Orthogonal Design of Experiments for Optimization of Lipid Nanoparticles for mRNA Engineering of CAR T Cells

Authors:
Margaret M. Billingsley[1], Alex G. Hamilton[1], David Mai[1,2], Savan K. Patel[1], Kelsey L. Swingle[1], Neil C. Sheppard[2,3], Carl H. June[2,3], and Michael J. Mitchell[1,2,4,5,6*]

Affiliations:
[1]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104, USA.
[2]Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
[3]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
[4]Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
[5]Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.
[6]Institute for Regenerative Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

Supplemental Figures

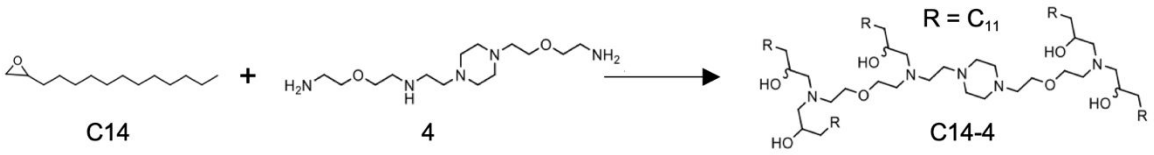

Figure S1. Michael addition chemistry is used to combine an excess of epoxide-terminated alkyl chains with this polyamine core (4) to generate the C14-4 ionizable lipid.

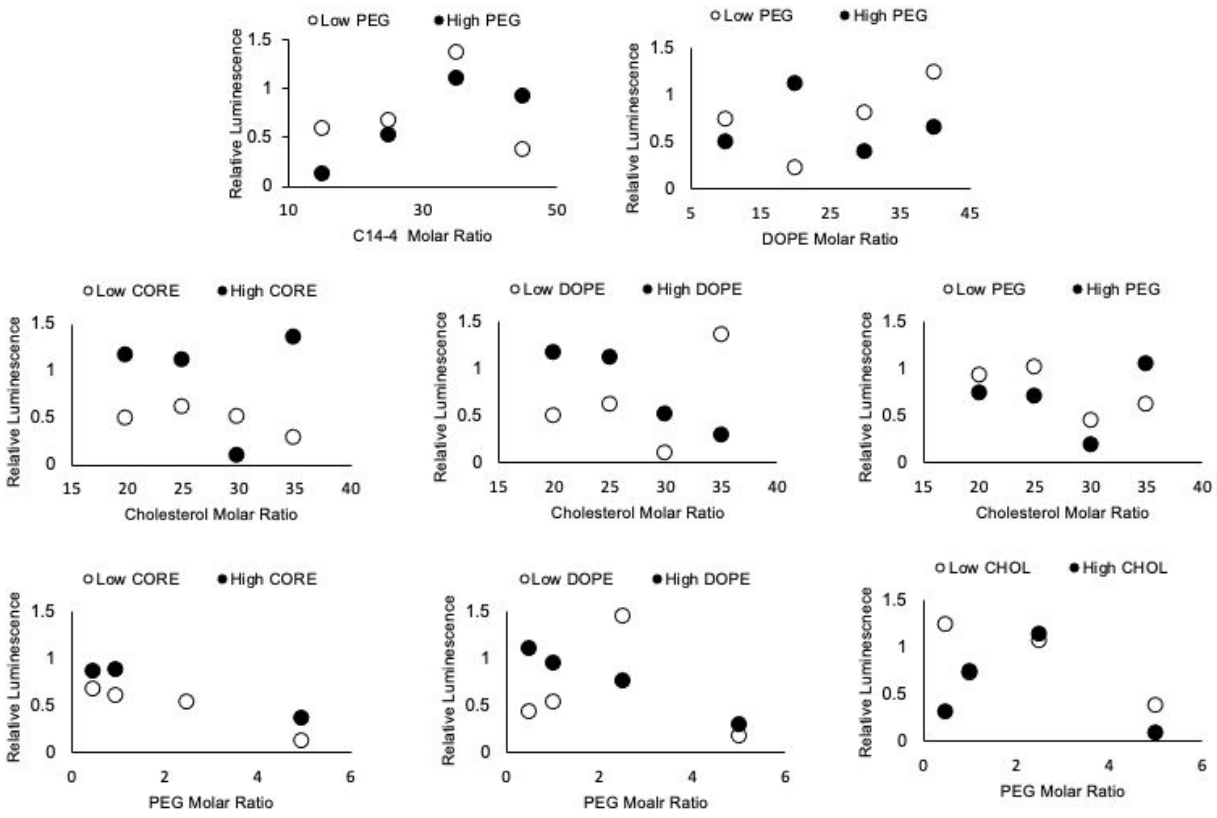

Figure S2. To observe the effects of two excipient amounts on mRNA delivery, each data point represents the average relative luminescence of two LNP formulations with the given excipient molar ratio with either the higher or lower molar ratios of the second excipient.

