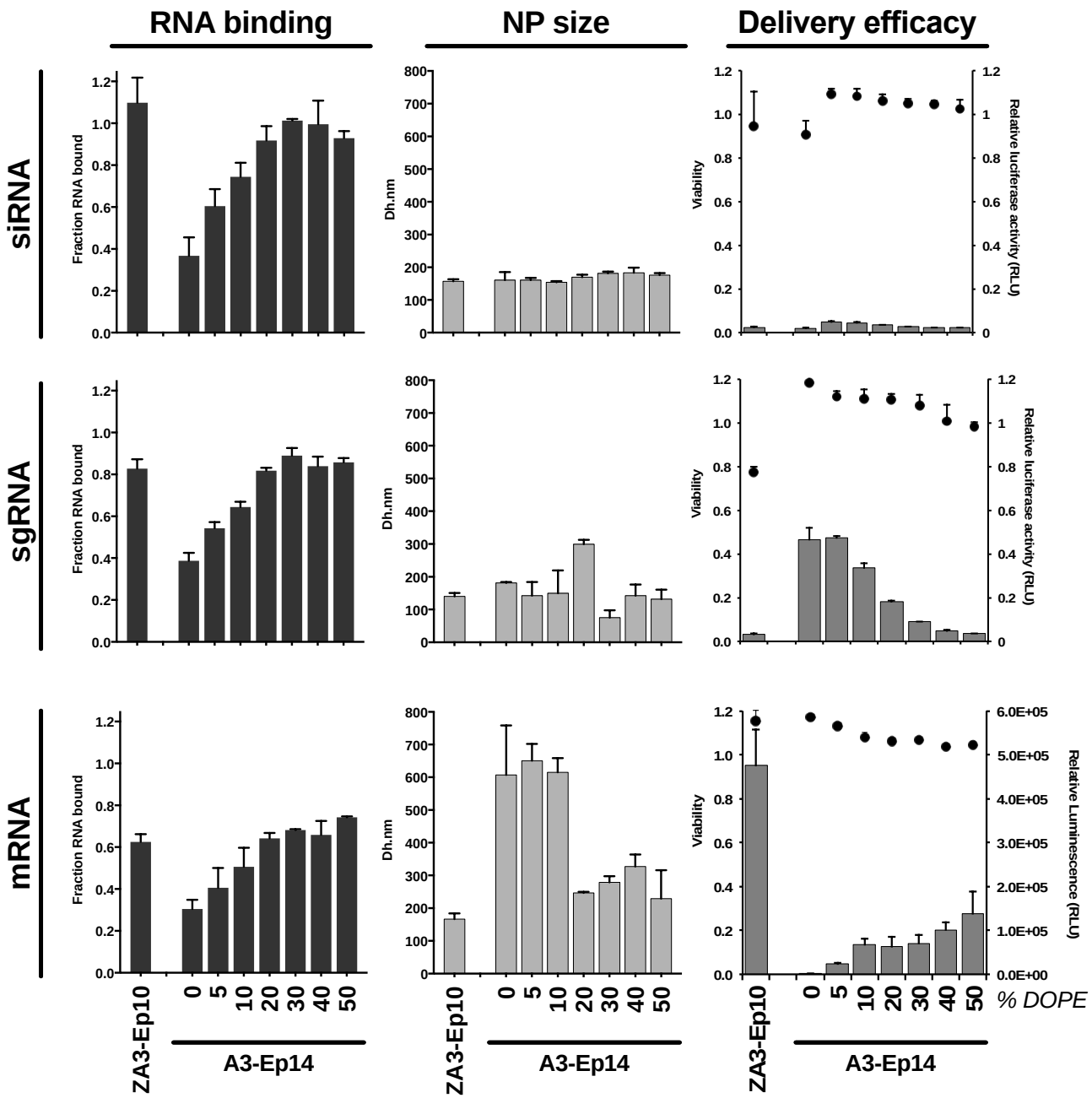


Figure S16. Comparing the RNA encapsulation, nanoparticle size, and delivery efficacy of ZA3-Ep10 and a cationic structural analogue (A3-Ep14, also referred to as C14-110 in the literature[4]), which is known to deliver small RNA. The ZNP or LNP formulation was fixed at 7.5:1 weight ratio ZAL or Cationic analogue to RNA. The lipid mixture for the NPs was 50:38.5:0.5 ZAL or cationic analogue: cholesterol: PEG-lipid, while for the A3-Ep14 NPs the zwitterionic phospholipid was titrated from 0 to 50% in the lipid mix. The nanoparticles were formulated by manual mixing using the *in vitro* formulation protocol. RNA binding was determined by the Ribogreen assay (N = 3 +/- standard deviation), while nanoparticle size was determined by dynamic light scattering (N = 3 +/- standard