NANO LETTERS

Letter

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Optimization of Lipid Nanoparticle Formulations for mRNA Delivery in Vivo with Fractional Factorial and Definitive Screening Designs

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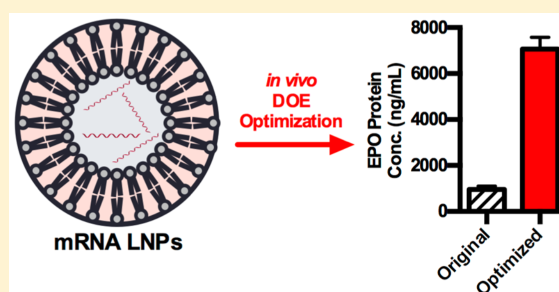
S Supporting Information

ABSTRACT: Intracellular delivery of messenger RNA (mRNA) has the potential to induce protein production for many therapeutic applications. Although lipid nanoparticles have shown considerable promise for the delivery of small interfering RNAs (siRNA), their utility as agents for mRNA delivery has only recently been investigated. The most common siRNA formulations contain four components: an amine-containing lipid or lipid-like material, phospholipid, cholesterol, and lipid-anchored polyethylene glycol, the relative ratios of which can have profound effects on the formulation potency. Here, we develop a generalized strategy to optimize lipid nanoparticle formulations for mRNA delivery to the liver in vivo using Design of Experiment (DOE) methodologies including Definitive Screening and Fractional Factorial Designs. By simultaneously varying lipid ratios and structures, we developed an optimized formulation which increased the potency of erythropoietin-mRNA-loaded C12-200 lipid nanoparticles 7-fold relative to formulations previously used for siRNA delivery. Key features of this optimized formulation were the incorporation of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and increased ionizable lipid:mRNA weight ratios. Interestingly, the optimized lipid nanoparticle formulation did not improve siRNA delivery, indicating differences in optimized formulation parameter design spaces for siRNA and mRNA. We believe the general method described here can accelerate in vivo screening and optimization of nanoparticle formulations with large multidimensional design spaces.



KEYWORDS: *Lipid nanoparticle, mRNA, design of experiment, nucleic acid, in vivo*

Nucleic acids have tremendous therapeutic potential to modulate protein expression in vivo but must be delivered safely and effectively. Because the delivery of naked nucleic acids results in poor cellular internalization, rapid degradation, and fast renal clearance,[1,2] lipid nanoparticles (LNPs) have been developed to encapsulate and deliver nucleic acids to the liver. Most notably, the field has seen orders-of-magnitude potency advances in the delivery of 21−23 nucleotide-long double stranded small interfering RNAs (siRNAs) due in part to the creation of new synthetic ionizable lipids and lipid-like materials.[2] Whereas some of these novel lipids were synthesized with rational design approaches by systematically varying the lipid head and tail structures (e.g., DLin-KC2-DMA, DLin-MC3-DMA, L319),[3−5] other materials were discovered by creating large combinatorial libraries of lipid-like materials (e.g., C12-200, cKK-E12, 503O13).[6−8] When formulated into LNPs, these amine-containing ionizable lipids and lipid-like materials electrostatically complex with the negatively charged siRNA and can both facilitate cellular uptake and endosomal escape of the siRNA to the cytoplasm.[6,9] In particular, the ionizable lipid-like material C12-200 has been widely used to make siRNA-LNP formulations for various therapeutic applications in vivo to silence protein expression.[10−12]

In addition to the ionizable material, three other excipients are also commonly used to formulate LNPs: (1) a phospholipid, which provides structure to the LNP bilayer and also may aid in endosomal escape;[2,13] (2) cholesterol, which enhances LNP stability and promotes membrane fusion;[14,15] and (3) lipid-anchored polyethylene glycol (PEG), which reduces LNP aggregation and "shields" the LNP from nonspecific endocytosis by immune cells.[16] The particular composition of the LNP can also have profound effects on the potency of the formulation in vivo. Several previous efforts to study the effect of formulation parameters on siRNA-LNP potency utilized the one-variable-at-a-time method,[17,18] in which formulation parameters were individually

