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【意見の内容】
１．　はじめに
令和１年６月１１日起案（発送日　令和１年６月１８日）の拒絶理由通知書において、平成３１年１月９日付け手続補正書でした補正が特許法第１７条の２第３項に規定する要件を満たしていないこと（理由１）と、請求項１～１１に係る発明が特許法第２９条第２項の規定により独狐を受けることができないこと（理由２）が指摘されています。しかしながら、出願人はこれらの指摘に対して異存があります。
２．　本願発明が特許されるべき理由
２．１　理由１（新規事項）について
審査官殿は、平成３１年１月９日付け手続補正書により請求項１に付加した「８０ｎｍ～１５０ｎｍの平均粒径を有し」との特定に関して、補正の根拠として挙げられた明細書の段落０９４７に記載の内容が本願発明に係る脂質ナノ粒子に関するものでもなければ、８０ｎｍ～１５０ｎｍの平均粒径という範囲を示すものでもないと述べています。また、表５６、５７、１４６、１４７、１５８、１５９、１６４、１６５を参照しても、８０ｎｍ及び１５０ｎｍという境界値は明記されておらず、８０ｎｍ～１５０ｎｍの平均粒径を有する粒子であれば、それ以外の平均粒径を有する粒子よりも優れた効果を奏しているともいえないと述べています。
しかしながら、審査官殿の指摘とは異なり、「８０ｎｍの平均粒径を有する静脈内製剤が、腫瘍学関連ポリヌクレオチド、一次構築物、またはｍｍＲＮＡを肝細胞に送達するのに有効であり得る」及び「内皮窓（ｆｅｎｅｓｔｒａｅ）のサイズに起因して、１５０ｎｍ未満の粒径が有効な肝細胞送達のために所望され得る」と述べる本願明細書の段落０９４７の記載は、以下で説明する本願明細書中の実施例の内容と組み合わせて考慮した場合、脂質ナノ粒子が８０ｎｍ～１５０ｎｍの平均粒径を有するという本願請求項１における限定を明確にサポートするものであると思料します。
まず、脂質ナノ粒子の平均粒径が８０ｎｍを下回る場合、脂質ナノ粒子で封入されたポリヌクレオチドによりコードされるタンパク質の発現レベルが低下する原因になります。以下に表Ａ及び表Ｂを示していますが、表Ａは本願明細書の表５３及び表５４中のデータの一部を組み合わせて作成したものであり、表Ｂは本願明細書の表５６及び表５７中のデータの一部を組み合わせて作成したものです。表Ａからは、平均粒径が８０ｎｍを下回る「NPA-074-1」の場合、平均粒径が８０ｎｍ以上である「NPA-071-1」、「NPA-072-1」及び「NPA-073-1」の場合に比べて、Ｇ-ＣＳＦの発現レベルが少なくとも一桁低いことが分かります。同様に表Ｂからも、平均粒径が８０ｎｍを下回る「NPA-073-1」の場合、平均粒径が８０ｎｍ以上である「NPA-071-1」及び「NPA-075-1」の場合に比べて、Ｇ-ＣＳＦの発現レベルが少なくとも一桁低いことが分かります。逆に言えば、表Ａ及び表Ｂに示す結果からは、脂質ナノ粒子の平均粒径が８０ｎｍ以上である場合、平均粒径が８０ｎｍを下回る場合に比べてタンパク質の発現レベルが増大することが分かります。

表Ａ

製剤番号	**NPA-071-1**	**NPA-072-1**	**NPA-073-1**	**NPA-074-1**
脂質	PEG-DMG 1.5%	PEG-DMG 3%	PEG-DSA 1.5%	PEG-DSA 3%
平均粒径	95 nm PDI: 0.01	85 nm PDI: 0.06	95 nm PDI: 0.08	75 nm PDI: 0.08
pH7.4でのゼータ	-1.1 mV	-2.6 mV	1.7 mV	0.7 mV
カプセル封入 **(RiboGreen)**	88%	89%	98%	95%
8時間での**G-CSF**発現 **(pg/mL)**	357,944	354,994	6,162	567

