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Supplemental Information

mRNA Delivery for Therapeutic Anti-HER2 Antibody Expression *In Vivo*

Yulia Rybakova, Piotr S. Kowalski, Yuxuan Huang, John T. Gonzalez, Michael W. Heartlein, Frank DeRosa, Derfogail Delcassian, and Daniel G. Anderson

SUPPLEMENTAL MATERIALS

Supplemental Figure 1. *Full trastuzumab construct used in the study.*

human immunoglobulin light chain kappa signal peptide

protein coding sequence without signal peptide

Trastuzumab heavy chain mRNA

GGACAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCG
GGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTG
CCAAGAGTGACTCACCGTCCTTGACACGATGAGGGCTTGGATCTTCTTTCTGCTCTG
CCTGGCCGGGCGCGCCTTGGCCGAAGTTCAACTGGTAGAGAGTGGAGGTGGGCTTG
TGCAACCAGGCGGATCCTTGCGACTGTCCTGCGCCGCTTCAGGCTTCAACATCAAGG
ACACCTACATCCATTGGGTCCGCCAGGCACCAGGAAAAGGTCTTGAATGGGTGGCC
AGAATCTACCCTACTAACGGTTACACCAGATATGCAGACTCCGTTAAGGGGCGATTT
ACCATTTCAGCAGACACCTCTAAGAACACCGCTTACCTGCAGATGAACTCACTTCGA
GCTGAGGACACCGCCGTTTACTATTGCAGCAGATGGGGCGGTGACGGCTTCTACGCT
ATGGATTACTGGGGACAGGGGACTTTGGTAACTGTGAGTAGTGCATCTACAAAGGG
GCCAAGCGTGTTCCCACTTGCCCCATCTTCTAAAAGCACCTCAGGAGGGACTGCAGC
CTTGGGTTGCTTGGTTAAAGATTATTTTCCAGAGCCTGTAACTGTATCCTGGAATAGT
GGGGCCCTCACAAGCGGAGTACATACTTTCCCTGCAGTATTGCAGTCTAGTGGACTC
TACTCTCTCAGCAGTGTAGTGACCGTACCTTCCAGTTCACTTGGAACACAGACCTAT
ATTTGCAATGTGAATCATAAGCCATCTAATACTAAAGTGGATAAGAAAGTGGAGCC
TAAATCTTGTGACAAGACTCATACATGCCCTCCCTGCCCTGCCCCTGAACTGTTGGG
AGGGCCCTCTGTATTTCTCTTCCCCCCTAAACCAAAGGACACCCTGATGATCAGTCG
AACTCCTGAGGTGACTTGTGTGGTTGTTGACGTGTCACATGAGGATCCCGAAGTGAA
ATTCAACTGGTACGTCGATGGAGTAGAGGTACACAATGCAAAGACAAAACCTAGGG
AGGAACAGTATAATTCTACCTATAGAGTGGTGTCTGTTCTCACAGTTCTCCATCAAG
ACTGGTTGAACGGTAAAGAATATAAATGCAAAGTCTCCAATAAGGCTTTGCCCGCTC
CCATTGAAAAAACAATCAGTAAAGCCAAAGGCCAGCCACGCGAACCACAGGTCTAC
ACCCTTCCACCATCTCGCGAAGAAATGACTAAGAATCAAGTGTCACTCACATGCCTC
GTCAAGGGCTTTTACCCTTCTGATATTGCAGTTGAATGGGAGTCTAACGGGCAGCCC
GAGAATAATTACAAGACTACCCCCCCCGTCCTTGACTCTGATGGCAGCTTTTTCCTGT
ACTCAAAATTGACTGTGGACAAGTCTCGATGGCAACAGGGTAATGTTTTCTCCTGTA
GCGTAATGCACGAGGCTCTTCATAACCATTATACCCAAAAATCTCTTTCATTGTCCCC
TGGAAAATGACGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGG
AAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCAAGC
T