Received: June 23, 2015
Revised: October 12, 2015

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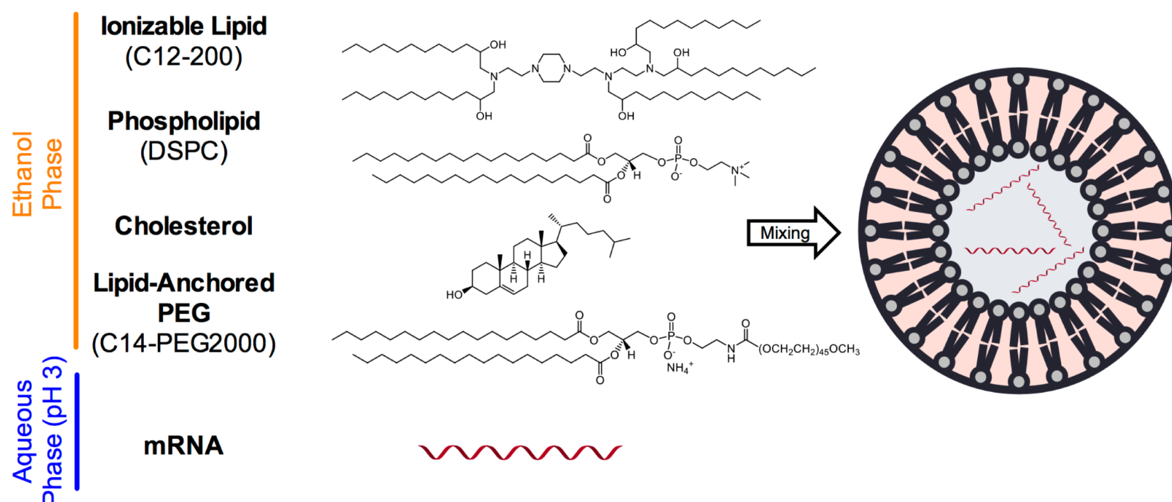

Figure 1. Formulation of lipid nanoparticles. Lipid nanoparticles (LNPs) are synthesized by the mixing of two phases: (1) a four-component ethanol phase containing ionizable lipid, helper phospholipid, cholesterol, and lipid-anchored PEG; (2) an acidic aqueous phase containing mRNA.

Table 1. Library A, B, and C Formulation Parameters

parameter	original formulation	Library A	Library B	Library C
C12-200:mRNA weight ratio	5:1	2.5:1 to 7.5:1	7.5:1 to 12.5:1	5:1 to 25:1
phospholipid	DSPC	DSPC, DSPE DOPC, DOPE	DSPC DOPE	DOPE
C12-200 molar composition	50%	40% to 60%	30% to 40%	35%
phospholipid molar composition	10%	4% to 16%	16% to 28%	16%
cholesterol molar composition	38.5%	21.5% to 55.5%	28.5% to 51.5%	46.5%
PEG molar composition	1.5%	0.5% to 2.5%	2.5% to 3.5%	2.5%

[a]Phospholipid abbreviations: DS = 1,2-distearoyl-*sn*-glycero- (saturated tail), DO = 1,2-dioleoyl-*sn*-glycero- (Δ9-cis unsaturated tail), PC = 3-phosphocholine (primary amine headgroup), PE = 3-phosphoethanolamine (quaternary amine headgroup).

varied to maximize LNP potency; this approach, however, does not allow for examination of potentially important second-order interactions between parameters. Inspired by statistical methodologies commonly used in the engineering and combinatorial chemistry literature,[19,20] we chose to utilize Design of Experiment (DOE) to better optimize LNP formulations for nucleic acid delivery. Using DOE, the number of individual experiments required to establish statistically significant trends in a large multidimensional design space are considerably reduced, which is particularly relevant for the economical screening of LNP formulations: in vitro screens are often poor predictors of in vivo efficacy with siRNA-LNPs,[21] and it would be both cost- and material-prohibitive to test large libraries of LNP formulations in vivo.

