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Research paper

The effect of mRNA-lipid nanoparticle composition on stability during microneedle patch manufacturing

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ABSTRACT

Messenger RNA (mRNA) encapsulated in lipid nanoparticles (LNPs) is a potent technology with broad applications. Microneedle patches (MNPs) can enhance the accessibility of mRNA-LNPs for vaccination or therapeutic applications. We evaluated the effects of LNP composition on the stability of mRNA-LNPs before and after MNP manufacturing, as assessed by changes in mRNA-LNP size, encapsulation efficiency, and protein expression *in vitro* and *in vivo*. The lipid molar ratio had a significant impact on LNP characteristics and ability to withstand stressors of MNP fabrication. An elevation in ionizable lipid content from 50% to 60% increased *in vitro* mRNA expression, measured by luciferase expression in RAW 264.7 cells, following extraction from an MNP. Among the three ionizable lipids tested, SM-102 had the highest expression before and after MNP manufacturing. Cholesterol analogues influenced *in vivo* expression of mRNA-LNPs before MNP manufacturing, but the effect was absent in mRNA-LNP MNPs. PEGylated lipid choice affected mRNA encapsulation in LNPs and had a strong effect on *in vitro* expression, but this effect was not seen *in vivo*. Phospholipid choice did not have a significant impact on most mRNA-LNP characteristics, but the use of DOTMA increased *in vitro* expression. These data suggest that LNP composition can affect mRNA-LNP characteristics and function both before and after MNP manufacturing. Future mRNA-LNP MNP designs should consider the effect of lipid molar ratios and favor higher ionizable lipid content.

1. Introduction

mRNA has gained scientific attention over the last 35 years for its rapid development time and simple manufacturing in vaccines and genetic therapies [1,2]. Ionizable LNPs improve mRNA transfection, endosomal escape, and *in vivo* stability and have been used in the first FDA-approved mRNA vaccines [3,4]. To widen the applications of mRNA-LNPs, researchers have developed additional methods for delivery, including aerosolized mRNA-LNPs and microneedle patches (MNPs), both of which involve physical stresses on the mRNA-LNP during nebulization and MNP manufacturing/drying process [5–8]. In these contexts, the impact of LNP composition on stability during manufacturing and delivery has not been extensively explored. This study therefore investigates the effects of LNP composition on mRNA-LNP characteristics prior to and following manufacturing in an MNP.

LNPs improve mRNA delivery by protecting mRNA from nucleases, enhancing cellular uptake, and enabling endosomal escape [9,10]. LNPs generally consist of four lipids: an ionizable lipid, a PEGylated lipid, and two helper lipids (e.g., cholesterol and a phospholipid) [3]. The molar ratio of amine groups on the ionizable lipid to the phosphate groups (i.e., N/P ratio) can influence on the structure of LNPs and the amount of mRNA encapsulated [11]. The N/P ratio can be experimentally modified by changing the ionizable lipid:mRNA ratio. The molar ratio between the four lipids is another factor that can change the LNP structure and function [12]. Finally, LNPs can be customized by adjusting the chosen lipids within the four categories.

The ionizable lipid is a key component that typically has a pKa of 6.2–6.9 and is cationic at acidic pH, allowing it to bind to the negatively charged backbone of mRNA [13]. The ionizable lipid contains an ionizable head group, a linker region, and a hydrocarbon tail [4]. While numerous ionizable lipids have been developed for use in LNPs, the ones with the most success in clinical applications share tertiary amines, branched tails, and ester linkages to enhance their biodegradability [14].

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The PEGylated lipid plays a key role in controlling mRNA-LNP size and prolongs circulation half-life [11]. However, hypersensitivity to PEGylated lipids is a known risk [15], and this may be a cause for the side effects seen in SARS-CoV-2 mRNA vaccines [16,17]. Consequently, PEGylated lipids are limited to less than 5 % of the total lipid concentration in mRNA-LNPs [18]. There are several factors that can be modified in PEGylated lipids, including the length of the hydrocarbon chain, the molecular weight (affected by the length of the PEG chain), and charge of the molecule [19,20].

Helper lipids facilitate the structural and functional integrity of the mRNA-LNPs. Cholesterol stabilizes the outer membrane of the LNP, and cholesterol analogues such as β-sitosterol can significantly change the shape of LNPs [21]. Phospholipids facilitate mRNA's packing, enhancing mRNA encapsulation and cellular delivery [22]. mRNA-LNPs can be fabricated with cationic, neutral, or anionic phospholipids, and the choice of phospholipid affects endosomal escape and organ tropism [23,24].

Dissolving MNPs are arrays of micron-scale needles that incorporate a drug of interest in a water-soluble polymer matrix [25,26]. They are designed to be inserted into the skin and release their cargo within minutes. MNPs offer several advantages for mRNA delivery. Their shallow insertion into the skin enables painless administration [27,28]. Upon dissolution in the skin, there is no sharps waste, which makes administration of MNPs simpler than hypodermic injections [29]. MNPs can even be self-administered [30,31], increasing accessibility of mRNA vaccines and therapies in parts of the world where healthcare personnel are limited or when home use is indicated. MNPs target the skin, which is an immune organ with unique antigen-presenting cells [32]. For many vaccines, intradermal delivery (such as with a MNP) can decrease the dose needed for a robust immune response and/or otherwise improve vaccine immunogenicity [33–36]. Finally, MNPs can increase thermostability of many vaccines and drugs [37,38].

Previous studies have shown that mRNA-LNPs delivered by MNP can effectively vaccinate mice [5,7]. Our previous work identified that MNPs incorporating mRNA-LNPs, formulated with 5 % polyvinyl alcohol (PVA) in RNase-free water, dried at 4 °C, and backed with an epoxy backing solution was an optimal MNP manufacturing method. Some remaining challenges to mRNA-LNP MNP design are a loss of mRNA encapsulation in LNPs and a reduction in *in vivo* expression of reporter mRNA compared to mRNA-LNPs delivered by injection. To address these challenges, we sought to evaluate the effects of LNP composition on mRNA-LNP MNP stability in this study. For our study design, we chose a baseline LNP composition from which we modified the LNP molar ratio and individual lipids to measure the effects of these components on a range of characteristics commonly tested in mRNA-LNPs. The lipids (SM-102, cholesterol, DSPC, and DMG-PEG2000) and lipid composition we used as the baseline formulation were based on the formulation of the Spikevax COVID-19 vaccine from Moderna, which we chose for its well-established clinical safety and efficacy.