Trastuzumab light chain mRNA

GGACAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCG
GGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTG
CCAAGAGTGACTCACCGTCCTTGACACGATGAGGGCTTGGATCTTCTTTCTGCTCTG
CCTGGCCGGGCGCGCCTTGGCCGATATTCAGATGACTCAGAGCCCCAGCAGCCTGTC
TGCTAGCGTTGGGGATAGAGTCACTATAACATGTCGGGCTTCCCAGGATGTGAATAC
TGCTGTCGCTTGGTATCAACAGAAGCCCGGCAAGGCACCAAAACTGCTGATATATA
GTGCCTCTTTCCTTTACTCCGGGGTTCCCAGTCGATTCTCTGGAAGCCGCAGCGGCA
CTGATTTCACACTTACTATAAGTAGTCTGCAACCTGAGGACTTTGCTACATACTACTG
CCAGCAGCACTATACAACACCCCCAACTTTCGGGCAAGGCACTAAGGTAGAAATTA

AAAGGACCGTTGCTGCTCCATCCGTCTTTATTTTTCCACCATCTGATGAACAGTTGAA
GAGCGGAACAGCAAGCGTCGTTTGTCTCCTGAACAATTTTTACCCACGAGAGGCAA
AAGTTCAATGGAAGGTAGACAATGCTCTTCAGAGCGGCAATTCCCAGGAGAGCGTA
ACCGAGCAGGATAGCAAAGACTCTACATACTCTTTGAGTTCAACCCTTACCCTGAGC
AAGGCTGATTACGAAAAACACAAGGTGTACGCTTGCGAGGTAACCCATCAGGGATT
GTCATCCCCAGTCACAAAATCATTCAATAGGGGCGAGTGCTGACGGGTGGCATCCCT
GTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCA
GCCTTGTCCTAATAAAATTAAGTTGCATCAAGCT

Supplemental Figure 2. *Alignment of trastuzumab peptides identified in serum of mice injected with trastuzumab mRNA-LNPs with full trastuzumab amino acid sequence.*

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Trastuzumab Heavy Chain
            10        20        30        40        50                60        70        80        90       100       110
QUERY   EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVAR---------YADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
        ::::::::::::::::::::::::::::::::::::::::::::::::::         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
T-HC    EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
             10        20        30        40        50        60        70        80        90       100       110       120

           120       130       140                                                                   150       160
QUERY   ASTKGPSVFPLAPSSKSTSGGTAALGCLVK-----------------------------------------------------------VDKKVEPKSCDKTHTCPPCPAPELLGG
        ::::::::::::::::::::::::::::::                                                           ::::::::::::::::::::::::::::
T-HC    ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG
            130       140       150       160       170       180       190       200       210       220       230       240

      170       180       190       200       210       220       230       240       250       260       270       280
QUERY   PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK--VSNKALPAPIEK------GQPREPQVYTLPPSREE
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::  ::::::::::::      ::::::::::::::::::
T-HC    PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE
            250       260       270       280       290       300       310       320       330       340       350       360

            290       300       310       320       330       340       350       360
QUERY   MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
T-HC    MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
            370       380       390       400       410       420       430       440       450


Trastuzumab Light Chain
             10              20        30        40        50              60        70        80        90              100
QUERY   DIQMTQSPSSLSASVGDR------ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR-----SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK----RTVAAPSVFIFPP
        ::::::::::::::::::      ::::::::::::::::::::::::::::::::::     ::::::::::::::::::::::::::::::::::    :::::::::::::
T-LC    DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP
             10        20        30        40        50        60        70        80        90       100       110       120

