Published in final edited form as:

Biomaterials. 2016 December ; 109: 78–87. doi:10.1016/j.biomaterials.2016.09.006.

Efficacy and Immunogenicity of Unmodified and Pseudouridine-Modified mRNA Delivered Systemically with Lipid Nanoparticles *in Vivo*

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Abstract

mRNA has broad potential for treating diseases requiring protein expression. However, mRNA can also induce an immune response with associated toxicity. Replacement of uridine bases with pseudouridine has been postulated to modulate both mRNA immunogenicity and potency. Here, we explore the immune response and activity of lipid nanoparticle-formulated unmodified and pseudouridine-modified mRNAs administered systemically *in vivo*. Pseudouridine modification to mRNA had no significant effect on lipid nanoparticle physical properties, protein expression *in vivo*, or mRNA immunogenicity compared to unmodified mRNA when delivered systemically with liver-targeting lipid nanoparticles, but reduced *in vitro* transfection levels. Indicators of a transient, extracellular innate immune response to mRNA were observed, including neutrophilia, myeloid cell activation, and up-regulation of four serum cytokines. This study provides insight into the immune responses to mRNA lipid nanoparticles, and suggests that pseudouridine modifications may be unnecessary for therapeutic application of mRNA in the liver.

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Introduction

Messenger RNA (mRNA) delivery is emerging as an attractive strategy for the treatment of a variety of diseases.¹ Nanoparticle delivery vehicles, including lipid nanoparticles (LNPs), polymer nanoparticles, viruses, and others, have been developed as tools to encapsulate and deliver RNAs into the cytoplasm, as naked nucleic acids cannot readily cross cell membranes.^{2–4} *In vitro* transcribed (IVT) mRNA is known to activate various pattern recognition receptors, including toll-like receptors^{5,6} (TLR3, TLR7, TLR8), RIG-I,⁷ and RNA-dependent protein kinase (PKR),⁸ leading to an antiviral immune response.⁹ Synthetic base modifications have been reported to mitigate immunogenicity and improve the stability of small interfering RNAs (siRNAs)¹⁰, and it has been hypothesized that similar strategies could also prove beneficial for mRNA therapeutics.¹¹ To reduce the immunogenicity and increase stability of therapeutic mRNAs, the use of naturally-occurring base modifications has been proposed, including pseudouridine,¹² 5-methylcytidine,¹³ 2-thiouridine,¹⁴ N⁶-methyladenosine¹⁵, and N¹-methylpseudouridine.¹⁶ However, one disadvantage of base-modified mRNAs is the potential for decreased translational capacity of the mRNA.^{12,17}

One common approach to mRNA base modifications is the 100% substitution of uridine with pseudouridine (PseudoU), which is sometimes used in combination with 5-methylcytidine replacement of cytidine.^{18,19} Karikó and co-workers performed pioneering work with PseudoU modification to mRNA, finding that PseudoU-modified mRNAs resisted ribonuclease (RNase) degradation,²⁰ reduced activation of TLRs⁶ and PKR⁸, and interestingly resulted in improved translational efficacy both *in vitro* and *in vivo* compared to unmodified mRNAs when delivered with both liposomal and non-liposomal formulations.^{12,21} However, Thess *et al.*²² recently reported that that PseudoU modifications significantly diminished mRNA translation and that codon-optimized unmodified mRNAs did not upregulate three measured pro-inflammatory cytokines *in vivo* when delivered intraperitoneally with a lipid/polymer non-liposomal transfection reagent. We hypothesize that these differing literature reports regarding the immunogenicity and efficacy of PseudoU-modified mRNAs *in vivo* (summarized in Table 1) may be due to 1) limited analysis²³ of the extracellular innate immune response to mRNAs *in vivo* and 2) the use of different delivery vehicles, methodologies, and material properties between studies. It has been previously demonstrated that delivery material properties^{24–27} can dramatically affect the potency of encapsulated nucleic acids, and Pardi *et al.* recently reported that 1-methylpseudouridine-modified mRNA-LNPs have different kinetics and efficacy *in vivo* depending on the route of administration.²⁸

The effect of PseudoU modifications on mRNA immunogenicity and efficacy remains an outstanding question with important implications for therapeutic application of mRNA. Therefore, in the present work we aimed to perform a thorough analysis of the extracellular innate immune response to and efficacy of mRNA with and without PseudoU modifications *in vivo* using mice. To maximize protein expression, mRNA was delivered intravenously²⁸ using LNPs based on the lipid-like material C12-200,^{24,29} which have recently been studied therapeutically in a hemophilia B mouse model.³⁰ Our results indicate that PseudoU modification of mRNA does not measurably change the LNP physical properties and neither improves the efficacy nor reduces the immunogenicity of the mRNA-LNP when delivered

systemically. Furthermore, at high mRNA doses we discovered indicators of a yet-uncharacterized, transient extracellular innate immune response to intravenously-delivered mRNA-LNPs, including cytokine elevation, neutrophilia, and myeloid cell activation in the blood and spleen. In all, these data do not indicate improved *in vivo* performance with PseudoU modification of mRNA, and suggest that unmodified mRNA may be at least as suitable for systemic mRNA-LNP administration.