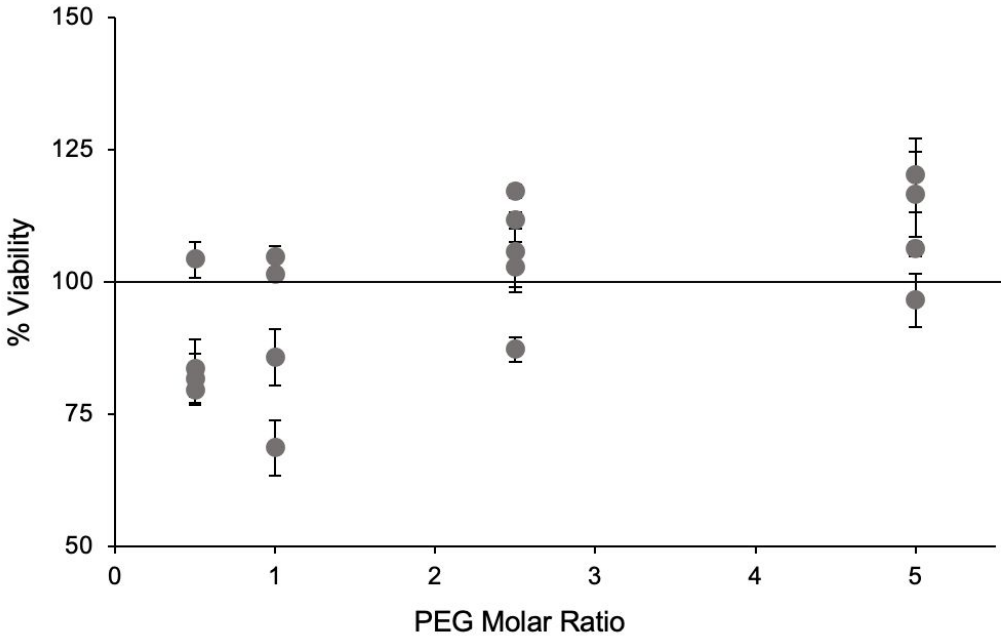

Figure S3. Jurkat cells treated with LNP formulations containing decreased PEG molar ratios show decreased viability. To assess the effects of PEG molar ratio on percent viability of Jurkat cells, each formulation from Library A is plotted by its PEG molar ratio and percent viability with error bars representing standard deviation. The line at 100% viability is used to show that in formulations with lower PEG molar ratios, LNPs are more likely to induce toxicity compared to formulations with higher PEG molar ratios. This trend was used to inform the design of Library B, which included only the 2.5 PEG molar ratio.

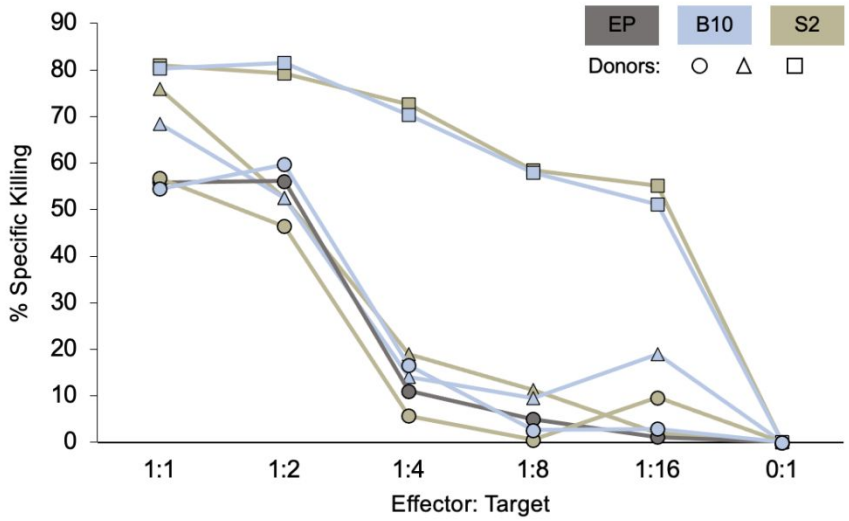

Figure S4. Results of CAR T and ALL cell co-culture assay after 48 hrs at different T cell to tumor cell ratios. The percent of cancer cell killing was determined by comparison to ALL cells co-cultured with untreated T cells as the negative control. Each shape represents a different donor

(n = 3 biological replicates for LNPs, n = 1 biological replicate for EP). All groups induce comparable cancer cell killing with notable variations based on donor.