          110       120           130       140       150       160       170       180
QUERY   SDEQLKSGTASVVCLLNNFYPR---VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTK
        ::::::::::::::::::::::   ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
T-LC    SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
             130       140       150       160       170       180       190       200       210
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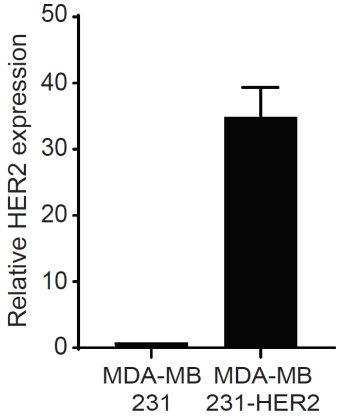
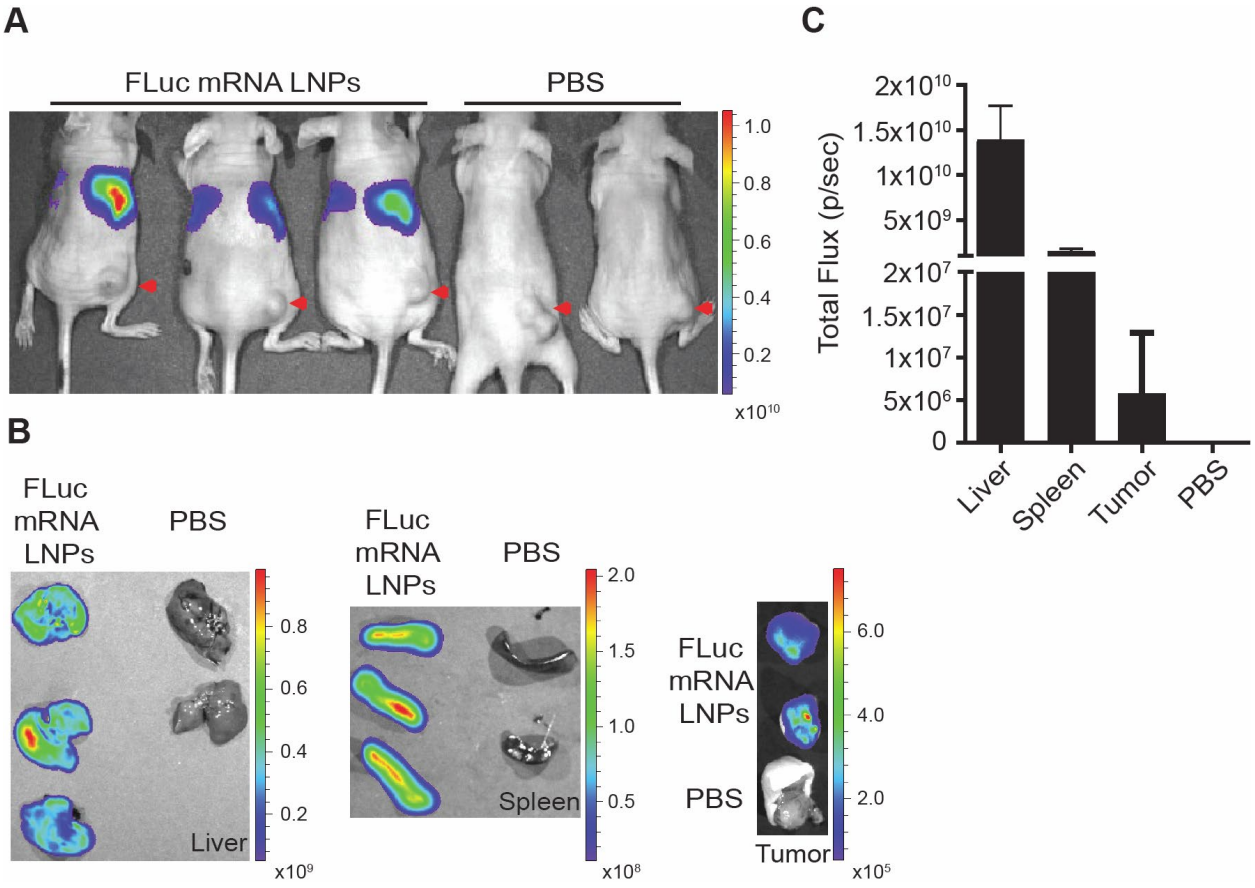
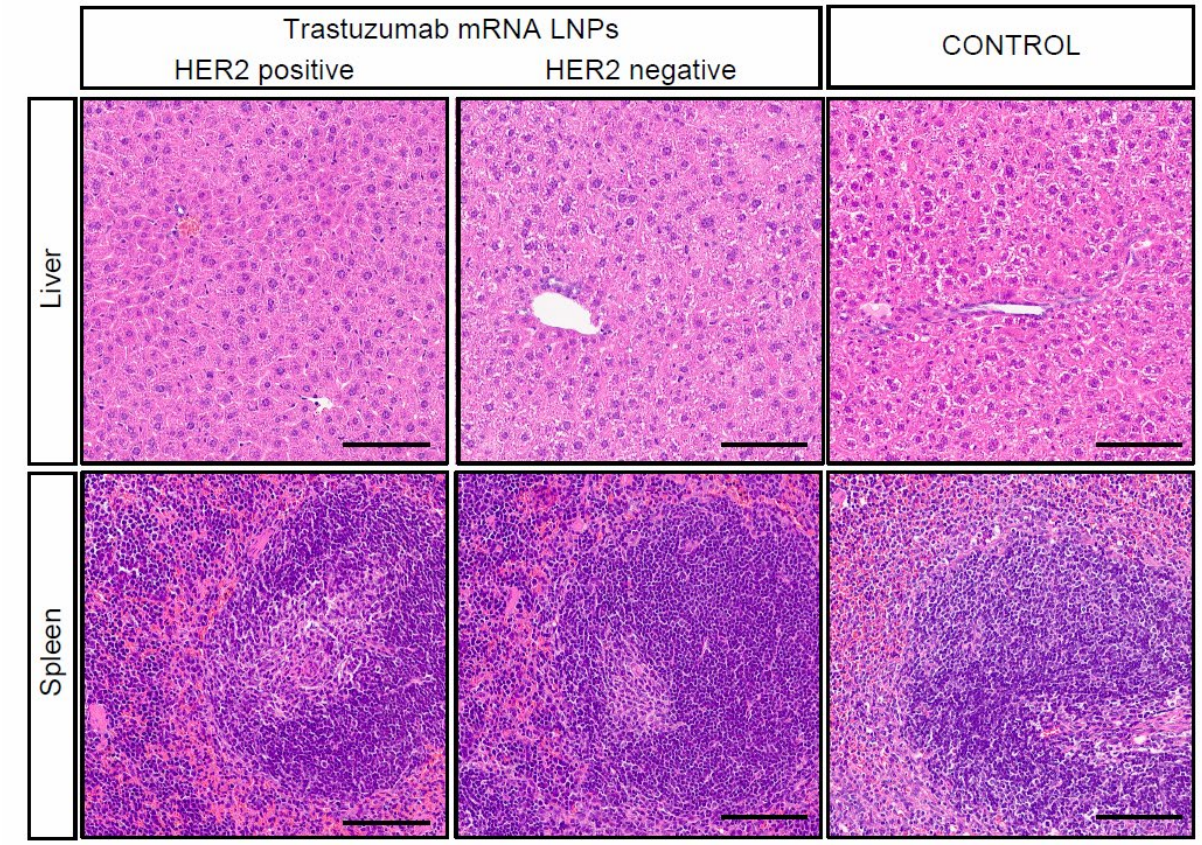
Supplemental Figure 3. *HER2 expression in MDA-MB-231-HER2 cells was measured using RT-qPCR and compared to control MDA-MB-231 cells. Mean±SD, relative to HER2-expression in MDA-MB-231 cells.*