To demonstrate the application of DOE to LNP formulation optimization in vivo, we formulated LNPs with a different type of nucleic acid than siRNA. Recently, messenger mRNA (mRNA) has been investigated for therapeutic protein production in vivo, including applications in cancer immunotherapy, infectious disease vaccines, and protein replacement therapy.[22,23] Unlike plasmid DNA, mRNA need only access the cytoplasm rather than the nucleus to enable protein translation and has no risk of inducing mutation through integration into the genome.[24] Because there are inherent chemical and structural differences between mRNA and siRNA in terms of length, stability, and charge density of the nucleic acid,[25] we hypothesized that LNP delivery formulations for mRNA may require significant variation from those developed for siRNA pack differently and with different affinity into nanoparticles than siRNA. To optimize LNP formulation parameters specifically for mRNA delivery, we developed a novel strategy in which we used DOE methodologies—including both Fractional Factorial and Definitive Screening Designs—to synthesize several smaller LNP libraries to screen in vivo. Using the formulation conditions of the original siRNA-LNPs as a starting point, each successive generation of library was designed to improve protein expression based upon the parameters in the previous library that were found to correlate with improved efficacy. Through this approach, we aimed to develop an optimized C12-200 LNP with increased protein expression over the original LNP formulation.

EPO mRNA Delivery with Original siRNA-Optimized LNP. The formulation process for synthesizing LNPs is described in Figure 1. The organic phase containing the lipids was mixed together with the acidic aqueous phase containing the nucleic acid in a microfluidic channel,[26] resulting in the formation of mRNA-loaded LNPs. We chose to use unmodified mRNA coding for erythropoietin (EPO), a secreted serum protein that has previously been successfully translated in vivo.[25,27] It has further been recently reported[28] that LNP-delivered unmodified EPO mRNA is more potent than EPO mRNA with pseudouridine and/or 5-methylcytidine modifications in vitro and in mice. To establish a baseline from which to improve, EPO mRNA was first formulated into LNPs using the original formulation parameters previously published[6] for siRNA delivery in vivo (Table 1). The formulation was dosed

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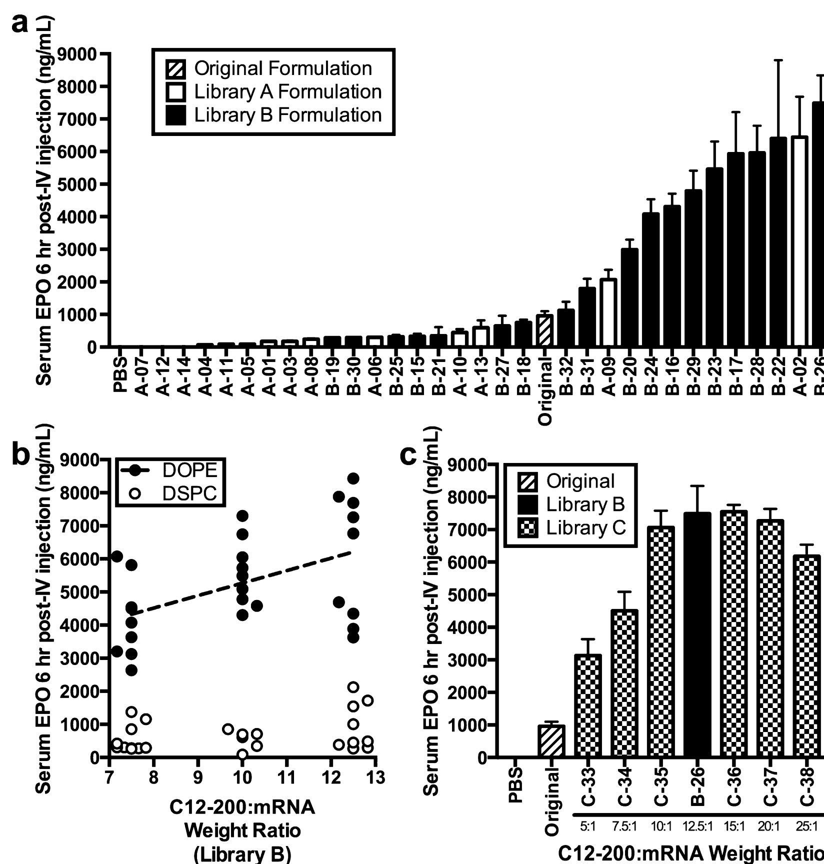