2. Methods

2.1. Materials

Firefly luciferase mRNA with N1′-methyl pseudouridine modification was purchased from ARNAV Biotech (Columbus, GA) for data shown in Figs. 1-4 and 8, and from GenScript Biotech (Piscataway, NJ) for data shown in Figs. 5-7. SM-102, ALC-0315, DLin-MC3-DMA, campesterol, beta-sitosterol, fucosterol, stigmasterol, DSPE-PEG-COOH (MW 2000), DSPE-PEG-COOH (MW 5000), 18:0 PEG PE (MW 2000), and C16 PEG Ceramide (MW 2000) were purchased from BroadPharm (San Diego, CA). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MA). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DOPS), 1,2-di-O-octadecenyl-3-trimethylammonium propane chloride salt (DOTMA), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), and DMG-PEG2000 were purchased from Avanti Lipids (Alabaster, AL). Epoxy resin was purchased from SEVGILI (St. Petersburg, FL). This epoxy is food-safe after curing but its safety for biomedical applications has not been evaluated. Polyvinyl alcohol 4–88 (PVA) was purchased from Millipore Sigma (Burlington, MA). Sucrose was purchased from Fisher Scientific (Waltham, MA). RNase-free water was purchased from Quality Biological (Gaithersburg, MD).

2.2. LNP fabrication

LNPs were fabricated using a microfluidic chip as described previously [39]. Briefly, the aqueous phase was prepared using a pH 3.0 citrate buffer (100 mM, Teknova, Hollister, CA) diluted to 20 mM in RNase-free water, with mRNA at 0.29 mg/mL. For the experiment in Fig. 1, the mRNA concentration was 0.015 mg/mL to accommodate large ionizable lipid:mRNA ratios. The organic phase was prepared with the ionizable lipid, cholesterol, phospholipid, and PEGylated lipid in the chosen molar ratios in ethanol. The volume ratio of aqueous to organic flow rates was 3:1, with a total flow rate of 800 μL/min. Pump 11 Elite syringe pumps (Harvard Apparatus, Holliston, MA) were used with FlowControl software (Harvard Apparatus) to control the flow rate.

2.2.1. MNP manufacturing

Metal microneedles measuring 600 μm in total length, 600 μm in diameter at the base, and 10 μm in diameter at the tip on a tapered

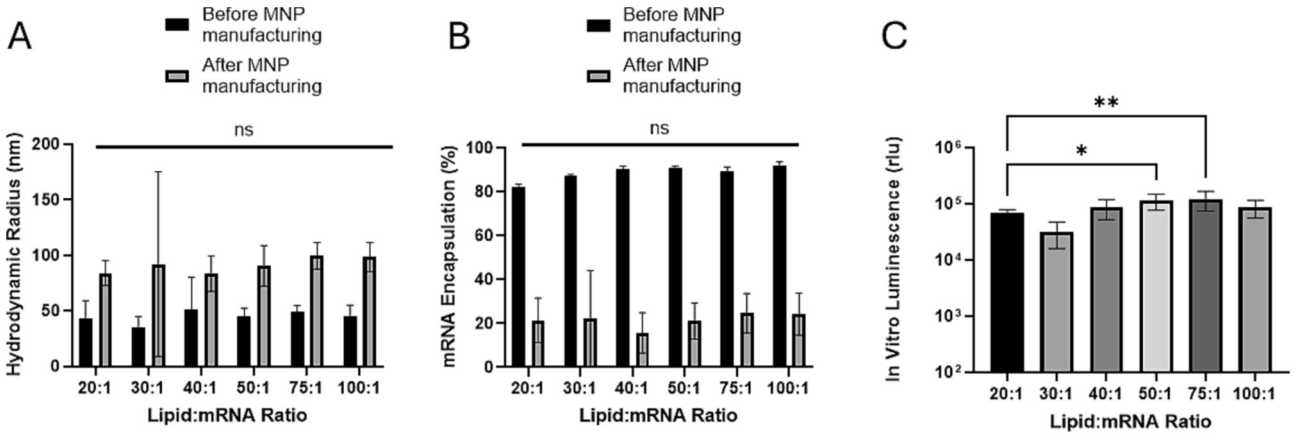

Fig. 1. Effect of lipid:mRNA mass ratio on stability of mRNA-LNP MNPs. (A) mRNA-LNP hydrodynamic radius. (B) mRNA encapsulation efficiency in LNPs. (C) *In vitro* expression of luciferase-encoding mRNA-LNPs after MNP manufacturing. (n = 5, ns = no significance, *p < 0.05, **p < 0.01, one-way and two-way ANOVA).

A

Group	SM-102	Chol	DSPC	DMG PEG2k
A	40%	48.5%	10%	1.5%
B	50%	38.5%	10%	1.5%
C	60%	28.5%	10%	1.5%
D	50%	23.5%	25%	1.5%
E	25%	23.5%	50%	1.5%
F	50%	37.5%	10%	2.5%