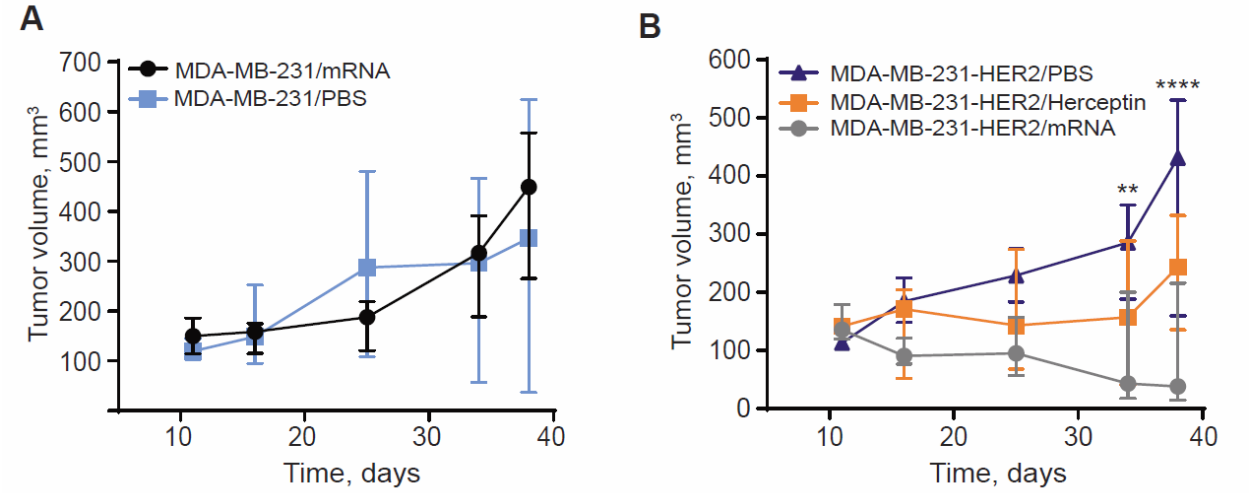
Supplemental Figure 4. *RNA expression in tumor-bearing mice after LNP administration. IVIS imaging of the whole-body (arrowheads point on tumor location) (**A**) and selected organs (**B**) from athymic nude mice 6h after i.v. injection with Firefly liciferase (Fluc) mRNA formulated into LNPs. Bioluminescence scale is radiance (p/sec/cm2/sr).* ***C.*** *Quantification of bioluminescence in selected tissues.*