deviation). Luciferase silencing or editing of siLuc and sgLuc NPs was assayed in HeLa-Luc-Cas9 cells (7.35 nM sgRNA, 17.9 nM siRNA), while luciferase expression by Luc mRNA NPs was evaluated in IGROV1 cells (0.77 nM mRNA). Cells were assays after 40 h incubation time by the One-Glo + Tox assay and plotted with viability (dots) and luciferase expression (bars) as mean +/- standard deviation (N = 4).

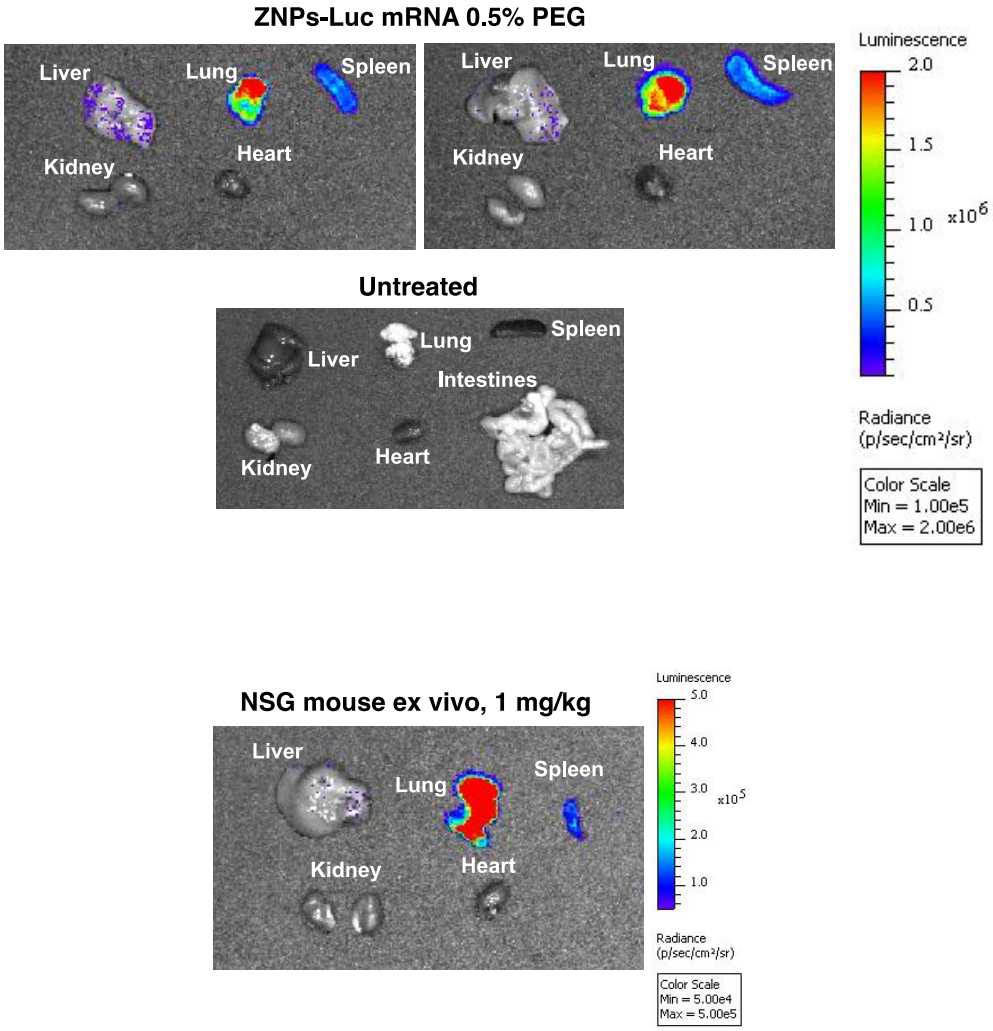


Figure S17. Bioluminescence imaging shows that *in vivo* expression of luciferase after Luc-mRNA administration by i.v. injection correlates with *in vitro* activity. Mice were injected with 1 mg/kg Luc mRNA and imaged 24 h after treatment. An untreated mouse was used as a negative control. The top right panel shows the *ex vivo* expression of the animal shown in Figure 3E.