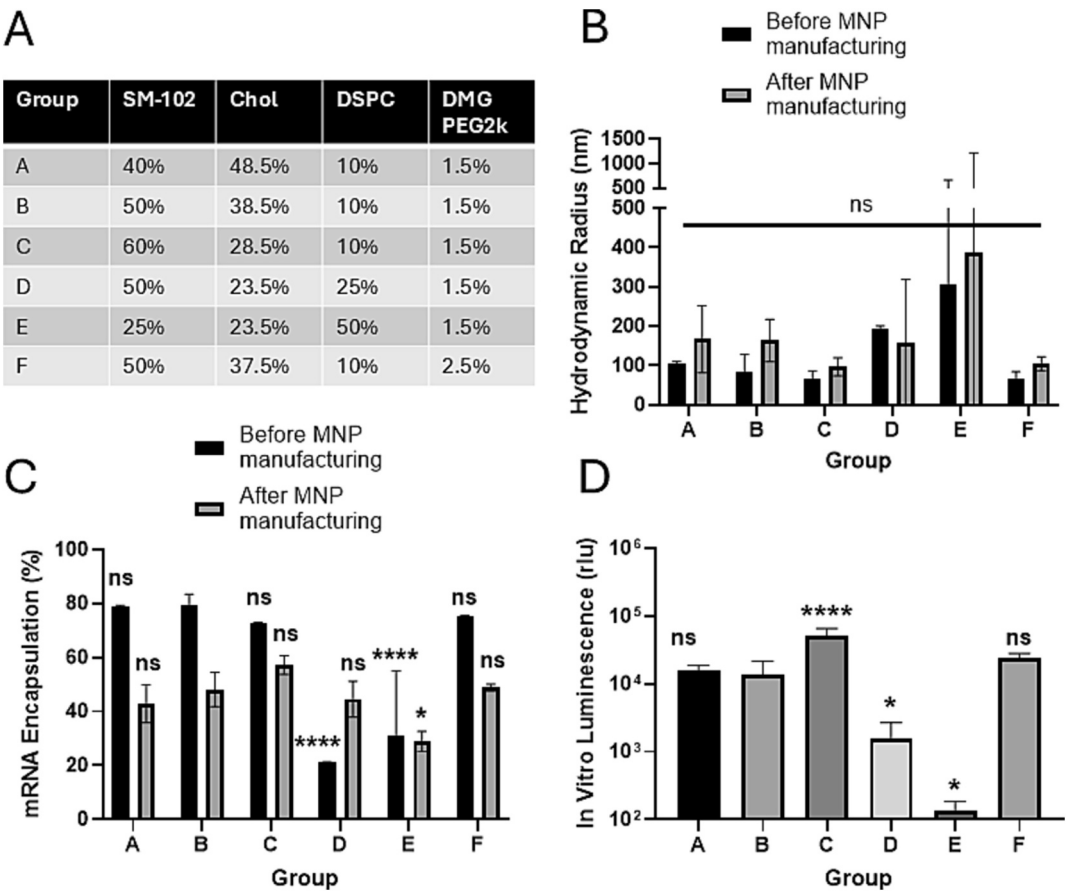

Fig. 2. Effect of lipid ratios on stability of mRNA-LNP MNPs. (A) Table of the molar composition of SM-102, cholesterol, DSPC, and DMG-PEG2k in mRNA-LNPs. (B) mRNA-LNP hydrodynamic radius. (C) mRNA encapsulation efficiency in LNPs. (D) *In vitro* expression of luciferase-encoding mRNA-LNPs after MNP manufacturing. (n = 3, ns = no significance, *p < 0.05, **p < 0.01, ****p < 0.0001, one-way and two-way ANOVA).

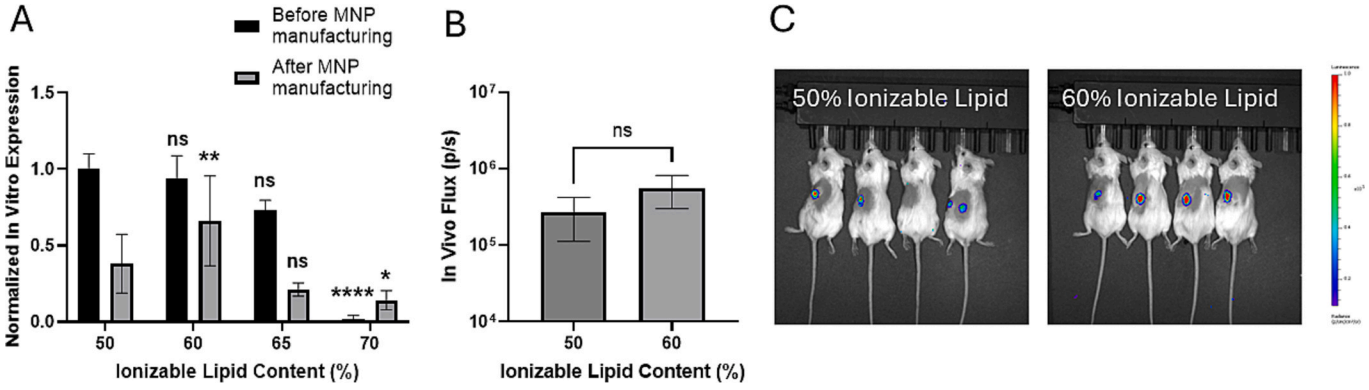

Fig. 3. Effect of ionizable lipid content on stability of mRNA-LNP MNPs. (A) *In vitro* luminescence of mRNA-LNPs before and after MNP manufacturing, normalized to mRNA-LNPs with 50 % SM-102. (B) *In vivo* expression of mRNA-LNPs made with 50 % and 60 % SM-102 after delivery from a MNP. (C) *In vivo* images of mice after application of MNPs containing mRNA-LNPs with 50 % or 60 % SM-102. (n = 4, ns = no significance, *p < 0.05, **p < 0.01, ****p < 0.0001, one-way and two-way ANOVA).

pedestal measuring 300 μm in length were manufactured in a circular array of 112 MNs in an area of ~ 1 cm^2. Twelve of these metal masters were aligned in a plastic case. Polydimethylsiloxane (PDMS; Ellsworth Adhesives, Germantown, WI) was poured onto the male metal masters, set overnight at 37 °C, and carefully removed to make a strip of twelve female molds.

Molds were placed on a device that allowed vacuum to be drawn from underneath. The microneedle casting solution was prepared by combining equal volumes of LNP suspension and 10 % w/v PVA solution in RNase-free water followed by addition of 10 % w/v sucrose (e.g. 20 μL of LNP suspension would be combined with 20 μL PVA solution and 4 mg of sucrose). Then, 30 μL of the microneedle casting solution was added onto the top of each PDMS mold and allowed to fill into the mold cavities and partially dry. After 10 min, the excess solution was carefully removed by wiping with a clean razor blade and dried for 30 min, followed by adding 150 μL of a backing casting solution. The backing casting solution was prepared by mixing epoxy resin and curing agent in a 1:1 vol ratio. After 4–5 h, an acrylic backing (laser cut from 1/16″

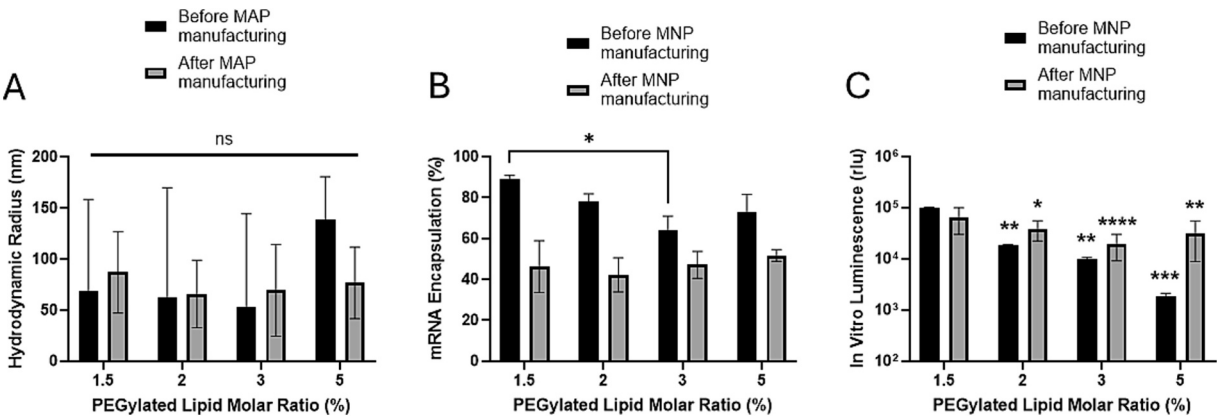

Fig. 4. Effect of PEGylated lipid content on stability of mRNA-LNP MNPs. (A) mRNA-LNP hydrodynamic radius. (B) mRNA encapsulation efficiency in LNPs. (C) *In vitro* expression of luciferase-encoding mRNA-LNPs before and after MNP manufacturing. (n = 6, ns = no significance, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA).