Supplemental Figure 5. Effects of treatment with trastuzumab mRNA LNPs on different organs. *Haematoxylin and eosin staining of livers and spleens from mice with HER-negative (MDA-MB-231) and HER2-positive (MDA-MB-231-HER2) tumors treated with the trastuzumab mRNA or PBS (control). Scale bar, 100 um.*



Supplemental Figure 6. *Growth (median±IQR) of HER2-negative (MDA-MB-231) and HER2-positive (MDA-MB-231-HER2) tumors in mice treated with four weekly injections of (**A**) 2 mg/kg trastuzumab mRNA (n=5) or saline (n=6); (**B**) 2 mg/kg trastuzumab mRNA (n=5), 8 mg/kg Herceptin (n=4) or saline (n=6). Two-way repeated measures ANOVA with Sidak multiple comparisons tests, ** p-value ≤ 0.01, **** p-value ≤ 0.0001 for HER2-positive tumors treated with mRNA compared to saline.*



Supplemental Table 1. *Signal peptide sequences used in the study.*

Protein name	Signal peptide sequence
Gaussian luciferase	ATGGGTGTAAAGGTGCTCTTCGCCCTGATATGTATAGCCGTG GCCGAGGCT
Human immunoglobulin light chain kappa	ATGAGGGCTTGGATCTTCTTTCTGCTCTGCCTGGCCGGGCGC GCCTTGGCC
Human immunoglobulin G1 heavy chain	ATGGACTGGACCTGGAGGTTCCTCTTTGTGGTGGCAGCAGCT ACAGGTGTCCAGTCC

Supplemental Table 3. Effects of treatment with trastuzumab mRNA LNPs on blood cell count parameters in athymic nude mice with MDA-MB-231 or MDA-MB-231-HER2 xenografts. *Female athymic nude mice inoculated with MDA-MB-231 or MDA-MB-231-HER2 cells received four weekly i.v. injections of 2 mg/kg trastuzumab mRNA formulated into cKK-E12 nanoparticles or PBS. The serum was collected one week after the final injection. One-way ANOVA followed by Dunnett's post hoc test, * p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001, **** p-value ≤ 0.001 compared to healthy mice.*