Results

1. mRNA Synthesis and LNP Formulation

mRNAs of three different sequences were synthesized with and without 100% pseudouridine (PseudoU) replacement of uridine (Fig. 1a). Following *in vitro* transcription (IVT), mRNA products were 5'-capped with Cap1 and 3'-polyadenylated, yielding mature mRNAs at their respective predicted lengths. (Fig. 1b, Fig. S1). Electrophoresis size fractionation was performed on all synthesized mRNAs to confirm the absence of smaller length fragments (Fig S2). In general, we found that IVT PseudoU-modified mRNAs had significantly lower yields, typically half those of unmodified mRNAs (Table S1).

PseudoU has an extra hydrogen-bond donor (shown as asterisk on Fig. 1a) that allows for increased local RNA stacking, more thermodynamically favorable duplex formation, and a more rigid sugar-phosphate backbone.^{31,32} We observed that PseudoU-modified mRNAs migrate at a slightly lower observed molecular weight than their unmodified counterparts on Bioanalyzer (Fig. 1b) and agarose gel (Fig. S1), which could be attributed to the tighter packing and thus smaller hydrodynamic volume of PseudoU-modified mRNAs. However, this difference in mRNA secondary structure imparted by PseudoU modifications does not affect mRNA loading into lipid nanoparticles (LNPs). When mRNAs were encapsulated into C12-200 LNPs (Fig. S3) using formulation parameters previously optimized specifically for mRNA delivery,²⁴ all LNPs had similar diameters (approximately 80 nm, Fig. 2a) and mRNA encapsulation efficiencies (approximately 55–65%, Fig 2b) regardless of mRNA length or PseudoU modification. Visualized by Cryogenic Transmission Electron Microscopy, both unmodified and PseudoU-loaded mRNA LNPs had similar, spherical morphologies (Fig. 2c,d).

2. Efficacy of Unmodified vs. PseudoU-modified mRNA LNPs

The effect of PseudoU modification on mRNA translation efficiency when delivered with LNPs was assessed both *in vitro* and *in vivo*. In this study, mRNAs coding for two proteins (i.e. mRNAs of different lengths) were tested: 1) Firefly luciferase (Luc), a model non-secreted luminescent protein, and 2) Erythropoietin (EPO), a secreted serum protein with therapeutic applications in anemia.²¹ In HeLa cells *in vitro*, unmodified mRNA-LNPs were approximately twice as potent as PseudoU-modified mRNA-LNPs at multiple doses for both EPO-encoding (Fig. 3a) and Luc-encoding (Fig. 3b) mRNAs. In a similar experiment, Thess *et al.*²² transfected HeLa cells with mRNA lipoplexes (Lipofectamine 2000) and reported that unmodified mRNAs led to significantly higher protein expression than PseudoU-modified mRNAs for both Luc and EPO. Here, we show a similar result across multiple

doses *in vitro* using LNPs, which have better serum stability, longer circulation times, and are less prone to aggregation than lipoplexes *in vivo*^{33,34}.

LNPs formulated with either unmodified or PseudoU-modified mRNA were next evaluated for efficacy *in vivo* following systemic delivery. Contrasting the *in vitro* findings, no significant difference in EPO expression between unmodified and PseudoU EPO mRNA-loaded LNPs was observed following intravenous administration in mice at doses ranging between 0.125 mg/kg and 0.5 mg/kg (Fig. 4a). To our knowledge, this is the first report that PseudoU modifications do not improve the potency of mRNA when delivered intravenously with LNPs in a mouse model. Similarly, no significant differences between unmodified and PseudoU Luc mRNA-loaded LNPs were observed for Luc expression in the spleen and liver (Fig. 4b), the only two organs which had measurable Luc expression for either treatment. Further, biodistribution was not altered by PseudoU modification of mRNA, as protein expression occurred predominately in the liver, consistent with previous reports.^{24,29}

3. Assessing Immunogenicity for Unmodified vs. PseudoU-modified mRNA-LNPs

The next objective was to determine if PseudoU modifications altered the immunogenicity of mRNA when delivered systemically *in vivo* using LNPs. Unmodified and PseudoU-modified mRNAs with a scrambled coding region of the same length as EPO were used to ensure that any immunogenicity was not in response to translated exogenous protein. Furthermore, LNPs formulated without mRNA were used as a control to parse out the immunogenicity of mRNA from that of the lipid delivery materials.

Previous studies^{12,21,22} investigating the immunogenicity of PseudoU-modified mRNAs were limited to measuring a small number of common pro-inflammatory cytokines *in vivo* following administration of non-LNP formulations (Table 1). Here, we measured a panel of over thirty cytokines in mouse serum six hours post-intravenous injection of mRNA-LNPs with and without PseudoU modifications (Fig. 5a). When mRNA-LNPs were injected intravenously at high doses, we observed varying degrees of elevation in the levels of a variety of interleukins, colony stimulating factors, and chemokines at six hours post-injection relative to a control of LNPs without mRNA. The most upregulated cytokines (G-CSF, MCP-1, RANTES, MIG) were confirmed to be dependent on dose of mRNA (Fig. 5b), and at sufficiently low doses of mRNA, cytokine response was minimal. Importantly, PseudoU-modified mRNA did not substantially reduce these serum cytokine levels compared to unmodified mRNA at six hours post-injection. Moreover, PseudoU modification of mRNA did not reduce mRNA-LNP liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) (Fig. S4).