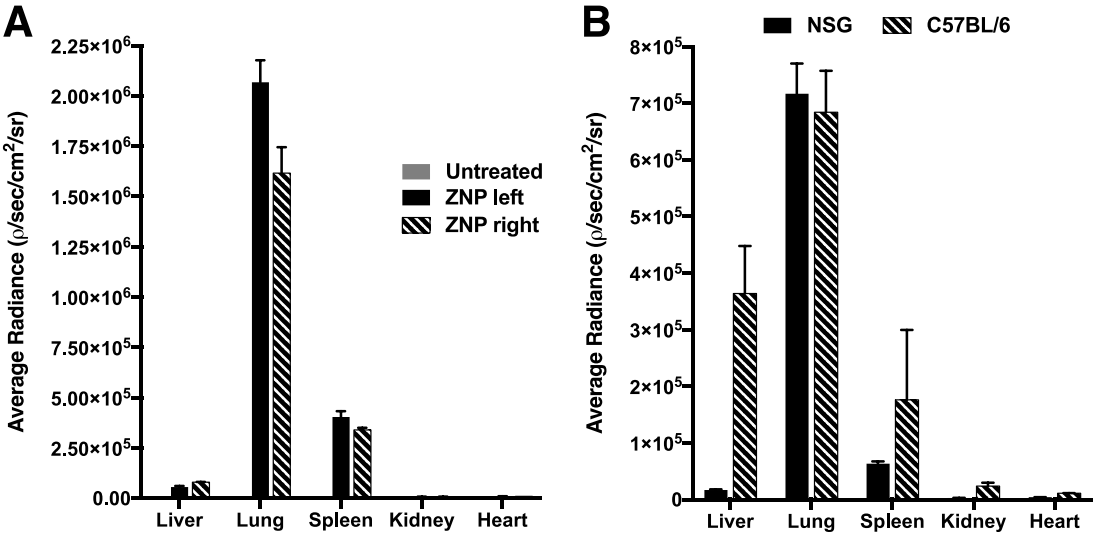


Figure S18. Quantitation of the ex vivo images by ROI analysis. **(A)** Quantitation of the athymic nude mice images shown in Figure S17 (top) and **(B)** quantitation images of the images in Figure S17 (bottom, NSG) and Figure 3F (C57BL/6). A minimum of 5 ROIs per organ was measured and plotted as mean +/- S.E.M.