Supplemental Tables

Table S1: Library A Characterization: Size, polydispersity index (PDI), and mRNA concentration (± standard deviations) and pKa values for each LNP formulation.

Library A				
Name	Size (nm)	PDI	Conc (ng/uL)	pKa
A1	125.0 ± 2.1	0.09 ± 0.01	10.4 ± 0.6	5.35
A2	78.6 ± 0.9	0.20 ± 0.01	29.1 ± 2.5	5.35
A3	80.1 ± 1.8	0.23 ± 0.01	45.0 ± 0.7	5.05
A4	57.5 ± 1.8	0.26 ± 0.04	42.2 ± 1.5	6.09
A5	82.0 ± 0.9	0.22 ± 0.01	36.9 ± 2.3	6.12
A6	58.4 ± 1.6	0.24 ± 0.01	35.8 ± 1.1	5.91
A7	126.2 ± 1.6	0.17 ± 0.01	11.4 ± 0.2	5.28
A8	101.1 ± 0.3	0.23 ± 0.02	15.7 ± 5.2	5.51
A9	57.3 ± 0.4	0.24 ± 0.01	35.2 ± 0.8	5.87
A10	95.0 ± 3.3	0.12 ± 0.03	28.0 ± 0.2	5.12
A11	103.4 ± 3.2	0.15 ± 0.03	10.8 ± 1.5	4.90
A12	151.0 ± 2.0	0.07 ± 0.04	18.1 ± 0.9	5.49
A13	110.7 ± 0.3	0.11 ± 0.02	15.3 ± 0.7	5.25
A14	142.1 ± 4.5	0.11 ± 0.04	2.9 ± 0.5	6.20
A15	105.6 ± 1.0	0.22 ± 0.02	25.7 ± 1.8	6.03
A16	102.6 ± 2.8	0.19 ± 0.01	18.5 ± 0.9	5.75
S2	93.9 ± 0.9	0.22 ± 0.02	31.5 ± 0.5	6.47

Table S2: Library B Characterization: Size, polydispersity index (PDI), and mRNA concentration (± standard deviations) for each LNP formulation.

Library B			
Name	Size (nm)	PDI	Conc (ng/uL)
B1	85.4 ± 0.1	0.24 ± 0.02	29.6 ± 1.0
B2	81.0 ± 3.7	0.22 ± 0.02	31.7 ± 0.5
B3	95.9 ± 0.8	0.22 ± 0.02	38.2 ± 0.2
B4	125.1 ± 3.9	0.19 ± 0.03	34.4 ± 1.1
B5	113.9 ± 0.8	0.22 ± 0.01	42.6 ± 1.1
B6	115.4 ± 1.2	0.13 ± 0.02	34.9 ± 0.8
B7	104.8 ± 0.5	0.16 ± 0.02	25.8 ± 0.4
B8	107.4 ± 0.6	0.14 ± 0.01	28.8 ± 0.5
B9	90.3 ± 2.8	0.17 ± 0.03	24.9 ± 1.6
B10	100.5 ± 0.2	0.18 ± 0.01	34.4 ± 1.9
B11	94.2 ± 1.0	0.18 ± 0.01	29.5 ± 0.5
B12	71.4 ± 0.9	0.27 ± 0.01	30.8 ± 0.2

Table S3: Formulations of libraries A and B listed as molar ratio percent.

Library A				
Name	C14-4	DOPE	Chol	PEG
A1	32.97	21.98	43.96	1.10
A2	24.59	32.79	40.98	1.64
A3	19.35	38.71	38.71	3.23
A4	15.79	42.11	36.84	5.26
A5	40.00	16.00	40.00	4.00
A6	35.71	28.57	28.57	7.14
A7	27.62	33.15	38.67	0.55
A8	26.04	41.67	31.25	1.04
A9	43.75	12.50	37.50	6.25
A10	37.84	21.62	37.84	2.70
A11	40.70	34.88	23.26	1.16
A12	34.83	39.80	24.88	0.50
A13	49.45	10.99	38.46	1.10
A14	47.12	20.94	31.41	0.52
A15	42.86	28.57	23.81	4.76
A16	41.86	37.21	18.60	2.33
S2	35.00	16	46.5	2.5