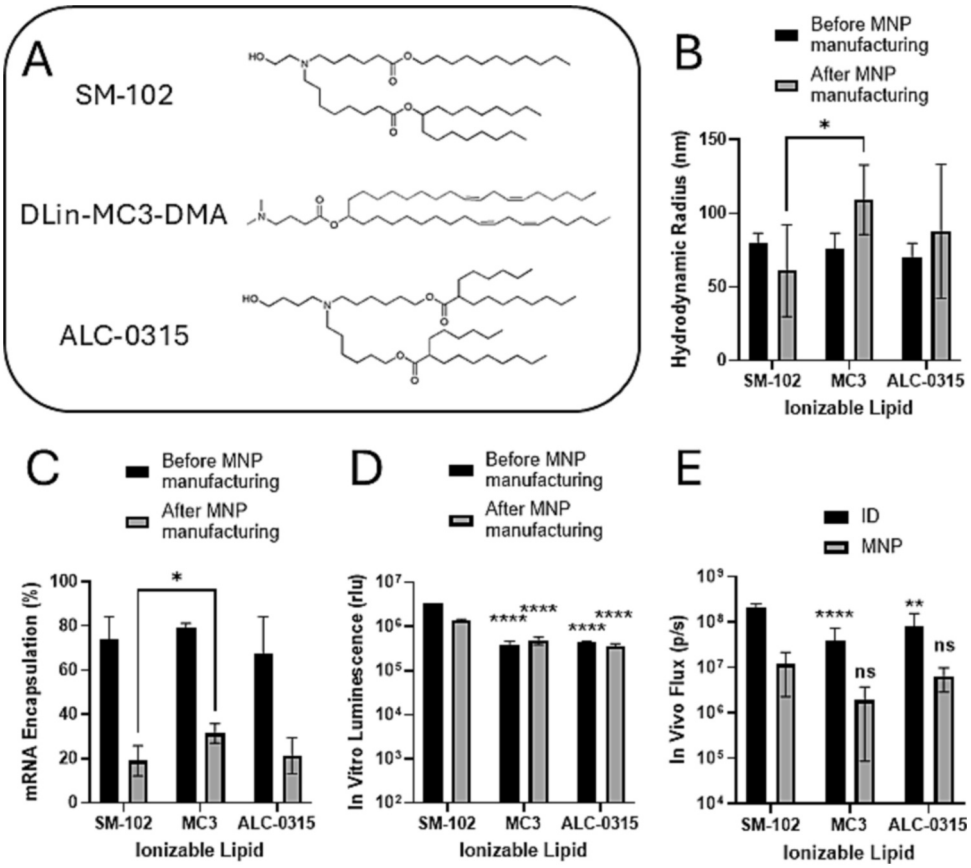

Fig. 5. Effect of ionizable lipid on stability of mRNA-LNP MNPs. (A) Chemical structures of the tested ionizable lipids. (B) mRNA-LNP hydrodynamic radius. (C) mRNA encapsulation efficiency in LNPs. (D) *In vitro* expression of luciferase-encoding mRNA-LNPs before and after MNP manufacturing. (E) *In vivo* expression of luciferase-encoding mRNA-LNPs injected intradermally (ID) or by MNP. (n = 4–6, ns = no significance, *p < 0.05, **p < 0.01, ****p < 0.0001, one-way and two-way ANOVA).

acrylic sheets (McMaster-Carr, Elmhurst, IL)) was affixed to the MNP backing, and the molds were removed from the vacuum and placed into a desiccator overnight at 4 °C. The following day, the MNPs were demolded from the PDMS molds and stored in the desiccator overnight at 25 °C.

2.2.2. MNP reconstitution

MNPs examined *in vitro* were reconstituted by placing them on a benchtop with the backing side down and the microneedles facing up. A volume of 100–200 μL of phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4; Corning, Glendale, AZ) was pipetted on top of each MNP. After 90 s, the PBS was mixed carefully with a pipette tip and then transferred into an Eppendorf tube. The MNPs were examined using light microscopy to determine that all microneedle tips had dissolved.