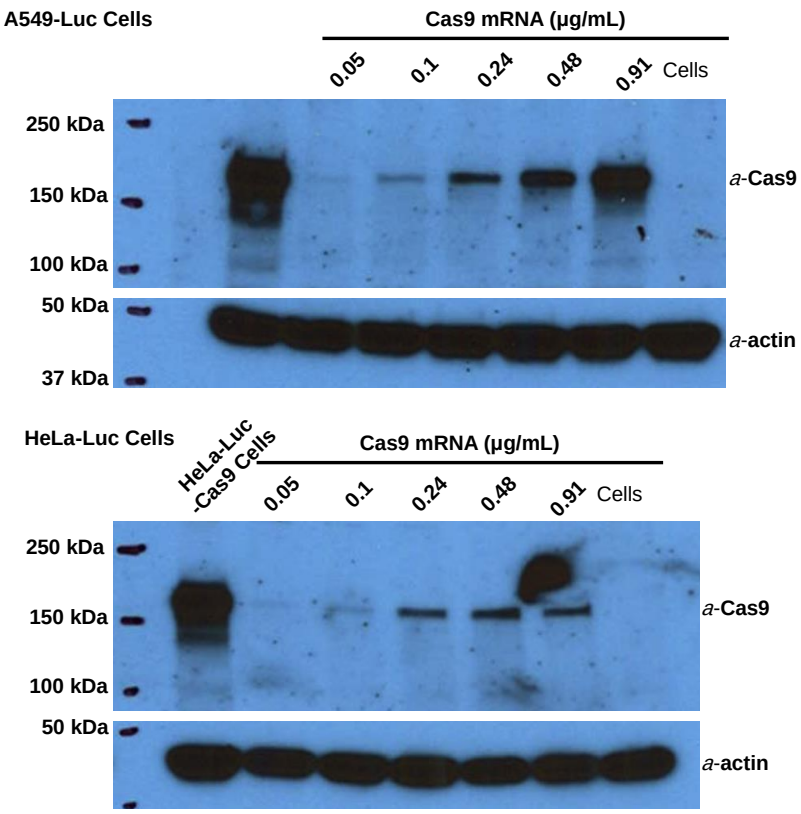


Figure S19. ZA3-EP10 Cas9 mRNA ZNPs enable dose responsive expression of Cas9 in both A549-Luc cells and HeLa-Luc cells with detectable Cas9 protein at 0.05 μg/mL mRNA concentration. Stable HeLa-Luc-Cas9 cells were used as a positive control while untreated cells did not show any bands.

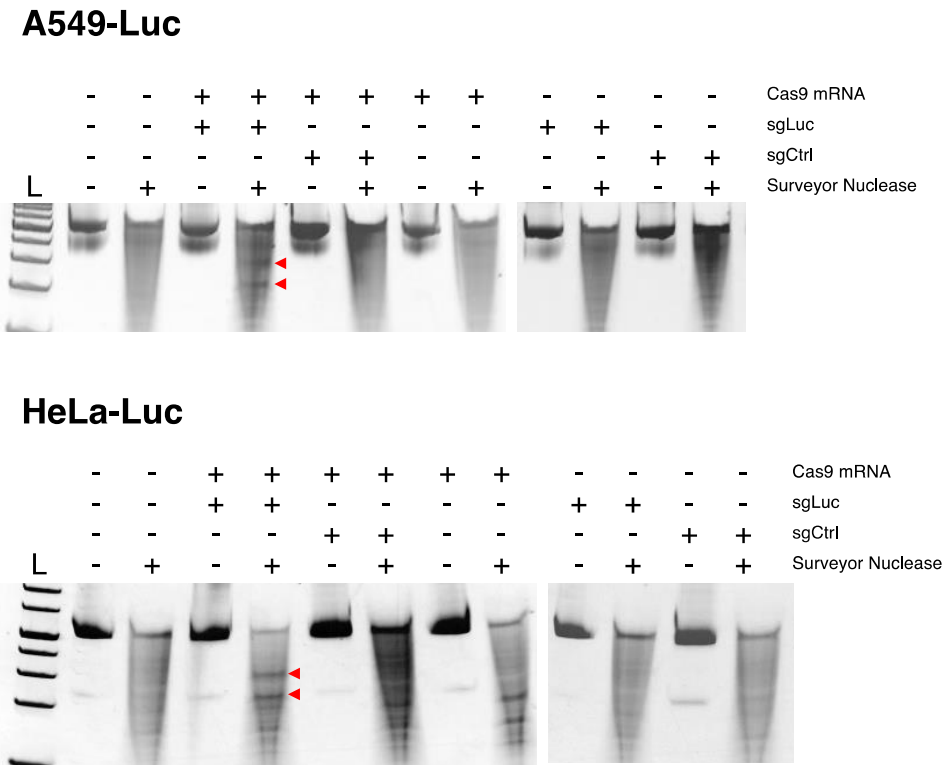


Figure S20. Co-delivery of Cas9 mRNA and sgLuc leads to editing in staged delivery at 2 μg per well Cas9 mRNA and 1 μg sgLuc in a 6-well plate in both A549-Luc and HeLa-Luc. Meanwhile, unguided

Cas9, Cas9-sgCtrl, or sgLuc alone do not show edited bands. The expected genomic DNA amplicon was 510 bp while the expected cut bands indicating editing are 233 bp and 277 bp (red arrows).