Library B				
Name	C14-4	DOPE	Chol	PEG
B1	40.00	34.29	22.86	2.86
B2	37.84	37.84	21.62	2.70
B3	35.90	41.03	20.51	2.56
B4	37.84	32.43	27.03	2.70
B5	35.90	35.90	25.64	2.56
B6	34.15	39.02	24.39	2.44
B7	43.24	32.43	21.62	2.70
B8	41.03	35.90	20.51	2.56
B9	39.02	39.02	19.51	244
B10	41.03	30.77	25.64	2.56
B11	39.02	34.15	24.39	2.44
B12	37.21	37.21	23.26	2.33

Materials and Methods

Ionizable lipid synthesis

As previously described[1], the C14-4 ionizable lipid was prepared via Michael addition chemistry (Figure S1). Briefly, an excess of epoxide terminated C14 alkyl chains (epoxytetradecane, Sigma Aldrich, St. Louis, MO) was reacted with the polyamine core (Enamine Inc., Monmouth Junction, NJ) under gentle stirring for 2 days at 80 ^{o}C. After drying with a Rotovap R-300 (Buchi, New Castle, DE) and resuspending in ethanol, the C14-4 was used for ionizable lipid nanoparticle (LNP) formulation.

mRNA synthesis

Luciferase mRNA was acquired from Trilink Biotechnologies (San Diego, CA) with N1-Methyl-PseudoU and 5-Methyl-C substitutions. Chimeric antigen receptor (CAR) mRNA was synthesized using standard *in vitro* transcription methods. Briefly, plasmid DNA encoding a second generation CD19-targeted CAR with CD3ζ and 4-1BB costimulatory domains was linearized overnight, and then used to produce mRNA with the T7 mMessage ULTRA kit (ThermoFisher, Waltham, MA) as per manufacturer instructions. The mRNA was then polyA tailed, capped, and purified using the RNeasy mini kit (Qiagen, Hilden, Germany).

Cell Culture

Jurkat cells (ATCC no. TIB-152), an immortalized human T cell line, were cultured in RPMI-1640 with L-glutamine (ThermoFisher) supplemented with 10% bovine serum and 1% penicillin-streptomycin. Primary human T cells (CD3+) were collected from the

peripheral blood of healthy volunteer donors and procured via the Human Immunology Core service. T cells were combined at a 1:1 CD4+ to CD8+ ratio and activated overnight with Human T-activator CD3/CD28 Dynabeads (ThermoFisher) at a 1:1 bead to cell ratio.

Lipid Nanoparticle (LNP) formulation and characterization

LNPs were synthesized by combining an aqueous phase containing mRNA with an ethanol phase containing the lipid and cholesterol components via microfluidic mixing devices[2]. The devices utilize chaotic mixing features to induce fluid folding in a state of laminar flow to reproducibly form homogeneous LNPs[2,3]. The aqueous phase was composed of 10 mM citrate buffer and mRNA at 1 mg/mL. The ethanol phase contained the ionizable lipid C14-4, 1,2-distearoyl-snglycero-3-phophoe-thanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL), cholesterol (Sigma, St. Louis, MO), and lipid-anchored polyethylene glycol (PEG) (Avanti Polar Lipids) at varying molar ratios. The aqueous and ethanol phases were then mixed in the microfluidic device as a 1:3 ratio using pump33DS syringe pumps (Harvard Apparatus, Holliston, MA). After synthesis, the LNPs were dialyzed against PBS for 2 hrs before sterilization with 0.22 μm filters.

To measure size, the LNPs were suspended in PBS and analyzed using dynamic light scattering (DLS) performed on a Zetasizer Nano (Malver Instruments, Malvern, UK). The diameter (z-average) and polydispersity index (PDI) of the LNPs were measured in triplicate. The mRNA concentration of the LNPs were measured via A260 absorbance on an Infinite M Plex plate reader (Tecan, Morrisville, NC). To determine the pK_a values of the LNPs, 6-(ptoluidinyl) naphthalene-2-sulfonic acid (TNS) assays were run. Briefly, buffered solutions of 150 mM sodium chloride, 20 mM sodium phosphate, 25 mM ammonium citrate, and 20 mM ammonium acetate were adjusted to reach pH values in increments of 0.5 from 2 to 12. LNPs were added to each pH-adjusted solution in a 96-well plate, and TNS was then added to each well for a final TNS concentration of 6 μM. The resulting fluorescence was read on a plate reader, and the pKa was calculated as the pH at which the fluorescence intensity reached 50% of its maximum value (representative of 50% protonation).