2.2.3. LNP characterization

Freshly made LNPs and reconstituted MNPs were diluted to 4 μg/mL

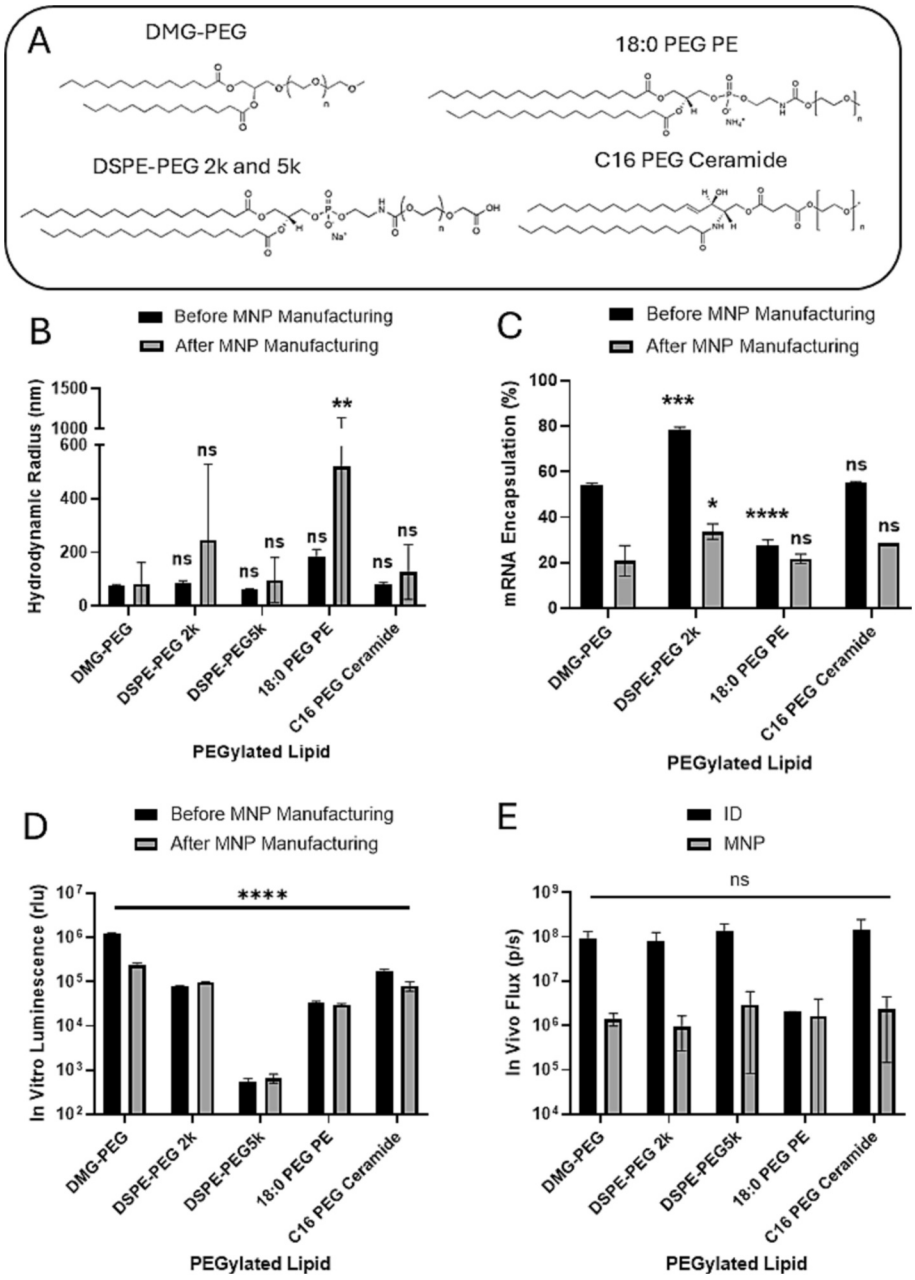

Fig. 6. Effect of PEGylated lipid on stability of mRNA-LNP MNPs. (A) Chemical structures of the tested PEG-linked lipids. (B) mRNA-LNP hydrodynamic radius. (C) mRNA encapsulation efficiency in LNPs. (D) *In vitro* expression of luciferase-encoding mRNA-LNPs before and after MNP manufacturing. (E) *In vivo* expression of luciferase-encoding mRNA-LNPs injected intradermally (ID) or by MNP. (n = 3, ns = no significance, *p < 0.05, ***p < 0.001, ****p < 0.0001, two-way ANOVA).

in PBS and added to an opaque well plate with a clear bottom. These samples were analyzed for hydrodynamic radius and polydispersity by dynamic light scattering (DLS) using a Dynapro Plate Reader (Wyatt Technology, Santa Barbara, CA).

LNPs were also evaluated for mRNA encapsulation efficiency and quantity of mRNA by Quant-it RiboGreen RNA Assay Kit (Invitrogen, Waltham, MA), according to manufacturer instructions. The samples were diluted in Tris-EDTA (TE) buffer (Invitrogen) and added to the first row of a well plate. Aliquots of the samples were mixed with TE buffer or 4 % v/v Triton X-100 (Millipore Sigma) in TE buffer to disrupt the LNPs and release the mRNA. A standard curve was also prepared using known concentrations of mRNA. The plate was stored for 10 min in an incubator at 37 °C. RiboGreen dye was added, and the plate was analyzed using a Synergy H4 Hybrid plate reader (BioTek, Winooski, VT) to determine the mRNA content in each well. The encapsulation efficiency was calculated using the following equation for each sample.

$$Encapsulation\ efficiency = \frac{Total\ mRNA - mRNA\ outside\ LNPs}{Total\ mRNA} = \frac{mRNA\ inside\ LNPs}{Total\ mRNA}$$

2.2.4. *In vitro expression*

MNPs were pressed by thumb into the backs of female BALB/c mice (3–18 months, 15–25 g, Charles River Laboratories, Wilmington, MA) for 1 min. Then, the MNPs were taped in place for an additional 14 min. After 24 h, D-luciferin (Gold Biotechnology, St. Louis, MO) in sterile saline was injected at a dose of 75 mg/kg intraperitoneally. Luminescence was measured using the In Vivo Imaging System (IVIS) Spectrum CT (Perkin Elmer, Shelton, CT). The Georgia Institute of Technology Institutional Animal Care and Use Committee approved all animal

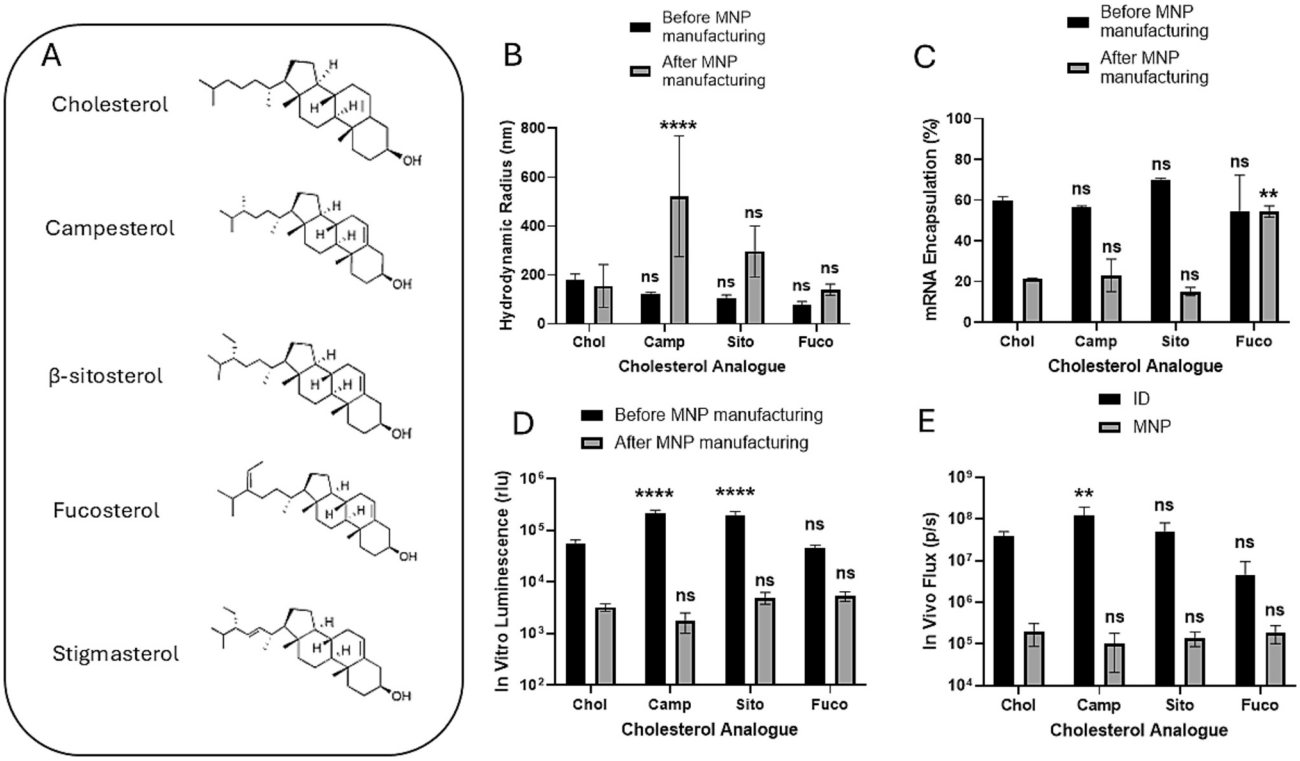

Fig. 7. Effect of cholesterol analogues on stability of mRNA-LNP MNPs. Cholesterol analogues were cholesterol (Chol), campesterol (Camp), β-sitosterol (Sito), fucosterol (Fuco), and stigmasterol. (A) Chemical structures of the tested cholesterol analogues. (B) mRNA-LNP hydrodynamic radius. (C) mRNA encapsulation efficiency in LNPs. (D) *In vitro* expression of mRNA-encoding LNPs before and after MNP manufacturing. (E) *In vivo* expression of luciferase-encoding mRNA-LNPs injected intradermally (ID) or by MNPs. (n = 3–4, ns = no significance, $^{**}p < 0.01$, $^{****}p < 0.0001$, one-way and two-way ANOVA).