表Ｂ

製剤番号	**NPA-071-1**	**NPA-073-1**	**NPA-075-1**
脂質	DLin-MC3-DMA	DLin-DMA	C12-200
修飾**RNA**	EPO	EPO	EPO
平均粒径	89 nm PDI: 0.07	70 nm PDI: 0.04	97 nm PDI: 0.05
pH7.4でのゼータ	-1.1 mV	-1.6 mV	1.4 mV
カプセル封入 **(RiboGreen)**	100%	99%	88%
2時間での**G-CSF**発現 **(pg/mL)**	304,190	73,852	413,010

次に、脂質ナノ粒子の平均粒径が１５０ｎｍを超える場合もまた、脂質ナノ粒子で封入されたポリヌクレオチドによりコードされるタンパク質の発現レベルが低下する原因になります。以下に表Ｃ、表Ｄ及び表Ｅを示していますが、表Ｃは本願明細書の表１４６及び表１４７中のデータの一部を組み合わせて作成したものであり、表Ｄは本願明細書の表１５８及び表１５９中のデータの一部を組み合わせて作成したものであり、表Ｅは本願明細書の表１６４及び表１６５中のデータの一部を組み合わせて作成したものです。表Ｃ～表Ｅに示す結果からは、平均粒径が１５０ｎｍを超える場合（すなわち、「111612-B」、「111612-C」及び「111612-A」の場合）、平均粒径が１５０ｎｍ以下である場合に比べて、タンパク質（ルシフェラーゼ）の発現レベルが少なくとも一桁低いことが分かります。

表C

製剤	**NPA-126-1**	**NPA-127-1**	**NPA-128-1**	**NPA-129-1**	**111612-B**
脂質	DLin-MC3-DMA	DLin-KC2-DMA	C12-200	DLinDMA	DODMA
脂質/mRNA比(重量/重量)	20:1	20:1	20:1	20:1	20:1
平均粒径	122 nm PDI: 0.13	114 nm PDI: 0.10	153 nm PDI: 0.17	137 nm PDI: 0.09	223.2 nm PDI: 0.142
pH7.4でのゼータ	-1.4 mV	-0.5 mV	-1.4 mV	2.0 mV	-3.09 mV
カプセル封入 **(RiboGr)**	95%	77%	69%	80%	64%
8時間での**I.V.**光束 **(p/s)**	1.47E+08	2.13E+08	3.72E+07	3.82E+07	5.62E+06
8時間での**I.M.**光束 **(p/s)**	5.83E+07	2.12E+08	2.60E+07	1.99E+07	Not Tested
8時間での**S.C.**光束 **(p/s)**	7.74E+07	2.00E+08	4.58E+07	9.67E+07	1.90E+07

表D

製剤	**NPA-130-1**	**NPA-131-1**	**NPA-132-1**	**NPA-133-1**	**111612-C**
脂質	DLin-MC3-DMA	DLin-KC2-DMA	C12-200	DLinDMA	DODMA
脂質/mRNA比(重量/重量)	20:1	20:1	20:1	20:1	20:1
平均粒径	120 nm PDI: 0.10	105 nm PDI: 0.11	122 nm PDI: 0.13	105 nm PDI: 0.14	221.3 nm PDI: 0.063
pH7.4でのゼータ	0.2 mV	-0.6 mV	-0.5 mV	-0.3 mV	-3.10 mV
カプセル封入 **(RiboGr)**	100%	100%	93%	93%	60%
8時間での**I.V.**光束 **(p/s)**	1.21E+07	1.23E+08	1.02E+07	5.98E+06	6.14E+06
8時間での**I.M.**光束 **(p/s)**	7.78E+06	2.85E+07	4.29E+06	2.22E+06	1.38E+05
8時間での**S.C.**光束 **(p/s)**	3.65E+07	1.17E+08	3.71E+06	9.33E+06	2.57E+06