Figure 2. Efficacy results of LNPs in Libraries A, B, and C. (a) Serum EPO concentration 6 h post-intravenous injection of 15 μg total mRNA for each formulation in Libraries A and B, including the original formulation (data presented as mean + SD, $n = 3$). (b) A statistically significant trend of increasing serum EPO concentration was observed with increasing C12-200:mRNA weight ratio and with DOPE phospholipid for Library B formulations, independent of the other formulation parameters. Furthermore, a statistically significant second-order effect was observed between DOPE and increasing weight ratio, as indicated by the larger relative slope of the DOPE best-fit line compared to the DSPC best-fit line. (1 data point = 1 mouse) (c) Serum EPO concentration 6 h post-intravenous injection of 15 μg total mRNA for formulation B-26 and Library C, which had similar formulation parameters as B-26 with differing C12-200:mRNA weight ratios. (Data presented as mean + SD, $n = 3$.)

in an average EPO serum level of 963 ± 141 ng/mL at 6 h post-injection.

Optimization of mRNA LNPs with Design of Experiment. Some previous efforts to optimize nanoparticle formulations have involved varying each of the important parameters individually and then possibly combining each optimized parameter for an overall optimized formulation.[17,18,29] Because pilot experiments suggested strong second-order effects between parameters in our system, we chose instead to vary all five independent parameters simultaneously. In an attempt to maximize EPO expression in mice and thereby optimize the C12-200 LNPs for mRNA delivery, we chose to simultaneously vary the C12-200:mRNA weight ratio, the phospholipid identity, and the molar composition of the four-component LNP formulation. Three additional phospholipids structurally similar to DSPC but with differing head groups (primary vs quaternary amine) and tail saturation (saturated vs Δ9-cis unsaturated) were incorporated into the LNP formulations.

Library A: Definitive Screening Design. We designed the first library, Library A, to be centered around the original siRNA-optimized LNP formulation parameters (Table 1). With four three-level quantitative factors (C12-200:mRNA weight ratio and three independent formulation molar compositions) large five-dimensional design space required DOE to reduce the number of formulations ($3 \times 3 \times 3 \times 3 \times 4 = 324$) to a reasonable number for in vivo experiments. An initial library of 14 formulations (coded A-01 through A-14, see Table S1 for parameters) was created using a Definitive Screening Design, a recently described economical DOE in which main effects are not confounded with two-factor interactions and nonlinear correlations can be detected.[30] The purpose of this first screen was to sample the large design space in a controlled fashion to eliminate unimportant formulation parameters and/or find a local maximum in efficacy from which a second-generation library could be generated.

Out of 14 formulations in Library A, two formulations (A-02 and A-09) resulted in higher EPO serum levels (6445 ± 1237 and 2072 ± 302 ng/mL, respectively) than the original formulation (Figure 2a). Although the results from Library A were insufficient to deduce statistically significant effects for EPO production in vivo, there were statistically significant ($p <$ 0.05) orthogonal trends (Figure S2). We hypothesize that the increased encapsulation efficiency with increasing C12-200:mRNA weight ratio (Figure S2a) is caused by better complexation of more positively charged ionized C12-200 lipid with negatively charged mRNA. We also observed decreased LNP size with increasing PEG composition (Figure S2b), a

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literature[18,31] and has been speculated to be caused by increased lipid bilayer compressibility and increased repulsive forces between liposomes.[32] The two top-performing formulations of Library A (A-02 and A-09) possessed similar attributes: increased weight ratio (7.5:1 vs 5:1), increased phospholipid content (16% vs 10%), and either DSPC or DOPE as the phospholipid; moreover, A-02 had decreased C12-200 content (40% vs 50%) and A-09 had increased PEG content (2.5% vs 1.5%).