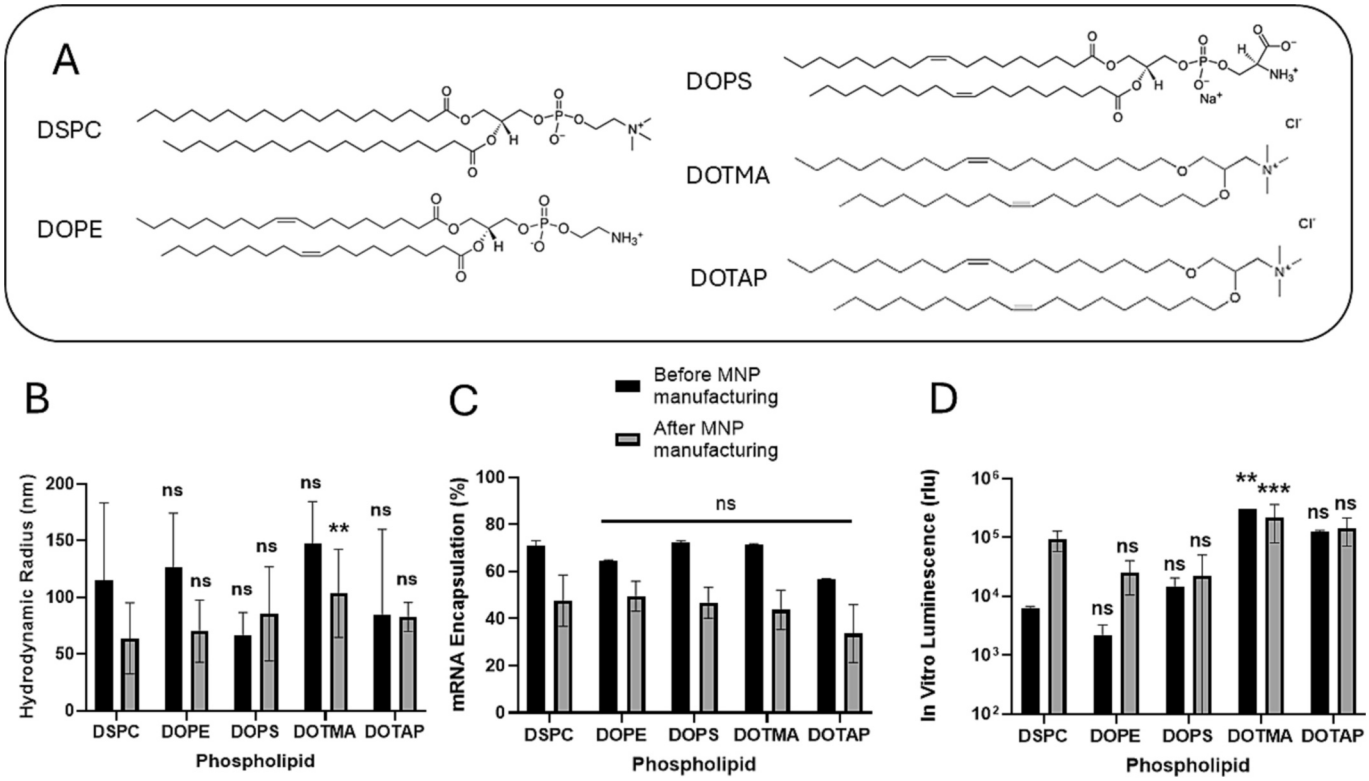

Fig. 8. Effect of phospholipid on stability of mRNA-LNP MNPs. (A) Chemical structures of tested phospholipids. (B) mRNA-LNP hydrodynamic radius. (C) mRNA encapsulation efficiency in LNPs. (D) *In vitro* expression of luciferase-encoding mRNA-LNPs before and MNP manufacturing. (n = 6, ns = no significance, $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA).

experiments.

2.2.5. Data analysis

Prism software (GraphPad, Boston, MA) was used to generate graphs and perform statistical analyses, including one-way and two-way analysis of variance (ANOVA) with multiple comparisons. Post-hoc analyses were completed with Dunnett's multiple comparisons test for one-way ANOVA and Tukey's multiple comparisons test for two-way ANOVA. When applicable, reported statistics denote the effect of the chosen lipid with reference to the control group.

3. Results

3.1. Microneedle patch manufacturing

In this study, MNPs were manufactured using a two-step process. In the first step, mRNA-LNPs were mixed with PVA and cast onto a silicone mold to form the microneedles. After drying, the second step involved administering an epoxy backing that connected the microneedles and formed the rest of the MNP. This process exposed mRNA-LNP to stressors including formulation with excipients, drying in an MNP mold, exposure to epoxy curing, and reconstitution for analysis.

3.2. Ionizable lipid:mRNA ratio

We first tested the effect of ionizable lipid:mRNA mass ratio over a range of 20:1 to 100:1 on mRNA-LNP stability both before and after MNP manufacturing. Over the range of conditions tested, lipid:mRNA mass ratio did not significantly influence size (Fig. 1**A**) or encapsulation efficiency (Fig. 1**B**) before or after MNP manufacturing. Following MNP manufacturing, there was a modest increase in *in vitro* mRNA expression from LNPs that had a 50:1 and 75:1 ionizable lipid:mRNA mass ratio (Fig. 1**C**). However, the low mRNA concentration necessary to attain such high ionizable lipid:mRNA ratios resulted in low mRNA loading that would generally be impractical for MNP applications. These data led us to conclude that a higher ionizable lipid:mRNA mass ratio would not sufficiently improve mRNA-LNP stability to justify its use in a MNP.

3.3. Lipid ratios

We next screened lipid ratios in LNPs comprising SM-102, cholesterol, DSPC, and DMG-PEG2k while maintaining steady mRNA content. In comparison to our standard LNP composition (group B), we varied ionizable lipid content (while correspondingly varying cholesterol content in groups A and C), increased phospholipid content (while correspondingly decreasing cholesterol or SM-102 content in groups D and E), and increased PEGylated lipid content (with a corresponding decrease in cholesterol content in group F) (Fig. 2**A**).

There were no significant differences in mRNA-LNP size amongst the lipid ratios tested, although group E, with increased phospholipid and decreased cholesterol and SM-102, had apparently larger size and high variability both before and after MNP manufacturing (Fig. 2**B**). Both of the groups with increased phospholipid content (groups D and E) had reduced mRNA encapsulation (Fig. 2**C**). After MNP manufacturing, *in vitro* mRNA-LNP expression was higher for the group with increased ionizable lipid content (group C) and lower for the groups with increased phospholipid content (groups D and E) (Fig. 2**D**). We concluded that a higher molar content of phospholipid should be avoided due to low encapsulation efficiency and low *in vitro* expression. Additionally, an increased content of ionizable lipid may be protective against the stressors of MNP manufacturing.