Parameter	Units	Healthy mice	Untreated tumor-bearing mice	MDA-MB-231	MDA-MB-231-HER2
		Mean±SD, n=3	Mean±SD, n=3	Mean±SD, n=3	Mean±SD, n=3
Leukocytes:					
WBC	K/ul	10.1 ± 1.6	5.1 ± 1.6	12.4 ± 9.4	5.6 ± 0.2
NE	K/ul	2.6 ± 0.7	1.7 ± 0.8	7.8 ± 7.5	2.7 ± 1
LY	K/ul	7 ± 0.7	3.0 ± 0.6**	3.9 ± 1.6*	2.7 ± 0.8**
MO	K/ul	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
EO	K/ul	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.0
BA	K/ul	0 ± 0.1	0.1 ± 0.1	0 ± 0	0.1 ± 0.1
Erythrocytes:					
RBC	M/ul	8.1 ± 0.3	6.8 ± 0.1***	8.5 ± 0.2	8 ± 0.2
Hb	g/dL	14.4 ± 0.8	11.3 ± 0.1****	13.3 ± 0.1*	12.8 ± 0.3**
HCT	%	47.4 ± 8.8	36.9 ± 5.6	46 ± 6	38.7 ± 0.5
MCV	fL	58.1 ± 8.2	54.1 ± 7.8	54.3 ± 6.1	48.2 ± 1.7
MCH	pg	17.7 ± 0.9	16.6 ± 0.1	15.7 ± 0.4**	16 ± 0.6*
MCHC	g/dL	30.9 ± 4.9	31 ± 4.1	29.3 ± 3.4	33.2 ± 0.6
RDW	%	15.6 ± 0.2	17.5 ± 0.7**	18.3 ± 0.1***	18.3 ± 0.5***
Thrombocytes:					
PLT	K/ul	688 ± 124	347 ± 270.5	866 ± 128	936 ± 203.5
MPV	fL	4.7 ± 0.1	5.5 ± 0.8	5.3 ± 0.3	5.3 ± 0.1

Supplemental Table 4. Effects of treatment with trastuzumab mRNA LNPs on parameters of serum chemistry in athymic nude mice. *Female athymic nude mice with MDA-MB-231 or MDA-MB-231-HER2 xenografts received four weekly i.v. injections of 2 mg/kg trastuzumab mRNA formulated into cKK-E12 nanoparticles or PBS. The serum was collected one week after the final injection. One-way ANOVA followed by Dunnett's post hoc test, * p-value ≤ 0.05 compared to healthy mice.*

Parameter	Units	Untreated tumor-bearing mice	MDA-MB-231	MDA-MB-231-HER2
		Mean±SD, n=4	Mean±SD, n=3	Mean±SD, n=3
Alk Phos	IU/L	27 ± 16.7	51 ± 15.6	45 ± 3.5
ALT	IU/L	102.3 ± 68.1	128 ± 46.1	71.7 ± 7.6
AST	IU/L	25 ± 4.7	28 ± 5.6	21.7 ± 4
CK	IU/L	499 ± 637	197.5 ± 2.1	118 ± 17.2
GGT	IU/L	0 ± 0	0 ± 0	0 ± 0
Albumin	g/dL	2.5 ± 0.1	2.4 ± 0.3	2.4 ± 0.2
Total Bilirubin	mg/dL	0.1 ± 0.1	0.2 ± 0.1	0 ± 0
Total Protein	g/dL	4.6 ± 0.3	4.2 ± 0.4	4.6 ± 0.3
Globulin	g/dL	2.1 ± 0.2	1.8 ± 0.2	2.2 ± 0.4
Bilirubin- Conjugated	mg/dL	0 ± 0	0 ± 0	0 ± 0
BUN	mg/dL	23.8 ± 4.4	24.7 ± 4.7	18.7 ± 3.8
Creatinine	mg/dL	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0
Cholesterol	mg/dL	81.3 ± 16.4	102 ± 4.4	87.7 ± 12.9
Glucose	mg/dL	219.5 ± 44.9	246 ± 66	251 ± 16.2
Calcium	mg/dL	7 ± 4.3	8.9 ± 0.9	8.9 ± 0.7
Phosphorus	mg/dL	7.8 ± 1.8	9 ± 1.9	5.9 ± 0.4
Bicarbonate	mEq/L	11 ± 0.8	13 ± 1.7	14.3 ± 1.2*
Chloride	mEq/L	110.3 ± 3.1	112 ± 9.1	111.3 ± 5.8
Potassium	mEq/L	5.9 ± 3.5	5.4 ± 0.9	4.3 ± 0.5
ALB/GLOB ratio	ratio	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.3
Sodium	mEq/L	148 ± 2.6	148 ± 13.1	147 ± 8.6
BUN/Creatinine ratio	ratio	107.5 ± 79.8	177 ± 157	187 ± 37.9
Bilirubin-Unconjugated	mEq/L	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0
NA/K	ratio	30.3 ± 12	28 ± 6.6	34.3 ± 6
Anion Gap	mEq/L	32.8 ± 3.6	28.7 ± 3.5	25.3 ± 2.1*