表Ｅ

製剤	NPA-137-1	NPA-134-1	NPA-135-1	NPA-136-1	111612-A
脂質	DLin-MC3-DMA	DLin-MC3-DMA	DLin-KC2-DMA	C12-200	DODMA
脂質/mRNA比 (重量/重量)	20:1	20:1	20:1	20:1	20:1
平均粒径	111 nm PDI: 0.15	104 nm PDI: 0.13	95 nm PDI: 0.11	143 nm PDI: 0.12	223.2 nm PDI: 0.142
pH7.4でのゼータ	-4.1 mV	-1.9 mV	-1.0 mV	0.2 mV	-3.09 mV
カプセル封入 (RiboGr)	97%	100%	100%	78%	64%
8時間でのI.V.光束 (p/s)	1.60E+09	3.22E+09	2.38E+09	1.11E+09	1.17E+07
8時間でのI.M.光束 (p/s)	2.16E+08	6.14E+08	1.00E+09	8.77E+07	7.05E+06
8時間でのS.C.光束 (p/s)	5.55E+08	9.80E+08	4.93E+09	1.01E+09	8.04E+07

以上を考慮した場合、「８０ｎｍ～１５０ｎｍの平均粒径を有し」という事項は本願の当初明細書等に記載された事項の範囲内のものであり、したがって、平成３１年１月９日付け手続補正書でした補正は特許法第１７条の２第３項に規定する要件を満たすものであると思料します。

２．２　理由２（進歩性）について

審査官殿は、請求項１～１１に係る発明が原出願である引用文献６（特表２０１５-５１８８１６号公報）により進歩性に欠けると指摘しています。この指摘は、本願が分割の実体的要件を満たすものではないという審査官殿の主張に依拠するものであるところ、先に述べたとおり、「８０ｎｍ～１５０ｎｍの平均粒径を有し」という事項は本願の当初明細書等に記載された事項の範囲内のものであり、したがって、平成３１年１月９日付け手続補正書でした補正は特許法第１７条の２第３項に規定する要件を満たすものです。よって、本願は分割の実体的要件を満たすものであり、引用文献６に基づく進歩性欠如の拒絶理由は取り下げられるべきものと思料します。

審査官殿はまた、請求項１～１１に係る発明が引用文献１～５により進歩性に欠けるとも指摘しています。これに対して出願人は以下に述べるとおり異存があります。

まず、審査官殿は請求項１～１１に係る発明が引用文献１～５により進歩性に欠けることを説明する中で、引用文献２（特表２０１２-５０５２５０号公報）が平均粒径９０～１３０ｎｍの脂質ナノ粒子を開示する旨のことを述べています。しかしながら、引用文献２の開示はほぼ全体にわたり低分子干渉ＲＮＡ（ｓｉＲＮＡ）の細胞内送達に焦点を当てたものであり、代替の適用例としてＤＮＡまたはｍＲＮＡ配列の送達が段落０１９７において簡単に触れられているに過ぎません。また、引用文献２において開示されているデータは、平均粒径が６４～７２ｎｍのナノ粒子（表１０を参照）を使用したｓｉＲＮＡのカプセル化及び送達についてのもののみです。

先に述べたとおり、本願明細書中の表５３、表５４、表５６及び表５７には、脂質ナノ粒子の平均粒径が８０ｎｍを下回る場合、脂質ナノ粒子で封入されたポリヌクレオチドによりコードされるタンパク質の発現レベルが有意に低下することが開示されており、本願明細書中の表１４６、表１４７、表１５８、表１５９、表１６４及び表１６５には、脂質ナノ粒子の平均粒径が１５０ｎｍを超える場合もまた、脂質ナノ粒子で封入されたポリヌクレオチドによりコードされるタンパク質の発現レベルが低下することが開示されています。引用文献１～５のいずれにも、このような結果を示唆するような記載を認めることはできません。