Based on these findings, we investigated the effect of ionizable lipid (SM-102) content further over a range of 50 % to 70 %. *In vitro* expression increased after MNP manufacturing using LNPs with 60 % ionizable lipid, which was consistent with previous findings (Fig. 3**A**). However, additional increases to 65 % and 70 % ionizable lipid content while correspondingly decreasing cholesterol content produced no improvement in *in vitro* expression compared to 50 % ionizable lipid. *In vivo* application of mRNA-LNP MNPs to mice showed no significant difference between 50 % and 60 % ionizable lipid formulations (Fig. 3**B**). We concluded that there were effects of lipid ratios on mRNA-LNP stability during the MNP manufacturing process, but these effects were modest for this set of lipids.

While the initial study did not show a significant effect from increasing the PEGylated lipid content from 1.5 % to 2.5 %, we carried out an additional study that increased PEGylated lipid content to 5 %. The higher PEGylated lipid levels had no effect on size (Fig. 4**A**), which is inconsistent with previous findings [11], but there was a high variability. There were no significant differences in mRNA encapsulation among samples after MNP manufacturing (Fig. 4**B**). However, higher PEGylated lipid content decreased *in vitro* expression both before and after MNP manufacturing (Fig. 4**C**). From these data, we conclude that an increase in PEGylated lipid content did not increase mRNA-LNP stability in an MNP and, in fact, decreased *in vitro* transfection.

3.4. Ionizable lipid

To expand on our studies using SM-102, we tested the effects of two additional ionizable lipids used in USFDA-approved formulations, DLin-MC3-DMA (MC3) and ALC-0315, on mRNA-LNP characteristics and function. Although all three ionizable lipids resulted in similar size and encapsulation efficiency in LNPs before MNP manufacturing, MC3 produced larger mRNA-LNPs (Fig. 5**B**) and superior encapsulation efficiency (Fig. 5**C**) after MNP manufacturing. LNPs formulated with SM-102 resulted in the highest *in vitro* expression of luciferase-encoding mRNA both before and after MNP manufacturing (Fig. 5**D**). Similarly, intradermal injection of LNPs prior to MNP manufacturing yielded maximum luciferase expression with SM-102, but this outcome was not seen in mRNA-LNPs delivered by MNP (Fig. 5**E**).

3.5. Pegylated lipid

In addition to mRNA-LNPs formulated with DMG-PEG2k, we screened several other PEGylated lipids to determine the effects of changes to hydrocarbon chain length, molecular weight, and charge on mRNA-LNP characteristics and function (Table 1). There were no significant differences among the sizes of the mRNA-LNPs fabricated with different PEGylated lipids before MNP manufacturing. However, after MNP manufacturing, the size of mRNA-LNPs made with 18:0 PEG PE increased significantly (Fig. 6**B**). While there was significant variation in mRNA encapsulation efficiency before MNP manufacturing, there was only a modest increase in encapsulation efficiency for mRNA-LNPs made with DSPE-PEG2k after MNP manufacturing. The other groups had similar encapsulation efficiency (Fig. 6**C**).

In vitro expression showed significant differences among all PEGylated lipids, with DMG-PEG2k exhibiting the highest expression before and after MNP manufacturing (Fig. 6**D**). These results suggested a

Table 1
PEG-linked lipids chosen to screen the effects of modifications to hydrocarbon chain length, molecular weight, and linker region.

PEG-linked lipid	Hydrocarbon Chain Length	Molecular Weight	Charge	End Functional Group
DMG-PEG2k	C14	2000	Neutral	Methyl
DSPE-PEG-COOH 2 k	C18	2000	Negative	Carboxylic acid
DSPE-PEG-COOH 5 k	C18	5000	Negative	Carboxylic acid
18:0 PEG PE	C18	2000	Neutral	Ether
C16 PEG Ceramide	C16	2000	Neutral	Methyl

disadvantage to using a PEGylated lipid with high molecular weight. Additionally, the two PEGylated lipids that led to the highest *in vitro* expression were neutral in charge and had shorter carbon chains. More research is needed to establish a causative relationship between these variables and higher expression. When delivered *in vivo,* there were no significant differences among the reporter protein expression in each group (Fig. 6**E**), contrasting with the *in vitro* results. Despite changes in mRNA-LNP characterization and *in vitro* expression, the PEGylated lipid choice did not appear to significantly affect *in vivo* expression.

3.6. Cholesterol

Cholesterol analogues are known to change the shape of LNPs [21], so we hypothesized that they would play a role in mRNA-LNP stability during MNP manufacturing. In addition to cholesterol, we tested four cholesterol analogues: stigmasterol, campesterol, β-sitosterol, and fucosterol. mRNA-LNPs that contained stigmasterol had an encapsulation efficiency of 0 % and were not further tested. mRNA-LNPs containing campesterol increased mRNA-LNP size after MNP manufacturing (Fig. 7**B**), indicating destabilization. mRNA-LNPs containing fucosterol had a higher mRNA encapsulation efficiency after MNP manufacturing (Fig. 7**C**), indicating a protective effect of fucosterol against mRNA leakage. However, these mRNA-LNP characteristics did not impact *in vitro* (Fig. 7**D**) or *in vivo* (Fig. 7**E**) expression, which had no significant differences across all groups after MNP manufacturing. Among mRNA-LNPs before MNP manufacturing, incorporation of campesterol and β-sitosterol increased *in vitro* expression, and for campesterol, also increased *in vivo* expression after intradermal injection.

3.7. Phospholipid

Due to the slight benefit seen with a greater content of ionizable lipid, we hypothesized that cationic phospholipids might aid in mRNA-LNP stability during MNP manufacturing, although there is some concern about their possible toxicity [40,41]. We tested two neutral phospholipids (DSPE and DOPE), one anionic phospholipid (DOPS), and two cationic phospholipids (DOTMA and DOTAP). The size of LNPs before MNP manufacturing did not differ significantly among the phospholipids used (Fig. 8**B**). The size of mRNA-LNPs made with DOTMA increased after MNP manufacturing more than the other groups. There was no significant difference among the groups in the encapsulation efficiency before or after MNP manufacturing.(Fig. 8**C**). *In vitro* expression was higher before and after MNP manufacturing for mRNA-LNPs containing DOTMA (Fig. 8**D**).

4. Discussion

This study assessed the effects of changing the composition of mRNA-LNPs on their stability before and after MNP manufacturing. Notably, MNP manufacturing involves drying mRNA-LNPs, whose structure is based on balancing hydrophilic and hydrophobic interactions, which are influenced by the presence or absence of water [42]. We believe this is the first study to broadly examine the effects of mRNA-LNP composition on stability during MNP manufacturing and contributes to the growing literature on mRNA-LNP stability overall.