Library B: Fractional Factorial Screening Design. A more robust second-generation library, Library B (coded B-15 to B-32, Table S1), was generated using a L18-Taguchi Fractional Factorial Design[29] with new parameter ranges which shifted in the direction of the two top-performing LNPs from the first library (Table 1). Out of 18 formulations in Library B, 11 formulations resulted in higher EPO serum levels than the original formulation (Figure 2a). The top-performing formulation was B-26 with an average serum EPO concentration of 7485 ± 854 ng/mL. A standard least squares linear regression model was applied to the data from Library B, and several statistically significant factors were found with respect to efficacy (Table S2). Several second-order effects were found to be statistically significant as well, including the second-order interaction between DOPE and C12-200:mRNA weight ratio as shown by the best-fit line ($p < 0.05$) for DOPE in Figure 2b. Additional description of the statistical model and significant effects may be found in the Supporting Information (Table S2, Figure S1).

The most apparent trend from Library B was that formulations with DOPE as the phospholipid resulted in significantly higher EPO production than formulations with DSPC, the original phospholipid (Figure 2b). In fact, the presence of DOPE in the formulation was the single strongest predictor of in vivo efficacy in our study. Whereas DSPC contains a quaternary amine headgroup and a fully saturated tail, DOPE contains a primary amine headgroup and a tail with one degree of unsaturation. It has been reported that conical lipids, such as DOPE, tend to adopt the less stable hexagonal phase, while cylindrical lipids, such as DSPC, tend to adopt the more stable lamellar phase.[33] Upon fusion with the endosomal membrane, LNPs containing DOPE may reduce membrane stability, ultimately promoting endosomal escape.[34,35] Another possible explanation involves their different encapsulation efficiencies: independent of other varying formulation parameters, formulations with DSPC entrapped mRNA on average significantly better than DOPE (51% vs 36%), so it may be possible that the stronger complexation of mRNA to lipid in DSPC LNPs hinders the subsequent decomplexation of mRNA from lipid once inside the cell, thus inhibiting translation of the mRNA to protein.

Library C: Maximizing Lipid:mRNA Weight Ratio with DOPE. As was initially hypothesized, we observed several second-order effects on EPO production between formulation parameters in Library B, most notably the synergistic effect between increasing the C12-200:mRNA weight ratio along with the use of DOPE as the phospholipid (Figure 2b). In an effort to further increase in vivo potency, a third and final library was generated (Library C, Table 1) to exploit this discovered second-order effect. The top-performing formulation (B-26) from Library B was reformulated with C12-200:mRNA weight ratios varying from 5:1 to 25:1 (coded C33−C38, Table S1). Surprisingly, increasing the weight ratio only increased the appears that increasing the weight ratio beyond 10:1 confers no significant efficacy advantage in vivo. Because no significant increases in EPO production were observed beyond 10:1 and to mitigate any concerns with possible lipid toxicity caused by increased lipid doses, we chose the 10:1 C12-200:mRNA weight ratio (C-35) as the final mRNA-optimized LNP formulation (Table 2).

Table 2. LNP Characteristics of C-35 Compared to the Original Formulation[a]

	original formulation	optimized formulation (C-35)
C12-200:mRNA weight ratio	5:1	10:1
phospholipid	DSPC	DOPE
C12-200 molar composition	50%	35%
phospholipid molar composition	10%	16%
cholesterol molar composition	38.5%	46.5%
C14 PEG 2000 molar composition	1.5%	2.5%
serum EPO (ng/μL)	962 ± 141	7065 ± 513
diameter (nm)	152	102
polydispersity index (PDI)	0.102	0.158
mRNA encapsulation efficiency (%)	24	43
pK_a	7.25	6.96
zeta potential (mV)	−25.4	−5.0

[a]Phospholipid abbreviations: DSPC = 1,2-distearoyl-*sn*-glycero-3-phosphocholine, DOPE = 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, Serum EPO reported as mean ± SD ($n = 3$) 6 h after 15 μg of total mRNA intravenous injection into mice.