The observation of elevated cytokines that are produced by and influence myeloid cells led us to question the effect of mRNA-LNPs on blood and splenic immune cell distribution and activity. Immune cell distributions in the blood and spleen were therefore measured at six hours post-intravenous injection of mRNA-LNPs. At a sufficiently high dose of mRNA (0.5 mg/kg), we observed neutrophilia (increased percentage of neutrophils among total immune cells) in both the blood (Fig. 6a) and the spleen (Fig. 6b). In addition, neutrophils in both the blood and spleen expressed significantly higher levels of cell-surface proteins that are markers for neutrophil activation, including CD14 and CD69, with CD11b also increased in

the spleen (Fig. 6c). Notably, neutrophil levels returned to baseline (Fig. S5a, b) and activation markers were no longer upregulated 72 hours post-injection (Fig. S6c), suggesting that neutrophilia induced by mRNA administration is a transient response. Correspondingly, a small but statistically significant drop in non-neutrophil myeloid cells was observed at 6 hours post-intravenous administration of mRNA-LNPs (Fig. 6a, b), with a return to baseline 72 hours post-injection (Fig. S5a, b).

In the spleen, dendritic cells and macrophages expressed higher levels of the activation marker CD86, but not other markers such as MHC-II and CD80, at 6 hours post-injection (Fig. 6c) with a return to baseline 72 hours post-injection (Fig. S5c). Similar increased CD86 expression has been observed on bone marrow-derived dendritic cells following lipoplexed antigen-encoding mRNA administered subcutaneously in mice (Pollard *et al.*³⁵). At 72 hours, the spleen mass had increased for both unmodified and PseudoU-modified mRNA-LNPs (Fig. S6), but no other differences were observed at 72 hours post-injection compared to vehicle-injected or PBS-injected mice. Prominently in this study, unmodified and PseudoU-modified mRNA-LNP administration resulted in similar changes in myeloid cell distribution and activation, suggesting that PseudoU modification does not reduce immune activation when mRNAs are delivered systemically with LNPs.

Discussion

The broad use of mRNA in the clinic requires the development of safe and effective delivery systems. One method to vary mRNA potency and immunogenicity is through RNA base modifications, with 100% pseudouridine (PseudoU) replacement of uridine (Fig. 1) being the most commonly reported. The systemic use of mRNA requires a delivery vehicle to ensure cellular uptake, target specific tissues/cell types, and prevent mRNA degradation.⁴ Recently, LNPs have been demonstrated as efficient mRNA delivery vehicles and have been studied *in vivo* in a therapeutic context^{22,35,36}. Findings here demonstrate that the properties of mRNA-loaded LNPs – specifically, encapsulation efficiency, diameter, and morphology – do not change as a result of PseudoU modification to the mRNA payload (Fig. 2) in spite of the different secondary structure imparted through PseudoU-modification of mRNA.

The literature contains different effects of PseudoU modification on mRNA immunogenicity and potency when delivered using a nanoparticle vehicle. While Karikó and colleagues reported that PseudoU modifications increased the efficacy of intravenously-administered, spleen-targeting lipofectin-complexed mRNA¹² and intraperitoneally-administered, spleen-targeting TransIT-complexed (a commercial non-liposomal lipid/polymer transfection reagent) mRNA²¹, Thess *et al.* have reported that PseudoU modifications *decreased* the efficacy of intraperitoneally-administered, codon-optimized mRNA delivered with TransIT²². As shown in Table 1, we hypothesize that these differences on the effect of PseudoU modifications result from some combination of four variables that are different from experiment-to-experiment: 1) delivery material (LNP vs. liposomal vs. non-liposomal lipid/polymer reagent), 2) route of administration (intravenous vs. intraperitoneal), 3) transfected cell type (liver vs. spleen), and 4) mRNA properties (different UTR's, codon-optimized,³⁷ HPLC-purified³⁸).

In our experiments, we chose a delivery material (LNP) and route of administration (intravenous) with clinical potential and relatively high protein expression per dose of mRNA. Using these conditions, we sought to evaluate the effect of PseudoU mRNA modifications on efficacy by using two different mRNA sequences both *in vitro* (Fig. 3) and *in vivo* (Fig. 4). Our results indicate that 100% PseudoU modified and unmodified mRNAs have similar efficacies when delivered intravenously using these LNPs in wild-type mice, but interestingly, PseudoU mRNA-LNPs resulted in significantly lower translation *in vitro* in HeLa cells. *In vitro* experiments do not fully recapitulate the complexities of the *in vivo* environment, and a previous study found that the efficacy of siRNA-LNPs *in vitro* did not always correlate with the efficacy found *in vivo*.³⁴ In the case of mRNA-LNPs, the increased resistance imparted by PseudoU modifications to mRNA degradation may be more significant *in vivo*, or perhaps HeLa cells and hepatocytes process PseudoU-modified mRNAs differently.