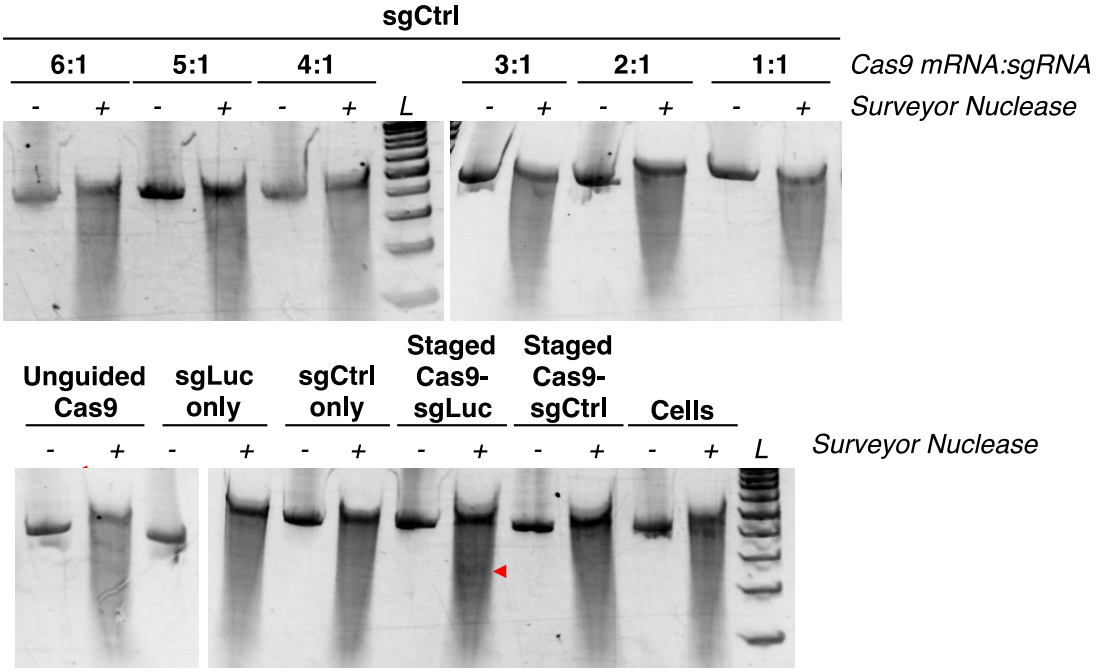


Figure S21. Control ZNPs (Cas9+sgCtrl, unguided Cas9, sgLuc only and sgCtrl only) did not show editing of luciferase target in A549-Luc cells. Staged co-delivery shows editing with sgLuc under similar conditons with 2:1 Cas9 mRNA:sgLuc wr.

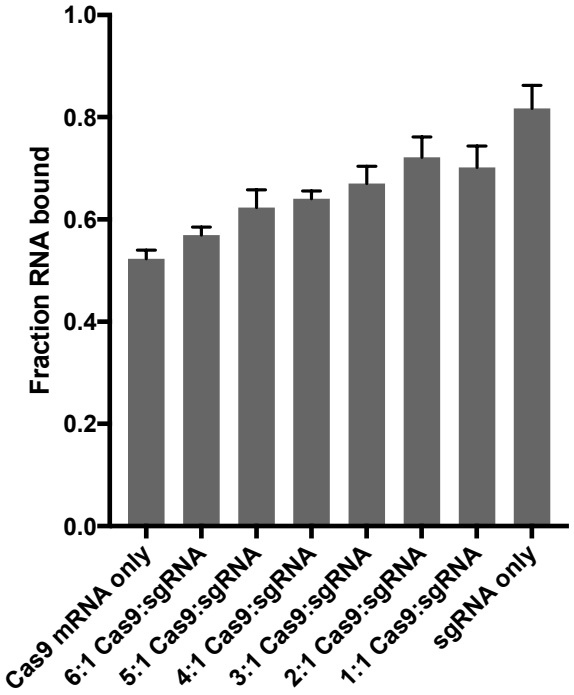


Figure S22. The encapsulation of Cas9 mRNA and sgRNA in co-delivery ZNPs. ZAL: total RNA was fixed at 7.5:1, with a lipid mixture of 50:38.5:0.5 ZA3-Ep10: cholesterol: PEG-lipid. Data are plotted as mean +/- standard deviation (N = 4).