また、平均粒径が８０～１５０ｎｍである脂質ナノ粒子を使用してポリヌクレオチドを封入することにより奏される本願発明の有利な効果は当業者が予期しうるものではありません。審査官が引用している引用文献２の段落０１８７には、「好ましくは、本明細書において提供する組成物は平均直径約７０ｎｍから約２００ｎｍ、より好ましくは約９０ｎｍから約１３０ｎｍにサイズ化される。」との記載が確かにあります。しかしながら、引用文献２には「約９０ｎｍから約１３０ｎｍ」の組成物が好ましいことを裏付けるような結果の開示はありません。また、「約９０ｎｍから約１３０ｎｍ」の組成物が好ましいとすると、引用文献２に開示のある平均粒径が６４～７２ｎｍのナノ粒子の実施例はその範囲から外れてしまうことになります。一方、引用文献の段落０１８０には、「一実施形態では、脂質混合物を、核酸の緩衝水溶液と組み合わせ、脂質粒子内にカプセル化された核酸を含有する、中間混合物を作製し、上記カプセル化核酸は、核酸／脂質の比が約３ｗｔ％対約２５ｗｔ％、好ましくは５ｗｔ％対１５ｗｔ％で存在する。上記中間混合物は、場合により、脂質-カプセル化核酸粒子が得られるようにサイズ化でき、上記脂質部分は、好ましくは直径３０ｎｍから１５０ｎｍ、より好ましくは約４０ｎｍから９０ｎｍを有す単層ベシクルである」との記載があり、さらに段落０１８１には、「上記ベシクルは、約３０ｎｍから約１５０ｎｍ、より好ましくは約３０ｎｍから約９０ｎｍの範囲のサイズを有する」との記載があり、引用文献２に開示のある平均粒径が６４～７２ｎｍのナノ粒子の実施例はこれらの範囲から外れていません。してみると、引用文献２の教示は本願発明の「８０ｎｍ～１５０ｎｍ」よりも小さい平均粒径の脂質ナノ粒子を使用する動機づけを当業者に与えるものであると思料します。

さらに言えば、引用文献２は、脂質ナノ粒子で封入されたｓｉＲＮＡに関するものであることから、脂質ナノ粒子で封入されたポリヌクレオチドによりコードされるタンパク質を発現させようとする場合に、脂質ナノ粒子の平均粒径の重要性について何らかの指針を提供するものではありません。参考文献として、KauffmanらによるNano. Lett. 2015, 15, 7300-7306を添付しています。この添付の参考文献の中で述べられているように、ｓｉＲＮＡを封入する脂質ナノ粒子とｍＲＮＡを封入する脂質ナノ粒子について、粒径を始めとする種々の特徴を最適化した場合、結果として得られる脂質ナノ粒子は、ｓｉＲＮＡの場合とｍＲＮＡの場合とでは異なるものになります。この参考文献のABSTRACTには、「the optimized lipid nanoparticle formulation did not improve siRNA delivery, indicating differences in optimized formulation parameter design spaces for siRNA and mRNA.」と記載されています。Kauffmanらは、最適化した脂質ナノ粒子を用いることにより、ｍＲＮＡの送達が有意に改善される一方、ｓｉＲＮＡの送達については何の改善ももたらされない旨のことも見出しており、「siRNA-loaded LNPs may be more tolerant than mRNA-loaded LNPs of design space differences.」と述べています。したがって、当業者であれば、ｍＲＮＡの送達に関する指針を求めて引用文献２に目を向けることはあり得ません。また、引用文献１，３～５はいずれも、脂質ナノ粒子を使用してｍＲＮＡを送達することを開示していないため、引用文献２が開示していない本願発明の特徴を補うものでもありません。

以上の理由から、本願請求項１に係る発明は、引用文献１～５に基づいて当業者が容易に発明をすることができたものではなく、進歩性を有すると思料します。また、請求項１を引用する請求項２～１１に係る発明についても少なくとも同じ理由で進歩性を有すると思料します。

３．　むすび

以上説明したとおり、本願に対する拒絶理由はすべて解消したものと思料します。よって、本願に対し特許すべき旨の査定を賜りたく存じます。

［参考文献］　Nano. Lett. 2015, 15, 7300-7306

NANO LETTERS

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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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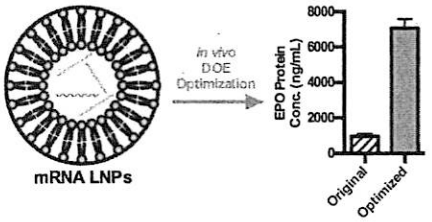
Supporting Information