In addition, increased levels of cytokines including MCP-1, RANTES, G-CSF, and MIG (Fig. 5) were observed following administration of high doses of both unmodified and PseudoU-modified mRNA-LNPs. Elevation of these cytokines has been previously implicated in the innate immune response to foreign nucleic acids: in human fetal membranes, viral ssRNA was found to upregulate MCP-1, RANTES, and G-CSF, among other cytokines (MIG was not tested).³⁹ Additionally, it was reported that MIG and RANTES were both significantly elevated following mRNA transfection of A549 cells *in vitro*,⁴⁰ and the RANTES secretion from A549 cells did not appear to be significantly different between lipoplexed unmodified and PseudoU mRNA.¹⁶ These four cytokines are involved in neutrophil recruitment and function: G-CSF controls neutrophil proliferation, differentiation, and trafficking,⁴¹ and the other three (MCP-1, RANTES, MIG) are inflammatory chemokines, typically signaling recruitment of more myeloid cells such as neutrophils to sites of inflammation or infection.⁴²

Indeed, neutrophilia and neutrophil activation were also observed in response to both unmodified and PseudoU-modified mRNA-LNPs at 6 hours post-injection (Fig. 6). Neutrophilia and inflammatory cytokine response are commonly observed as part of an innate immune response to RNA-based viral infectious agents.⁴³ mRNA-loaded lipid nanoparticles share several structural similarities to some types of ssRNA viruses. Like mRNA-LNPs, ssRNA viruses can have spherical morphologies, a diameter on the order of 100 nm, and single-stranded RNA decorating the outside of the particle.⁴⁴ Cytokine production and neutrophilia have been observed in response to ssRNA viruses (including West Nile Virus,⁴⁵ SARS,⁴⁶ and Hantavirus⁴⁷) through the activation of receptors that recognize pathogen-associated and damage-associated molecular patterns. Our data suggests that regardless of PseudoU modification, the innate immune system may interpret mRNA-laden LNPs at high enough doses through similar molecular pattern recognition mechanisms. However, of importance is the observation that these responses are hyper-acute (observed 6 hours post-injection) and transient (returned to baseline by 72 hours).

These findings provide additional perspective on the existing literature (Table 1) to provide a more comprehensive description of the *in vivo* extracellular innate immune response to mRNA-loaded LNPs. Our results suggest that intravenously-injected mRNA-LNPs can

interact with phagocytic myeloid cells in the bloodstream and neutrophils specifically are activated and secrete several cytokines. Circulating neutrophils are also observed in higher percentages and are activated in the spleen. We postulate that either activated neutrophils or blood monocytes which interact with mRNA-LNPs activate dendritic cells and macrophages in the spleen. Moreover, encapsulated mRNA is expressed predominately in the liver with some expression occurring in the spleen (Fig. 7). Independent of conclusions based on mRNA modification, these results contribute mechanistic insight into the nature of the innate immune response elicited by therapeutic mRNAs in circulation. Further, this model suggests potential approaches to reduce this immune response by decreasing blood concentration of mRNA-LNPs and thereby minimizing their interaction with circulating myeloid cells. For example, it may be possible to mediate these effects through a gradual intravenous drip of mRNA-LNPs rather than bolus infusion.

Given that LNP encapsulation protects unmodified mRNAs from RNase degradation (Fig S7), the stability imparted by the PseudoU modifications against circulating RNases in the bloodstream may be unnecessary when the mRNA is delivered with LNPs. It is also noteworthy that incorporation of PseudoU into mRNA significantly reduces IVT yields (Table S1) and PseudoU triphosphate is currently much more expensive than uridine triphosphate when obtained commercially at small scales. Our results suggest that PseudoU modifications do not improve the efficacy or alter immunogenicity *in vivo*, and indicate that unmodified mRNAs may be preferred in future applications requiring systemic delivery of mRNA-LNPs. Future work will be necessary to determine if other mRNA base modifications do in fact improve efficacy or alter immunogenicity of the mRNA when delivered systemically with LNPs. For example, N(1)-methylpseudouridine has recently been reported¹⁶ to result in significantly higher translation *in vivo* compared to unmodified mRNAs when delivered with lipofectamine/mRNA complexes via intramuscular and intradermal administration; however, it remains to be seen if these results change with different delivery vehicles, administration routes, and mRNA properties as listed in Table 1.

For certain therapeutic applications such as mRNA-based vaccines, an immune response to mRNA-LNPs may be desired. For other applications, an inappropriate immune response could result in unwanted toxicity. The immune responses observed here were measurable but transient in nature, with nearly all markers of immunogenicity returning to baseline levels after 72 hours. Additionally, while transient neutrophilia can be a sign of infection, it is not necessarily harmful in and of itself, as significant increases in neutrophil counts are also observed after vigorous exercise⁴⁸ or during pregnancy⁴⁹. Furthermore, at low doses of mRNA-LNPs (0.125 mg/kg), no significant immune response was observed.

In conclusion, we have evaluated PseudoU-modified and unmodified mRNA when encapsulated in C12-200 LNPs by measuring nanoparticle physical properties, *in vitro* and *in vivo* potency, and *in vivo* immunogenicity. PseudoU modifications to mRNA did not result in changes to LNP size, morphology, or encapsulation efficiency. Furthermore, PseudoU modification of mRNA did not improve the efficacy of the mRNA-LNP *in vitro* or *in vivo*. Lastly, we identified three previously-unreported indicators of a modest and transient extracellular innate immune response to mRNA-LNPs – myeloid cell activation, neutrophilia, and cytokine elevation – but determined that PseudoU modification of mRNA

does not reduce this immune response. In all, we report no benefit in these studies for using PseudoU-modified mRNAs when delivered systemically with lipid nanoparticles.