Supplemental Table 5. *Physicochemical properties of RNA nanoparticles. Particle size and the Polydispersity index were analyzed by dynamic light scattering using Zetasizer Nano (Malvern). Encapsulation efficacy was determined with the Quant-iT Ribo-Green assay (Invitrogen). HC – heavy chain, LC – light chain. Data are presented as Mean ±SD, n=3.*

Trastuzumab HC mRNA to LC mRNA ratio	Polydispersity	Size Intensity mean (nm)	Encapsulation efficacy (%)
1:1	0.14 ± 0.02	103 ± 3	71 ± 6
1:2	0.13 ± 0.01	104 ± 5	76 ± 3
1:4	0.15 ± 0.02	101 ± 3	72 ± 4

Supplemental Methods

Generation of MDA-MB-231-HER2 cell line. MDA-MB-231 cells were retrovirally transduced with human HER2 (MSCV-human Erbb2-IRES-GFP, Addgene #91888). Green fluorescent protein (GFP) positive cells were sorted by FACS, HER2 expression in the selected cells was confirmed by RT-qPCR using HER2-FWD GAAGCCTCACAGAGATCTTG and HER2-REV CCTTACACATCGGAGAACAG primers (from (57)).

Cell-based assays. For transfection with mRNA 500-700 000 cells were seeded per well in 6-well plates. Within 24h the cells were transfected with mRNA using Lipofectamine™ MessengerMAX™ Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Breast cancer cell survival was analyzed using CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacturer's protocol. Briefly, 15 000 cells were plated per well in 96 well plate. Next day cells were treated with trastuzumab isolated from mouse blood or with Herceptin (Genentech). Two days after the treatment CellTiter was added to the cells and luminescence was measured by microplate reader Tecan Infinite® 200 PRO.

IVT-mRNA synthesis. IVT-mRNA synthesis was performed as previously described (Kauffman et al, 2015, 2016). Briefly, DNA plasmids containing a T7 promoter upstream of the sequences of trastuzumab heavy or light chain were linearized and transcribed using the HiScribe T7 RNA Synthesis Kit (New England Biolabs (NEB). mRNA was capped with the Vaccinia Capping System (NEB), and polyA tails were added to the RNA using a Poly(A) Polymerase Kit (NEB).). All mRNAs were purified after the transcription and tailing steps using MEGAClear RNA purification kit according to manufacturer protocol (Life Technologies). Final purified mRNAs contained a 5' cap (Cap1), a 5' UTR consisting of a partial sequence of the cytomegalovirus (CMV) immediate early 1 (IE1) gene, a coding region as described below, a 3' UTR consisting of a partial sequence of the human growth hormone (hGH) gene, and a 3' polyA tail estimated to be approximately 120 nucleotides long.

IVT-mRNA formulation into LNPs. LNPs were prepared by mixing ethanol and aqueous phase at a 1:3 volumetric ratio in a microfluidic device, using syringe pumps as previously described (Chen, Love et al., 2012). The ethanol phase was prepared by solubilizing a mixture of ionizable lipidoid cKK-E12, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti), cholesterol (Avanti), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (ammonium salt) (C14-PEG 2000, Avanti) at a molar ratio of 35:16:46.5:2.5 The aqueous phase

was prepared in 10 mM citrate buffer (pH 3) with either trastuzumab mRNA or luciferase mRNA (Firefly luciferase mRNA, TranslateBio). LNPs were dialyzed against PBS in a Slide-A-Lyzer™ G2 Dialysis Cassettes, 20,000 MWCO (Thermo Fisher) for 2h at RT. The concentration of mRNA encapsulated into LNPs nanoparticles was analyzed using Quant-iT RiboGreen assay (Thermo Fisher), according to manufacturer's protocol. The efficacy of mRNA encapsulation into LNPs was calculated by comparing measurements in the absence and presence of 1% (v/v) Triton X-100. Nanoparticle size, polydispersity (PDI), and ζ-potential were analyzed by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). LNP hydrodynamic diameters are reported in the volume weighting mode and are an average of three independent measurements (Supplemental Table 5).