Evaluation of Methodology. Although only 14% (2 of 14) of the Library A formulations resulted in increased potency compared to the original parameters, 61% (11 of 18) of the Library B formulations and 100% of Library C formulations (6 of 6) did so (Figures 2a,c). This suggests that formulation parameters can be optimized and are critically important for efficient mRNA delivery with C12-200 LNPs. Furthermore, the increasing percentage of formulations that performed better than the original in each subsequent library demonstrates the predictive success of the generated statistical models (Table S2). A flowchart of the complete methodology we developed for in vivo nanoparticle optimization can be found in Figure S3.

Characterization of mRNA-Optimized LNP. The optimized formulation C-35 had the following formulation parameters: 10:1 C12-200:mRNA weight ratio with 35% C12-200, 16% DOPE, 46.5% cholesterol, and 2.5% C14-PEG2000 molar composition. The average efficacy of C-35 with 15 μg of total EPO mRNA injection in vivo, 7065 ± 513 ng/mL, was increased over 7-fold compared to the original traditional LNP formulation (963 ± 141 ng/mL). C-35 was further characterized and compared to the original formulation with regard to size, polydispersity, encapsulation efficiency, and pK_a (Table 2). No significant morphological differences were observed between the two formulations with transmission electron microscopy (TEM) (Figure S4). Although others have reported increases in siRNA nanoparticle potency with decreasing size,[36] we found no such trend with all 38 mRNA formulations tested in our LNP system. Jayaraman et al.[4] found that pK_a was an important characteristic in predicting the efficacy of liver-targeting siRNA LNPs with an optimal pK_a of

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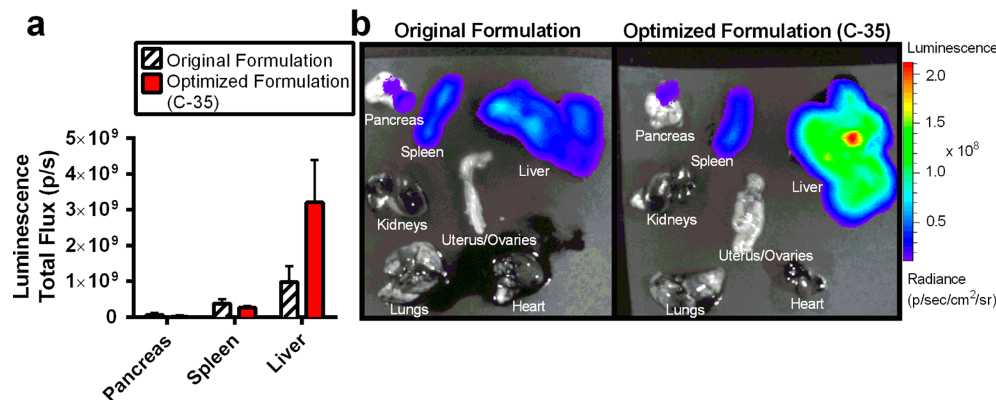

Figure 3. Efficacy and biodistribution of original and C-35 formulation with Luc mRNA. (a) Efficacy of original and C-35 LNP formulations synthesized with mRNA coding for luciferase in three organs of interest as measured by total flux from luminescence 6 h after intravenous injection of 15 μg total mRNA. (Data presented as mean + SD, $n = 3$). (b) Representative biodistribution image of luciferase expression for original and C-35 LNP in seven organs as measured with an IVIS imaging system 6 h after intravenous injection of 15 μg of total mRNA.