Materials and Methods

mRNA Synthesis

DNA plasmids (Invitrogen) containing a T7 promoter upstream of the sequences for luciferase (Luc), or erythropoietin (EPO), or scrambled EPO coding region (scramble) mRNA were used as templates for mRNA synthesis. DNA plasmids were linearized using restriction enzyme XbaI (New England Biolabs, Ipswich, MA) and transcribed using the HiScribe T7 RNA Synthesis Kit (New England Biolabs). To make pseudouridine-modified mRNA, uridine triphosphate was replaced with pseudouridine triphosphate (Trilink, San Diego, CA) during the transcription step. mRNA was capped with the Vaccinia Capping System (New England Biolabs), and the cap was modified to Cap1 using mRNA Cap 2'-O-Methyltransferase (New England Biolabs). PolyA tails were added to the RNA using a Poly(A) Polymerase Kit (New England Biolabs). All mRNAs were purified after the transcription and tailing steps using MEGAClear RNA purification columns (Life Technologies, Beverly, MA). Concentration was determined using a NanoDrop 1000 (Thermo Scientific, Cambridge, MA).

mRNA Sequences

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 100 nucleotides long.

EPO—

AUGGGGGUGCACGAAUGUCCUGCCUGGCUGUGGCUUCUCCUGUCCCUGCUGU
CGCUCCCUCUGGGCCUCCCAGUCCUGGGCGCCCCACCACGCCUCAUCUGUGAC
AGCCGAGUCCUGGAGAGGUACCUUGGAGGCCAAGGAGGCCGAGAAUAUCACGA
CGGGCUGUGCUGAACACUGCAGCUUGAAUGAGAAUAUCACUGUCCCAGACACC
AAAGUAAAUUUCUAUGCCUGGAAGAGGAUGGAGGUCGGGCAGAGCCGUAGAA
GUCUGGCAGGGCCUGGCCCUGCUGUCGGAAGCUGUCCUGCGGGGCCAGGCCCU
GUUGGUCAACUCUCCCAGCCGUGGGAGCCCCUGCAGCUGCAUGUGGAUAAAG
CCGUCAGUGGCUCGCAGCCUACCCACUCUGCUUCGGGCUCUGGGAGCCCAGAA
GGAAGCCAUCUCCCCUCCAGAUGCGGCCUCAGCUGCUCCACUCCGAACAAUCA
CUGCUGACACUUUCCGCAAACUCUUCGGAGCUAUCCAAUUCUCCGGGGAAA
GCUGAAGCUGUACACAGGGGAGGCCUGCAGGACAGGGGACAGAUGA

Scramble—

AUGGUUCGAGGGUGAACGAAGCGACUGUCUCGGCGGUUCCCCUAGCCACGGG
UGAGAGAGUUGACGCCGCGAGUCGCGAGUGGACAGUGCGGCUGCGGCUCGGAC
GUUGACCGACAGUGAGAGGACGGCACAGCAGAGCCCCACACUCCCGUCAGCCA
UUCGCUACCUUGUGAGGCGUUGGGACUCUUUCCGGUGGGGAUCCGCCGAUC
ACGACGCCUUGCUAGACCGGGGGCUGUGAAUCCACCCAAGAUCUUAUCUGGCU

GGGUGGCGACUGCGCGCUUGACGCGCGACCGACCCCGAGCAAAGUCAUAACAUA
AGUGGAGCCUGGUUGUGUGGCGUGUCGAAUACCCCUUUGGCGUGUUCAGGACG
AAGGGGCGUCAUUGUCACCGGUGAUCACCAUCGUGCCAGACCGAGCGCACACU
ACACGAGCUGCUUAGACCGCAUAUCAUACGGAAGGGUCUCCCUACUUCCACG
ACUCCUUUUGACAGUUCUCACAUCUCGUGAGUAGCGCCCGAGCCUUAUUU
ACGUGGCACUAGGCCUCCGACCCAGUCGCCUCACCACACGAAACCCUGUA