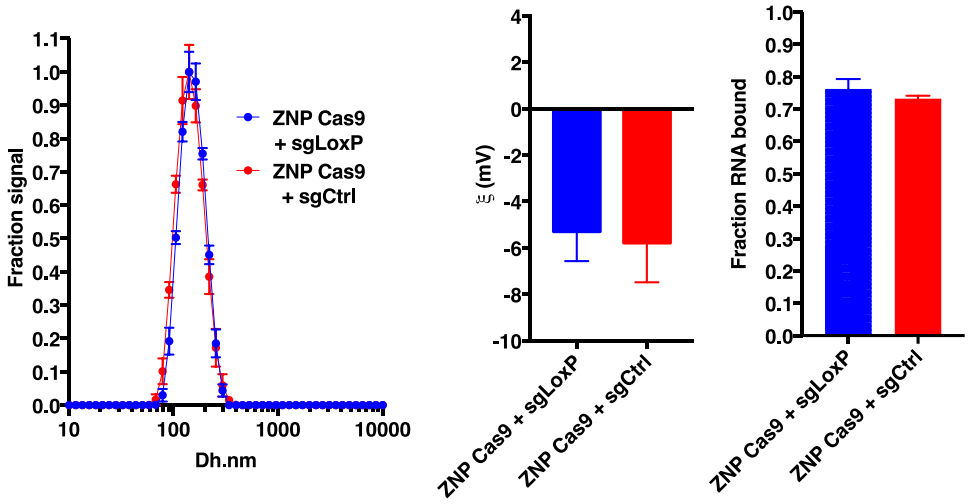


Figure S23. Particle properties of *in vivo* administered ZNPs encapsulating Cas9 mRNA and sgRNA. For size and zeta potential measurements, N = 5 for RNA encapsulation N = 4. Data are plotted as mean +/- standard deviation.

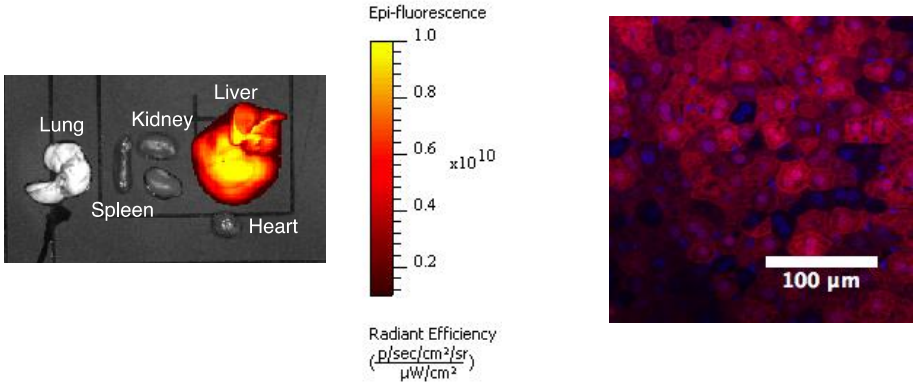


Figure S24. The Cre recombinase AAV positive control demonstrates expression of tdTomato in liver ex vivo at the whole organ level and in cells from tissue sections.

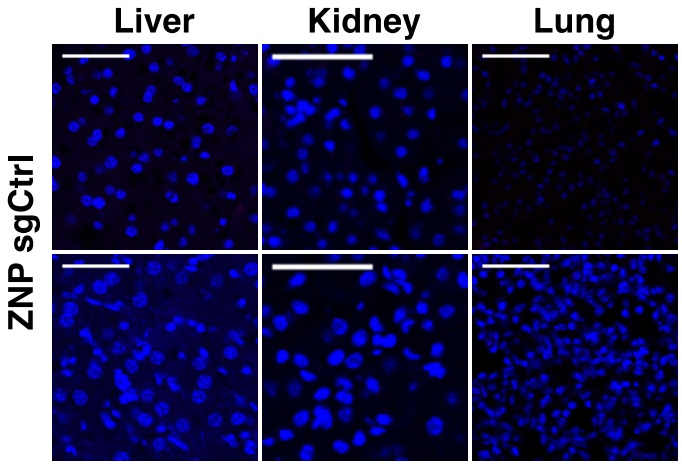


Figure S25. Delivery of ZA3-Ep10 ZNPs encapsulating Cas9 mRNA and sgCtrl does not show any tdTomato positive cells in sectioned tissue slides.