system, the in vivo efficacy is not significantly correlated with pK_a of the LNP, although the slightly lower pK_a of C-35 (pK_a = 6.96) compared to the original formulation (pK_a = 7.25) may partially explain its improved efficacy. The surface charge of the LNP may also partially explain differences in efficacy: the optimized formulation C-35 is less negatively charged (zeta potential = −5.0 mV) than the original formulation (−25.4 mV). C-35 contains twice the amount of amine-rich ionizable lipid C12-200 than the original formulation, which is likely the predominant reason C-35 is more positively charged. Although one study found no relationship between surface charge and hepatocellular delivery in vivo with siRNA-loaded lipid nanoparticles,[21] other reports have noted that more positively charged nanoparticles bind better to negatively charged cellular membranes and this electrostatic interaction might facilitate uptake.[37]

In order to determine whether C-35 would similarly improve the efficacy of mRNAs with different lengths, we formulated LNPs with firefly luciferase (Luc) mRNA, an mRNA which has a coding region roughly three times longer than that of EPO mRNA (1653 vs 582 nucleotides). Luciferase protein generated by C-35 LNPs was expressed predominately in the liver and likewise resulted in a statistically significant, approximately 3-fold increase in luciferase expression as measured by liver luminescence compared to the original formulation (Figure 3). Although LNPs made with Luc mRNA had similar encapsulation efficiencies as those made with shorter EPO mRNA (Tables 1, S3), we anticipate that significantly longer mRNAs would eventually become too large to effectively load into LNPs.

siRNA Delivery with mRNA-Optimized LNP. Having optimized the formulation for mRNA delivery, we then wanted to examine the potential for siRNA delivery with C-35 as compared to the original siRNA-optimized formulation. We formulated siRNA coding for Factor VII (FVII), a serum clotting factor expressed exclusively in hepatocytes, using both the C-35 LNP and the original LNP formulation to determine their relative silencing in hepatocytes. FVII levels were measured 72 h after intravenous injection of siRNA-loaded LNPs ranging from 0.01 mg/kg to 0.1 mg/kg, and there was no significant difference between the original and optimized formulations at any dose (Figure 4, Table S4) despite having significantly different formulation parameters. The ED_{50} of both

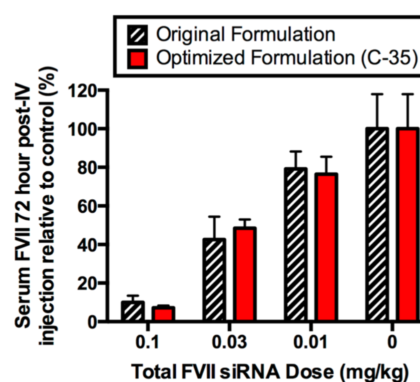

Figure 4. Efficacy of original and C-35 formulation with siRNA. Efficacy of original versus optimized C-35 formulation made with C12-200 and siRNA coding against Factor VII (FVII) protein as measured by serum FVII levels 72 h post-intravenous injection of various doses of total siRNA. FVII levels were normalized with respect to PBS-injected control mice. (Data presented as mean + SD, $n = 3$.)

approximately 0.03 mg/kg of total siRNA content, consistent with previous reports.[6]

Interestingly, siRNA-loaded LNPs may be more tolerant than mRNA-loaded LNPs of design space differences. Over the past decade in the siRNA delivery field, many groups have focused on developing new ionizable lipids to increase the potency of siRNA-LNPs but have generally used the same standard formulation parameters in consecutive studies.[3,4,6−8] The discovery of new ionizable lipids and lipid-like materials, however, is an endeavor which is often time- and material-intensive, requiring large-scale combinatorial libraries or chemically difficult rational design approaches. Meanwhile, we have shown that for one of the most commonly used ionizable materials for siRNA delivery, C12-200, merely changing the formulation parameters can significantly increase the potency of the LNP when loaded with two different mRNAs of varying lengths, EPO or Luc (Table 2, Figure 3).

In this study, we have demonstrated a new general method for optimizing previously used siRNA lipid nanoparticle technology for a new class of RNA therapeutics and identified a lead optimized formulation for mRNA delivery, coded C-35. To the best of our knowledge, this study represents the first optimization of nanoparticle potency in vivo using Design of

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