Luc—

AUGGAAGAUGCCAAAAACAUAAGAAGGGCCCAGCGCCAUUCUACCCACUCGA
AGACGGGACCGCCGGCGAGCAGCUGCACAAAGCCAUGAAGCGCUACGCCUGG
UGCCCGGCACCAUCGCCUUUAGACGCACAUUCGAGGUGGACAUUACCUACGC
CGAGUACUUCGAGAUGAGCGUUCGGCUGGCGAGAAGCUAUGAAGCGCUAUGGGC
UGAAUACAAACCAUCGGAUCGUGGUGUGCAGCGAGAAUAGCUCAGUUCUUCAU
GCCCGUGUUGGGUGCCUGUUCUUCGUGUGGCGUGGCCCCAGCUAACGACA
UCUACAACGAGCGCGAGCUGCUGAACAGCAUGGGCAUCAGCCAGCCACCGUC
GUAUUCGUAGAAGAAAGGGCUGCAAAAGAUCUCAACGUGCAAAAGAAGCUAC
CGAUCAUACAAAAGAUCAUCAUGGAUAGCAAGACCGACUACCAGGGCUUC
CAAAGCAUGUACACCUUCGUGACUUCUUUCCACCCGGCUUCAACGAGUACG
ACUUCGUGCCCAGAGCUUCGACCGGGACAAAACCAUCGCCCUCAUGAUAAC
AGUAGUGGCAGUACCGGAUUGCCCAAGGGCGUAGCCCUACCGCACCGCCCGCU
GUGUCCGAUUCAGUCAUGCCCGCGACCCCAUCUUCGGCAACCAGAUCAUCCCC
GACACCGCUAUCCUCAGCGUGGUGCCAUUUACACCGGCUUCGGCAUGUUCAC
CACGCUGGGCUACUUUUCUGGGCUUUCGGGUCGUGCUAUGUACCGCUUCGAG
GAGGAGCUAUUCUUGCGCAGCUUGCAAGACUAUAAGAUUCAUUCUGCCCUGCU
GGUGCCCACACUAUUUAGCUUCUUCGCUAAGAGCAUCUCAUGACAAGUACGAC
CUAAGCAACUUGCACGAGAUCCGCGCGGGGGCGCCGCUACAGCAAGGAGGU
AGGUGAGGCCGUGGCCAAACGCUUCCACCUACCAGGCAUCCGCCAGGGCUACG
GCUGACAGAACAAACAGCGCCAUUCUGAUCACCCCCGAAGGGGACGACAAGCC
UGGCGCAGUAGGCAAGGUGGUGCCCUUCUUCGAGGCUAAGGUGGUGGACUUG
GACACCGGUAAGACACUGGGUGUAACCAGCGGGCGAGCUGUGCGUCCGUGGCC
CCAUGAUCAUGAGCGGCUACGUUAACAACCCCGAGGCUACAAACGCUCUCAUC
GACAAGGACGGCUGGCGUCACAGCGGCGACAUCGCCUACUGGGCGAGGACGAG
CACUUCUUCUUCGUGGACCGGCUAAGAGCCUGAUCAAAUACAAGGGCUACCA
GGUAGCCCCAGCCGAACUGGAGAGCAUCCUGCUGCAACACCCCAACAUCUUCG
ACGCCGGGGUCGGGCCUGCCCGACGACGAUGCCGGCGAGCUGCCCGCCGAGU
CGUCGUGCUGGAACACGGUAAAACCAUGACCGAGAAGGAGAUCGUGGACUAUG
UGGCCAGCCAGGUUACAACCGCCAAGAAGCUGGGGUGGUGUUGUGUUCGUGG
ACGAGGUGCCUAAAGGACUGACCGGCAAGUUGGACGCCCGCAAGAUCGCGGAG
AUUCUCAUUAAGGCCAAGAAGGGCGGCAAGAUCGCCGUGUAA

mRNA Characterization

mRNA samples were characterized using the E-Gel iBase Power System with E-Gel EX gels (ThermoFisher) under denaturing conditions with 90% formamide. Gels were imaged using a BioRad ChemiDoc MP imager. Size fractionation was performed with an Agilent 2100 BioAnalyzer (Santa Clara, CA) at an mRNA concentration of 0.6 µg/µL. An RNA ladder

(200, 500, 1000, 2000, 4000, 6000 nt) was used to generate a standard curve to convert Bioanalyzer results from migration time to number of bases.

LNP Synthesis

C12-200 lipid nanoparticles (LNPs) were prepared as previously described.²⁴ Briefly, an ethanol phase containing a mixture of C12-200 (WuXi AppTec, Shanghai, China), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Alabaster, AL), cholesterol (Sigma), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (C14-PEG 2000, Avanti) at a 35:16:46.5:2.5 molar ratio was mixed with an aqueous phase containing 10 mM citrate buffer (pH 3) with mRNA at a 1:3 volume ratio in a microfluidic chip device.⁵⁰ To formulate control LNPs (vehicle only), no mRNA was included in the aqueous phase. The LNPs were then dialyzed against PBS in a 20,000 MWCO cassette at 4°C for 2 hr and no further purification was performed. For consistency, unmodified mRNA-LNPs, PseudoU-modified mRNA LNPs, and control LNPs were formulated in parallel.

LNP Characterization

mRNA encapsulation efficiency (i.e. loading efficiency) was calculated by performing a modified Quant-iT RiboGreen RNA assay (Invitrogen) as previously described.⁵¹ The diameter and polydispersity (PDI) of the LNPs were measured using dynamic light scattering (ZetaPALS, Brookhaven Instruments). Diameter is reported as the largest intensity mean peak average. LNPs prepared for cryogenic transmission electron microscopy (Cryo-TEM) were dialyzed against 0.1x PBS, deposited onto a lacey copper grid coated with a continuous carbon film, and cooled continuously by liquid nitrogen. Using a minimum dose method, imaging was performed using a JEOL 2100 FEG microscope (Jeol, Freising, Germany) operated at 200 kV and a magnification setting of 60,000.

mRNA RNase Degradation

RNase A (ThermoFisher) was incubated with mRNA or mRNA-LNPs at 62.5 mU RNase/ μ g mRNA in a buffer made of 20 mM Tris-HCl, 2 mM EDTA, 1 M NaCl at room temperature for 30 min. RNase was inactivated by adding 6.4 mU Proteinase K / μ g mRNA and incubating at 55°C for 10 min. To extract mRNA from LNPs, an equal volume of phenol-chloroform was added, the tube was vortexed vigorously, and the aqueous phase (containing the mRNA) was extracted. mRNAs were characterized using E-Gel EX gels as previously described.