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

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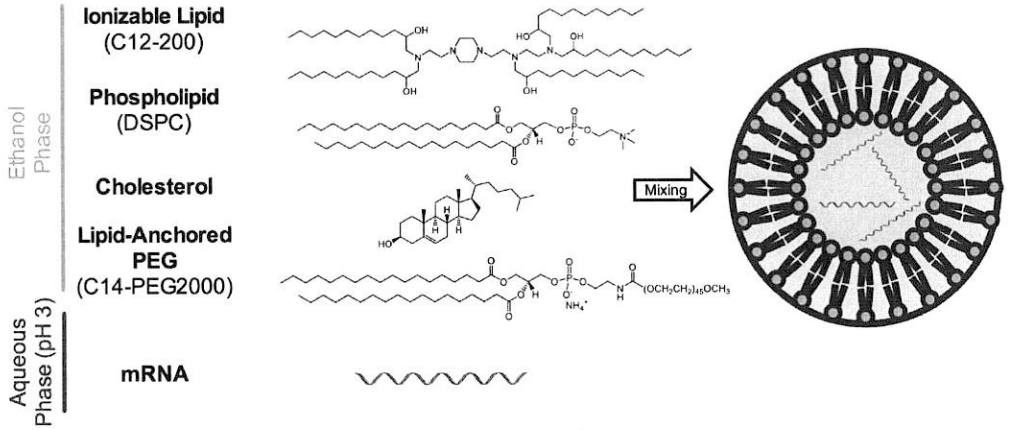

Figure 1. Formulation of lipid nanoparticles. Lipid nanoparticles (LNPs) are synthesized by the mixing of two phases: (1) a four-component ethanol phase containing ionizable lipid, helper phospholipid, cholesterol, and lipid-anchored PEG; (2) an acidic aqueous phase containing mRNA.

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 delivery. We further hypothesized that formulated mRNA may 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 intravenously at 15 μg of total mRNA per mouse and resulted

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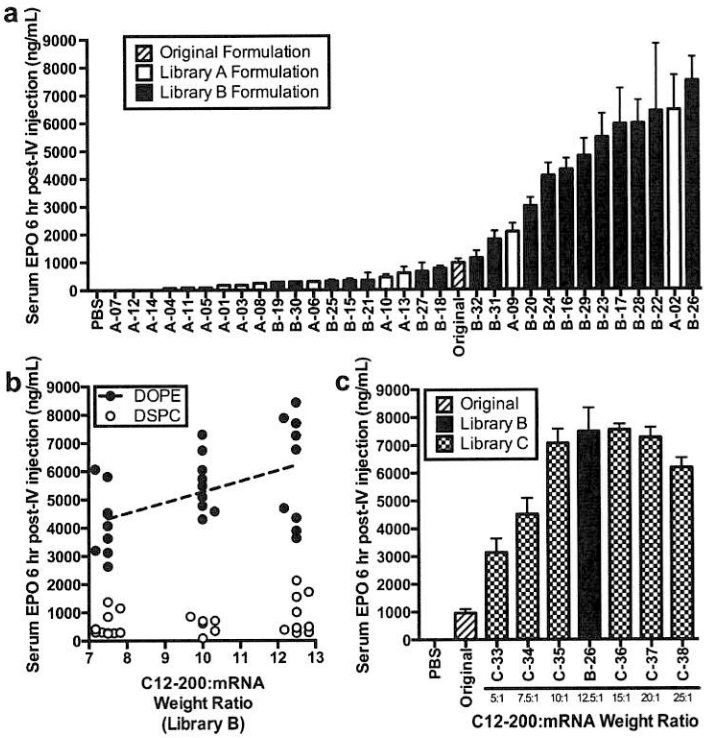