mRNA delivery to Jurkat Cells and primary human T cells

Jurkat cells or primary human T cells were plated in triplicate at 60,000 cells per 60 μL in 96-well plates and immediately treated with a set dose of mRNA via LNPs diluted in PBS or a positive control. For Jurkat cells, the positive control treatment was lipofectamine, which was generated via a 10 min incubation of lipofectamine MessengerMAX transfection reagent (ThermoFisher) and mRNA per manufacturer protocol. The combined lipofectamine and mRNA were then used to treat Jurkat cells at the same mRNA dose as the LNP treatments. For CAR mRNA delivery to primary human T cells, the positive control was electroporation (EP). For EP, T cells were washed three times with serum-free media, resuspended at 10^8 cells/mL, mixed with mRNA at a concentration matched to the set dose for LNP treatment, and electroporated in a 2 mm cuvette using an ECM830 Electro Square Wave Porator (Harvard Apparatus BTX).

Luciferase and Toxicity Assays

To determine luciferase mRNA delivery after 24 or 48 hrs of incubation with mRNA treatment, the cells were centrifuged at 300g for 7 min and resuspended in 50 μL of 1X lysis buffer (Promega, Madison, WI) and 100 μL of luciferase assay substrate (Promega). The luminescent signal was then measured on a plate reader, and the signal from each group was normalized as described—either to an untreated or control group. To quantify cytotoxicity after 24 or 48 hrs, the 60 μL of CellTiter-Glo (Promega) was added to each well. After 10 min of incubation, the luminescence corresponding to ATP production was quantified using a plate reader, and the signal from each group was normalized to untreated cells.

CAR Expression and Co-culture Killing Assays

As an additional positive control CAR T cell generation, viral vectors were used to transduce primary human T cells. High-titer, replication-defective lentiviral vectors were generated using 293T human embryonic kidney cells. In a T150 tissue culture flask, 10^7 cells were treated with 7μg of pMDG.1, 18 μg of pMDLg/p.RRE packaging plasmids, and 15 μg of transfer plasmid with 96 μL of Lipofectamine 2000 transfection reagent (Life Technologies, Grand Island, NY). The viral supernatant was collected at 24 and 48 hrs post transfection and concentrated via ultracentrifugation overnight at 25,000 rpm. After 24 hrs of Dynabead (ThermoFisher) activation at a 1:3 cell to bead ratio, T cells were combined with lentiviral vectors at ~3 infectious particles per cell.

To measure CAR expression, T cells were stained using an anti-mouse FMC63 scFv mAb conjugated to PE (BioSwan). Further, CD4 and CD8 stained with mouse anti-human CD4 conjugated to FITC (clone RPA-T4, BD Biosciences) and mouse anti-human CD8 conjugated to APC (clone RPA-T8, BD Biosciences). T cells were then washed, and the surface expression of CAR, CD4, and CD8 were evaluated on a BD LSRII Fortessa. Standard gating was performed with doublet exclusion, and cytotoxicity was measured using the CellTiter Glo assay described above.

To assess the functionality of the CAR T cells, a co-culture killing assay was performed using luciferase-expressing Nalm-6 cells—a CD19+ pre-B acute lymphoblastic leukemia cell line—plated with CAR T cells at varying ratios of effector to target cells.[4,5] After 48 hrs, 100 μL of luciferase assay substrate (Promega) was added to the cultures and luminescence was measured on a plate reader after a 10 min incubation. The percent of specific lysis was calculated using the control of target cells plated without effector cells.

References

1. Billingsley, M. *et al.* Ionizable Lipid Nanoparticle Mediated mRNA Delivery for Human CAR T Cell Engineering. *Nano Lett.* (2020). doi:10.1021/acs.nanolett.9b04246
2. Chen, D. *et al.* Rapid Discovery of Potent siRNA-Containing Lipid Nanoparticles Enabled by Controlled Microfluidic Formulation. *J Am Chem Soc* **134**, 6948–51 (2012).
3. Stroock, A. D. *et al.* Chaotic mixer for microchannels. *Science (80-.).* **295**, 647–651 (2002).
4. Barrett, D. M. *et al.* Treatment of Advanced Leukemia in Mice with mRNA Engineered T Cells. *Hum. Gene Ther.* **22**, 1575–1586 (2011).
5. Castella, M. *et al.* Development of a Novel Anti-CD19 Chimeric Antigen Receptor : A Paradigm for an Affordable CAR T Cell Production at Academic Institutions. *Mol. Ther. Methods Clin. Dev.* **12**, 134–144 (2019).