In vitro Experiments

HeLa cells (ATCC, Manassas, VA) were cultured in high glucose Dulbecco's Modified Eagle's Medium (ThermoFisher) containing 10% fetal bovine serum and 1% penicillin-streptomycin and maintained at 37°C and 5% CO₂. For transfection experiments, cells were plated at 20,000 cells/well in a 96-well plate. After 24 hr, the media in each well was replaced with 150 μ L of media containing LNPs. After another 24 hr, assays were performed as described below.

For wells containing EPO mRNA-LNPs, 100 μ L of supernatant was removed and measured for EPO concentration using an ELISA assay (Human Erythropoietin Quantikine IVD ELISA Kit, R&D Systems, Minneapolis, MD). Live cell number was quantified using the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, WI). For wells containing Luc mRNA-LNPs, the live cell number was first quantified using the MultiTox-Fluor Multiplex Cytotoxicity Assay. Then, luminescence was measured using the Bright-Glo Luciferase Assay System (Promega). All assays were performed according to manufacturer guidelines, and luminescence/fluorescence was measured using a Tecan infinite M200 Pro microplate reader.

In vivo 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. LNPs were administered to female C57BL/6 mice (Charles River Laboratories, 16 – 20 g) intravenously via the tail vein. For flow cytometry experiments, blood was collected via the tail vein into an EDTA-lined Microvette tube (Sarstedt, Nümbrecht, Germany). For experiments requiring serum, blood was collected via the tail vein into a serum separator tube and serum was isolated by centrifugation. Serum EPO concentration was measured using an ELISA assay as described above. Serum cytokine concentration was measured using the Bio-Plex Pro Mouse Cytokine kits (23-Plex Immunoassay and 9-Plex Assay, Bio-Rad, Hercules, CA) according to the manufacturer's instructions and read with a BioPlex-200 (Bio-Rad) plate reader. Serum liver enzyme concentrations were measured with a Beckman Olympus AU400 Serum Chemistry Analyzer by Charles River Laboratories (Wilmington, MA).

To determine luciferase levels, mice were administered an intraperitoneal injection of D-luciferin (130 μ L, 30 mg/mL in PBS). After 15 min, the mice were sacrificed, organs were collected, and organ luminescence was measured using an IVIS imaging system (PerkinElmer, Waltham, MA) and quantified using LivingImage software (PerkinElmer).

Flow Cytometry

To prepare blood single cell suspensions, 100 μ L of blood was mixed with 1 mL of RBC Lysis buffer and incubated on ice for 10 min. To prepare spleen single cell suspensions, spleens were harvested from mice following euthanasia, manually ground up with forceps in PBS, and passed through a 70 μ m filter. Following centrifugation and removal of supernatant, cells were re-suspended in 1 mL of RBC Lysis buffer and incubated for 10 min. Once single cell suspensions had been made, cells were fixed with 2% paraformaldehyde in flow buffer (PBS containing 0.5% BSA and 2 mM EDTA) for 15 min at 4°C.

Fixed cells were stained with up to eight of the following anti-mouse antibodies at a 1:300 dilution in flow buffer unless otherwise stated: TCR- β (clone H57-597), CD19 (clone 6D5), CD11b (clone M1/70), Ly-6G (clone 1A8), CD45 (clone 30-F11), CD69 (clone H1.2F3), CD14 (clone Sa14-2), MHC-II (I-A/I-E clone M5/114.15.2), CD80 (clone 16-10A1, 1:100 dilution), CD86 (clone GL-1, 1:100 dilution), F4/80 (clone BM8), CD11c (clone N418). All

antibodies were purchased from Biolegend (San Diego, CA). Data was collected using a BD LSR II cytometer (BD Biosciences).

The following identifications of cell populations were used: 1) T cells: CD45⁺, CD11b⁻, TCR-β⁺, 2) B cells: CD45⁺, CD11b⁻, CD19⁺, 3) Neutrophils: CD45⁺, CD11b⁺, Ly-6G⁺, 4) Non-neutrophil myeloid: CD45⁺, CD11b⁺, Ly-6G⁻, 5) Macrophages: CD45⁺, CD11b⁺, F4/80⁺, 6) Dendritic cells: CD45⁺, CD11b⁺, CD11c⁺.

Statistics

When comparing two groups, a Student's *t* test was used assuming a Gaussian distribution with unequal variances. When performing multiple *t* tests for two groups (e.g. dose response and cytokines), multiple comparisons were corrected for using the Holm-Šidák method. For mouse studies, data presented is representative of one independent experiment. Statistical significance was defined with an alpha level of 0.05. All statistical analyses were performed using GraphPad Prism 6 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Shire Pharmaceuticals (Lexington, MA). We wish to thank the Nanotechnology Materials Core Facility, Animal Imaging and Preclinical Testing Core, Flow Cytometry Core, and KI Genomics Core at the Koch Institute at MIT.

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Figure 1. mRNA modification strategy and characterization. (a) Chemical structures of uridine and pseudouridine (PseudoU) bases, where (*) denotes the extra hydrogen-bond donor of PseudoU. (b) Electrophoresis size fractionation performed by an Agilent Bioanalyzer on all six fully 5'-capped and 3'-tailed mRNA's used in this study. L = ladder, 1 = unmodified scramble mRNA, 2 = PseudoU scramble mRNA, 3 = unmodified EPO mRNA, 4 = PseudoU EPO mRNA, 5 = unmodified Luc mRNA, 6 = PseudoU Luc mRNA.