Figure 2. Efficacy results of LNPs in Libraries A, B, and C. (a) Serum EPO concentration 6 h post-intravenous injection of 15 μg total mRNA for each formulation in Libraries A and B, including the original formulation (data presented as mean + SD, $n = 3$). (b) A statistically significant trend of increasing 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) and one four-level qualitative factor (phospholipid type), this 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 phenomenon that has been previously observed in the

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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 serum EPO concentration up to a certain point (Figure 2c); it 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 between 6.2 and 6.5. It appears that in our C12-200 mRNA

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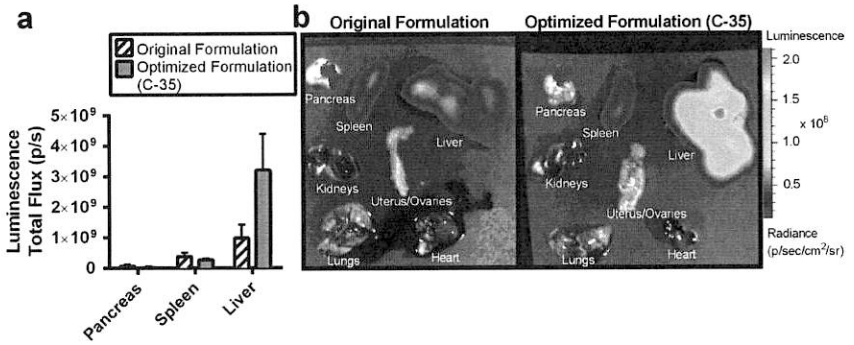

Figure 3. Efficacy and biodistribution of original and C-35 formulation with Luc mRNA. (a) Efficacy of original and C-35 LNP formulations synthesized with mRNA coding for luciferase in three organs of interest as measured by total flux from luminescence 6 h after intravenous injection of 15 μg total 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 C-35 and the original formulations with FVII siRNA were

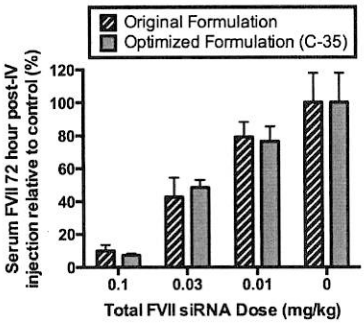

Figure 4. Efficacy of original and C-35 formulation with siRNA. Efficacy of original versus optimized C-35 formulation made with C12-200 and siRNA coding against Factor VII (FVII) protein as measured by serum FVII levels 72 h post-intravenous injection of various doses of total siRNA. FVII levels were 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 Experiment principles. Although C-35 significantly improved

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mRNA delivery with mRNA's of two different lengths, C-35 was surprisingly equally as efficacious for siRNA delivery as the original siRNA-optimized formulation. We believe that the optimized formulations described here may provide a basis for further formulation optimization with other mRNA delivery materials as well. Furthermore, the generalized approach we described for in vivo optimization of multicomponent nanoparticle formulations may accelerate the discovery of more potent formulations with other materials and drug payloads.

Methods. *Lipid Nanoparticle Synthesis.* The ethanol phase was prepared by solubilizing with ethanol a mixture of C12-200 (prepared as previously described,[6] courtesy of Alnylam Pharmaceutics, Cambridge, MA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids, Alabaster, AL), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE, Avanti), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti), cholesterol (Sigma), and/or 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (C14-PEG 2000, Avanti) at predetermined molar ratios. The aqueous phase was prepared in 10 mM citrate buffer (pH 3) with either EPO mRNA (human erythropoietin mRNA, courtesy of Shire Pharmaceuticals, Lexington, MA), Luc mRNA (Firefly luciferase mRNA, Shire), or FVII siRNA (Factor VII siRNA,[7] Alnylam). Syringe pumps were used to mix the ethanol and aqueous phases at a 3:1 ratio in a microfluidic chip device.[26] The resulting LNPs were dialyzed against PBS in a 20 000 MWCO cassette at 4 °C for 2 h.

mRNA Synthesis. mRNA was synthesized by in vitro transcription from a plasmid DNA template encoding the gene, which was followed by the addition of a 5′ cap structure (Cap 1) using a vaccinia virus-based guanylyl transferase system. A poly(A) tail of approximately 300 nucleotides was incorporated via enzymatic addition employing poly-A polymerase. Fixed 5′ and 3′ untranslated regions were constructed to flank the coding sequences of the mRNA.