Biodistribution analysis. cKK-E12 LNPs containing Firefly Luciferase mRNA (TranslateBio) were injected intravenously into female athymic nude mice via tail vein (2 mg kg-1). 6 h after the injection of the nanoparticles, mice were injected intraperitoneally with 130 μL of D-luciferin (30 mg mL-1 in PBS, Perkin Elmer). After 10 min, mice were sacrificed by CO2 asphyxiation and both tumors and organs (liver and spleen) were isolated and imaged with an IVIS Spectrum In Vivo Imaging System (PerkinElmer). Florescence signals were quantified using Living Image software v4.4 (PerkinElmer).

Histological analysis. Freshly collected tissues were fixed in 4% paraformaldehyde and embedded into paraffin. Four-micrometer-thick sections were subjected to hematoxylin and eosin staining.

ELISA. Tratsuzumab levels were measured using Invitrogen™ IgG1 Human ELISA Kit (cat. #EHIGG1) (Figure 1) or AffinityImmuno Trastuzumab (Herceptin®) Pharmacokinetic ELISA (cat. #EL-1611-201) (Figure 2) according to manufacturers' instructions.

Mass Spectrometry studies were performed by Swanson Biotechnology Center at David. H Koch Institute for Integrative Cancer Research at MIT. *IgG enrichment*: Herceptin was isolated from the samples using the Pierce MS-compatible IP kit protein A/G per manufacturer's instructions. 25 uL of beads were used for 1 ug of Herceptin (determined via ELISA). Beads were washed with lysis buffer, added to the Herceptin containing samples, and incubated at r.t. for 1 h on a rotator. The beads were then washed three times with wash buffer A, three times with wash buffer B, and three times with 100 mM ammonium bicarbonate, pH 8. *Reduction, Alkylation, and Tryptic Digestion*: On-bead reduction, alkylation, and digestion was performed. Proteins were reduced with 10 mM dithiothreitol (Sigma) for 1h at 56ºC and then alkylated with 55 mM iodoacetamide (Sigma) for 1h at 25ºC in the dark. Samples were incubated with PNGaseF for 2 h at 37ºC. Proteins were then digested with modified trypsin (Promega) at an enzyme/substrate ratio of 1:50 in 100 mM ammonium bicarbonate, pH 8 at 25ºC overnight. Trypsin activity was halted by addition of formic acid (99.9%, Sigma) to a final concentration of 5%. Peptides were desalted using C18 SpinTips (Protea, Morgantown, WV) then vacuum centrifuged and stored at −80 °C. *LC-MS/MS*: Peptides were loaded on a precolumn and separated by reverse phase HPLC using an EASY- nLC1000 (Thermo) over a 75 minute gradient before nanoelectrospray using a QExactive HF-X mass spectrometer (Thermo). The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 70,000 across 350-2000 m/z, AGC 3e6, and maximum IT 50 ms. The full MS scan was followed by MS/MS for the top 15 precursor ions in each cycle with a NCE of 28 and dynamic exclusion of 30 s. Raw mass spectral data files (.raw) were searched using Proteome Discoverer 2.2 (Thermo) and Mascot version 2.4.1 (Matrix Science). Mascot search parameters were: 10 ppm mass tolerance for precursor ions; 15 mmu for fragment ion mass tolerance; 2 missed cleavages of trypsin; fixed modification was carbamidomethylation of cysteine; variable modifications were methionine oxidation, asparagine

deamidation. Only peptides with a Mascot score greater than or equal to 25 and an isolation interference less than or equal to 30 were included in the data analysis.

Gene expression analysis. RNA was isolated using Omega Bio-tek The E.Z.N.A.® Total RNA Kit I isolation kit according to manufacturers' instructions. Reverse transcription reaction was performed using Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit and 1 ug of RNA. Levels of mRNAs were assessed by qPCR using Roche LightCycler 480. β-actin mRNA was used as housekeeping controls. The mRNA levels were normalized to the level of β-actin gene and to an average value of control group.

The analysis of pharmacokinetic properties of trastuzumab. Pharmacokinetic parameters of Trastuzumab were calculated using PKSolver 2.0 software (58). Non-compartmental analysis was performed on ELISA datasets (Affinity Immuno, Pharmacokinetic Trastuzumab ELISA) measuring trastuzumab concentration in mouse serum samples collected over the course of 30 days.