Figure 2. mRNA-loaded LNP synthesis and characterization. (a) Representative size distributions of all mRNA-loaded LNPs and LNP vehicle without mRNA. The legend includes average diameters, presented as mean $\pm$ std. dev. ($n = 5$ for scramble mRNA's and vehicle, $n = 3$ for EPO and Luc mRNAs). (b) mRNA encapsulation efficiency of all mRNA-loaded LNPs. Data presented as mean $\pm$ std. dev. ($n = 5$ for scramble mRNA's, $n = 3$ for EPO and Luc mRNA's). (c) Representative images of unmodified mRNA LNPs visualized by Cryo-EM. (d) Representative images of PseudoU mRNA LNPs visualized by Cryo-EM.

Figure 3.

Unmodified mRNA LNPs are significantly more efficacious *in vitro*. (a) Supernatant EPO concentration as a function of dose for unmodified and PseudoU-modified EPO mRNA LNPs in HeLa cells 24 hr post-transfection. (b) Luciferase expression as a function of dose for unmodified and PseudoU-modified Luc mRNA LNPs in HeLa cells 24 hr post-transfection. Data presented as mean $\pm$ std. dev. (n = 4). Asterisk represents a statistically significant difference.

Figure 4.

Unmodified mRNA LNPs are similarly efficacious *in vivo*. (a) Serum EPO concentration as a function of dose for unmodified and PseudoU-modified EPO mRNA LNPs in mice 6 hr post-intravenous injection. Data presented as mean $\pm$ std. dev. ($n = 4$). (b) Luciferase expression in spleen and liver for unmodified and PseudoU-modified Luc mRNA LNPs 6 hr post-intravenous injection at 0.1 mg/kg. Bar represents mean ($n = 4$).

Figure 5.

Both unmodified and PseudoU-modified mRNA-LNPs elicit a cytokine response. (a) Serum cytokine concentration 6 hr post-intravenous administration of mRNA-LNPs relative to the control (LNPs without mRNA) at an mRNA dose of 0.5 mg/kg (control injected at equivalent lipid dose). Data presented as mean $\pm$ std. dev. ($n = 5$). Asterisk represents a statistically significant difference. (b) Serum G-CSF, MCP-1, RANTES, or MIG concentration 6 hr post-intravenous administration of mRNA-LNPs relative to the control (LNPs without mRNA) at various mRNA doses (control injected at equivalent lipid dose). Bar represents mean ($n = 5$). The legend in (a) applies to all panels.

Figure 6.

Neutrophilia and myeloid cell activation is similar for unmodified and PseudoU-modified mRNA-LNPs. Distribution of immune cell subsets in the blood (a) and spleen (b) measured by flow cytometry at 6 hour post-intravenous injection of mRNA-LNPs at 0.5 mg/kg mRNA dose (control LNPs without mRNA injected at equivalent lipid dose). (c) Increased expression of activation markers on neutrophils in the blood and spleen, and dendritic cells and macrophages in the spleen as measured by flow cytometry at 0.5 mg/kg mRNA dose (control LNPs without mRNA injected at equivalent lipid dose). All data presented as mean +/- std. dev. (n = 4).

Figure 7.
Hypothesized extracellular innate immune response to mRNA-LNPs injected systemically, regardless of PseudoU modification of mRNA.

Table 1

Comparison of studies in the literature comparing efficacy of unmodified and PseudoU mRNA's in mice

Publication	Kariko et al. ¹² , 2008	Kariko et al. ²¹ , 2012	Thess et al. ²² , 2015	Present, 2016
Delivery Vehicle	Lipofectin	TransIT	TransIT	LNP
Route of Administration	i.v.	i.p.	i.p.	i.v.
Primary Organ of Expression	Spleen	Spleen	Spleen	Liver
mRNA Codon-Optimized	No	Yes	Yes	No
mRNA HPLC-purified	No	Yes	No	No
mRNA 5' UTR	<i>Xen.</i> β -globin	TEV	HSD17B4	CMV
mRNA 3' UTR	<i>Xen.</i> β -globin	<i>Xen.</i> β -globin	ALB	hGH
Efficacy Findings	PseudoU more efficacious	PseudoU more efficacious	Unmodified more efficacious	Equal efficacy
Innate Immunogenicity Findings	Unmodified is immunogenic; PseudoU is non-immunogenic.	Unmodified is immunogenic; PseudoU is non-immunogenic.	Unmodified is non-immunogenic (PseudoU not tested)	Equal immunogenicity (high doses); equally non-immunogenic (low doses)
Criteria for Determining Innate Immunogenicity	1 cytokine tested (IFN- α)	3 cytokines tested (TNF- α , IL-6, IFN- γ)	3 cytokines tested (TNF- α , IL-6, IFN- γ)	30+ cytokines tested, blood/spleen neutrophilia, blood/ spleen myeloid cell activation

Note: TransIT is a commercial non-liposomal lipid/polymer transfection reagent. Abbreviations: CMV: cytomegalovirus, hGH: human growth hormone, HSD17B4: hydroxysteroid (17- β) dehydrogenase 4, ALB: albumin, TEV: tobacco etch virus, *Xen.*: Xenopus.