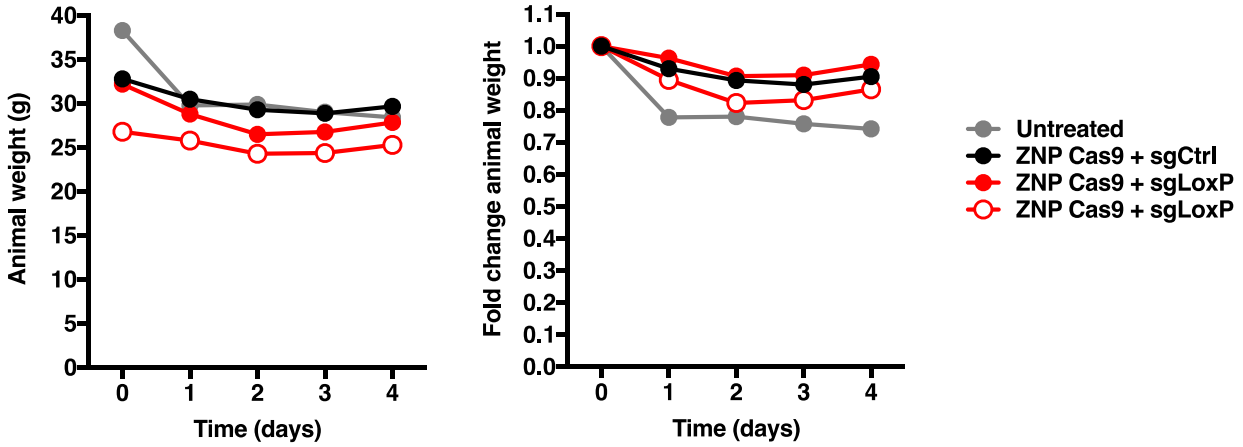


Figure S26. Measurement of animal body weight after systemic administration of ZA3-Ep10 ZNPs encapsulating Cas9 mRNA and sgRNA at 5 mg/kg total RNA dose.

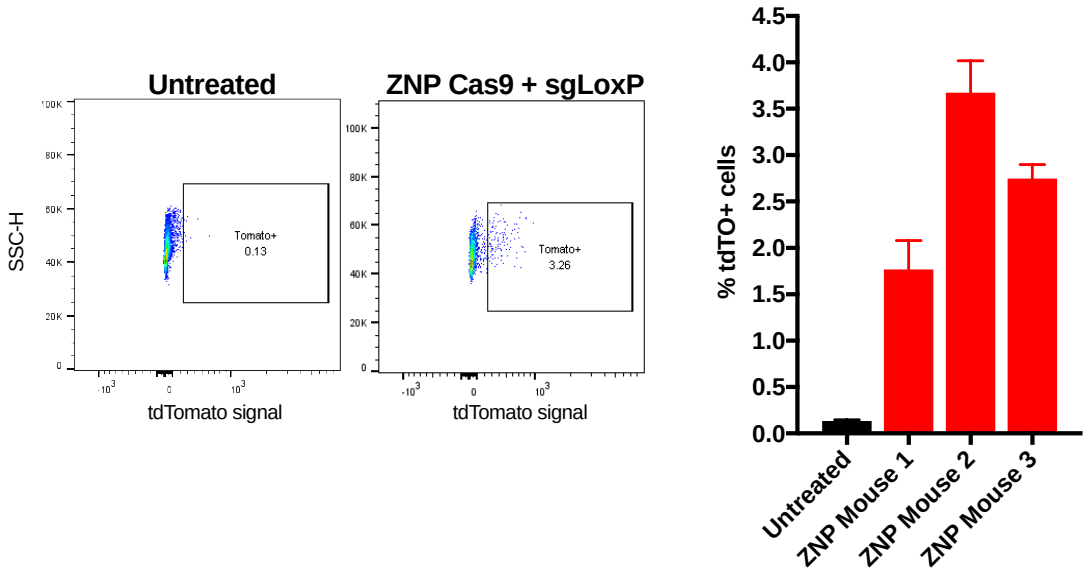


Figure S27. Quantification of tdTomato positive hepatocytes in animals treated with ZNPs as determined by flow cytometry of isolated primary hepatocytes. The left panel shows representative plots of samples from an untreated LSL-tdTO mouse and a ZNP-Cas9 mRNA-sgLoxP treated mouse. Mouse 1 and mouse 2 were treated at 2 mg/kg total RNA 2 times on consecutive days, while mouse 3 received a single dose at 5 mg/kg total RNA and all animals were harvested ~ 1 week after ZNP administration. Each sample was run four times and values are plotted as mean +/- standard deviation.