LNP Characterization. To calculate the nucleic acid encapsulation efficiency, a modified Quant-iT RiboGreen RNA assay (Invitrogen) was used as previously described.[38] The size and polydispersity (PDI) of the LNPs were measured using dynamic light scattering (ZetaPALS, Brookhaven Instruments). Zeta potential was measured using the same instrument in a 0.1× PBS solution. Size data is reported as the largest intensity mean peak average, which constituted >95% of the nanoparticles present in the sample. The pK_a was determined using a TNS assay as previously described.[38] To prepare LNPs for Transmission Electron Microscopy (TEM), LNPs were dialyzed against water and negative staining was performed with 2% uranyl acetate. LNPs were then imaged with a Tecnai Spirit transmission electron microscope (FEI, Hillsboro, OR).

Animal Experiments. All animal studies were approved by the M.I.T. Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. Female C57BL/6 mice (Charles River Laboratories, 18–22 g) were intravenously injected with LNPs via the tail vein. After 6 or 72 h, blood was collected via the tail vein with serum separation tubes, and the serum was isolated by centrifugation. Serum EPO levels were measured using an ELISA assay (Human Erythropoietin Quantikine IVD ELISA Kit, R&D Systems, Minneapolis, MD). Serum FVII levels were measured using a chromogenic assay (Biophen FVII, Aniara Corporation, West Chester, OH) and compared with a standard curve obtained from control mice. Six hours after administration of Luc mRNA LNPs, mice were administered an intraperitoneal injection of 130 μL of D-luciferin (30 mg/mL in PBS). After 15 min, the mice were sacrificed, and eight organs were collected (liver, spleen, pancreas, kidneys, uterus, ovaries, lungs, heart). The organs' luminescence were analyzed using an IVIS imaging system (PerkinElmer, Waltham, MA) and quantified using LivingImage software (PerkinElmer) to measure the radiance of each organ in photons/sec.

Statistics. Design of Experiment (DOE) was performed, and statistical data were analyzed using JMP software (SAS, Cary, N.C.). In this study, statistical significance was defined as p-values less than 0.05. Three mice per formulation/dose ($n = 3$) were used for all in vivo experiments. For Library A, a $3^4 \times 2^2$ Definitive Screening Design[30] was used with 4 three-level quantitative factors (C12-200 RNA weight ratio, C12-200 mol %, phospholipid mol %, and PEG mol %) and 2 two-level qualitative factors for phospholipid tail group (DS = 1,2-distearoyl-*sn*-glycero- and DO = 1,2-dioleoyl-*sn*-glycero-) and phospholipid headgroup (PC = 3-phosphocholine and PE = 3-phosphoethanolamine). For Library B, a $3^4 \times 2^1$ L-18 Taguchi Fractional Factorial Design[29] was used with 4 three-level quantitative factors (C12-200 RNA weight ratio, C12-200 mol %, phospholipid mol %, and PEG mol %) and 1 two-level qualitative factor for phospholipid (DSPC or DOPE). To make the Standard Least Squares regression model for Library B, a full model with all orthogonal and second-order effects was generated and subsequently reduced until only statistically significant effects remained in the model as determined by ANOVA. A posthoc Tukey test was performed using JMP to verify that the two levels of phospholipid effect were statistically different ($p < 0.0001$). When comparing means between two groups, a Student's t test was used assuming a Gaussian distribution and unequal variances. Further details about statistics and models used in this study, including ANOVA results, parameter estimates, residuals, etc., can be found in Table S2, Figure S1, and the Supplementary Methods section.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.5b02497.

Description of Library B statistical model, nanoparticle characterization for all LNP formulations (including formulation composition, encapsulation efficiency, size, polydispersity, and efficacy measurements), additional structure/function relationships for Library A, and a detailed description of the statistical methodologies used including a flowchart (PDF)

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Notes

The authors declare no competing financial interest.

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