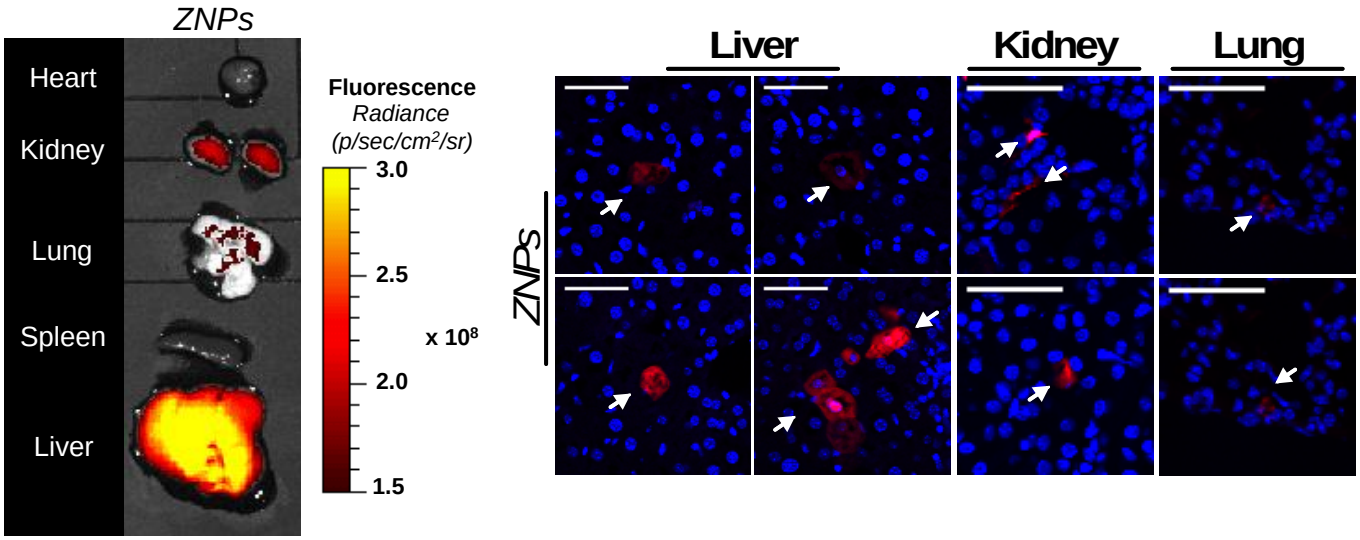


Figure S28. A ZNP treated tdTomato mouse shows significant fluorescent signal in the liver and kidneys 2 months after editing by ZA3-Ep10 ZNPs encapsulating Cas9 mRNA and sgLoxP (5 mg/kg).

REFERENCES

[1] K. Zhou, L. H. Nguyen, J. B. Miller, Y. Yan, P. Kos, H. Xiong, L. Li, J. Hao, J. T. Minnig, H. Zhu, D. J. Siegwart, *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 520-525.
[2] F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott, F. Zhang, *Nat. Protoc.* **2013**, *8*, 2281-2308.
[3] J. Hao, P. Kos, K. Zhou, J. B. Miller, L. Xue, Y. Yan, H. Xiong, S. Elkassih, D. J. Siegwart, *J. Am. Chem. Soc.* **2015**, *137*, 9206-9209.
[4] K. Love, K. Mahon, C. Levins, K. Whitehead, W. Querbes, J. Dorkin, J. Qin, W. Cantley, L. Qin, T. Racie, M. Frank-Kamenetsky, K. Yip, R. Alvarez, D. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D. Anderson, *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1864-1869.