4.1. Ionizable Lipid:mRNA ratio

Despite the known effect of the N/P ratio on LNP structure [11], we determined that the ionizable lipid:mRNA mass ratio did not have a meaningful impact on the stability of mRNA-LNPs during MNP manufacturing.

4.2. Lipid composition

We determined that lipid composition affected mRNA-LNP characteristics before and after MNP manufacturing. We found that LNPs with an increased phospholipid concentration had low mRNA encapsulation. This effect was less pronounced after MNP manufacturing and was only statistically significant at the highest phospholipid concentration of 50 %. Likewise, LNPs made with 40 %, 50 %, or 60 % ionizable lipid content had no difference in size or encapsulation efficiency before MNP manufacturing. However, after MNP manufacturing, LNPs with a 60 % ionizable lipid molar ratio had higher *in vitro* expression. This may be because the higher concentration of positively charged ionizable lipids led to greater bonding with negatively charged mRNA that stabilized the mRNA-LNPs while undergoing the stress of MNP manufacturing.

Despite this, greater than 60 % ionizable lipid content did not further improve *in vitro* expression, and the improvement seen with 60 % ionizable lipid *in vitro* did not translate *in vivo*. In addition to its effect on LNP characteristics such as size and encapsulation before MNP manufacturing, this work shows that lipid composition could also affect LNP stability under stressors such as those involved in MNP manufacturing. These experiments were limited to LNPs made of SM-102, cholesterol, DSPC, and DMG-PEG2k, so future work is needed to determine optimal lipid composition for other lipids.

4.3. Ionizable lipid

We tested three ionizable lipids present in the USFDA-approved LNP-mRNA formulations: SM-102, DLin-MC3-DMA, and ALC-0315. mRNA-LNPs made with SM-102 had the highest expression before MNP manufacturing both *in vitro* and *in vivo*. After MNP manufacturing, the differences between the groups diminished and there was no significant difference *in vivo*. The findings indicate that ionizable lipids that led to high expression in a liquid state (before MNP manufacturing) were also favored in a dry state (after MNP manufacturing).

4.4. Pegylated lipid

We determined the effects of hydrocarbon chain length, molecular weight, and charge of PEGylated lipids. mRNA-LNPs containing PEGylated lipids with shorter hydrocarbon chain length, lower molecular weight, and neutral charge resulted in higher expression *in vitro*. However, these *in vitro* differences were not seen *in vivo,* leading us to conclude that PEGylated lipid choice did not play a significant role for mRNA-LNPs *in vivo*. The differences between *in vitro* and *in vivo* results may be attributed to different cell types (such as keratinocytes and dermal dendritic cells versus RAW 364.7 cells), *in vivo* immune responses that are not present *in vitro*, and other complexities of the *in vivo* environment that are not captured *in vitro.* Additionally, we only screened five PEGylated lipids, and further research is needed to isolate the most important factors for *in vitro* expression.

4.5. Cholesterol

We evaluated four cholesterol analogues (stigmasterol, campesterol, β-sitosterol, and fucosterol) and compared them to cholesterol in mRNA-LNPs. mRNA-LNPs made with campesterol had higher *in vitro* and *in vivo* expression, and LNPs made with β-sitosterol had higher *in vitro* expression before MNP manufacturing. Cholesterol analogues also played a role in mRNA-LNP characteristics after MNP manufacturing, with campesterol increasing LNP size and fucosterol increasing mRNA encapsulation. However, these changes to mRNA-LNP characteristics did not translate to differences in mRNA expression after MNP manufacturing either *in vitro* or *in vivo*. We determined that cholesterol influenced the structure and stability of mRNA-LNPs, although cholesterol analogues did not enhance expression from mRNA-LNP MNPs.

4.6. Phospholipid

We hypothesized that cationic phospholipids would stabilize mRNA-LNPs during MNP manufacturing. While we found that mRNA-LNPs containing the cationic phospholipid DOTMA had a higher *in vitro* expression both before and after MNP manufacturing, we did not assess their *in vivo* expression from mRNA-LNP MNPs due to toxicity concerns for permanently cationic lipids [40,41]. Recent studies have addressed toxicity concerns for LNPs containing DOTMA and found them to be safe [43,44], but regulatory questions about these lipids may still need to be addressed.

4.7. Limitations and future work

These studies focused on testing a limited number of LNP compositions among the many that could be tested. Our positive control in all experiments was a 50:38.5:10:1.5 molar ratio of SM-102, cholesterol, DSPC, and DMG-PEG2k, respectively. In most experiments, the positive control produced mRNA-LNP expression that was as good as or better than the other compositions tested. This may indicate that this mRNA-LNP composition, which was optimized by Moderna for intramuscular injection as a liquid form may also be well-suited for drying during MNP manufacturing and delivery to the skin. Our experimental design generally involved making incremental modifications that changed one mRNA-LNP parameter at a time, which can optimize a formulation similar to the starting formulation but may miss superior formulations that are dramatically different from the positive control. Future research should involve a broader screening of different LNP compositions not based on the Moderna formulation, with a focus on those with demonstrated safety and efficacy *in vivo*. A constrained mixture design would more completely identify optimal composition of lipid components. This work also only addressed mRNA-LNP stability during the formulation and fabrication process; future studies should address long-term stability during storage. Finally, this work focused on *in vitro* characterizations that were not always followed up with *in vivo* expression results. Given the inconsistent correlation between *in vitro* and *in vivo* findings in this study, more confirmatory studies should be performed *in vivo*.

5. Conclusions

With the goal of understanding the effects of mRNA-LNP composition on stability during MNP manufacturing, we investigated the ionizable lipid:mRNA ratio, the lipid ratios, and the choice of each type of lipid used among the four lipids that make up the LNP. The lipid ratios had a significant impact on mRNA-LNP stability after MNP manufacturing. Specifically, high phospholipid content had a detrimental effect. The ionizable lipid SM-102 led to the greatest *in vivo* expression of mRNA-LNP MNPs. The PEGylated lipid choice resulted in differences *in vitro* that did not translate to *in vivo* expression. Cholesterol analogues played a role in *in vivo* expression of intradermally injected mRNA-LNPs but did not affect *in vivo* expression from a mRNA-LNP MNP. The phospholipid DOTMA resulted in the highest *in vitro* expression of mRNA-LNPs from MNPs. mRNA-LNP stability was affected to a greater extent by the lipid molar ratio than individual lipid identity. Overall, this study broadly investigated the effects of LNP composition, which should be considered in future design and development of mRNA-LNP MNPs.

CRediT authorship contribution statement

Sophia H. Sakers: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gianna Fiduccia:** Investigation. **Katherine E. Byrne:** Investigation. **B.Pradeep K. Reddy:** Methodology. **James E. Dahlman:** Conceptualization. **Mark R. Prausnitz:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mark R. Prausnitz is an inventor of patents, a paid advisor to companies, and a founder/shareholder of companies developing microneedle-based products. This potential conflict of interest has been disclosed and is managed by Georgia Tech.

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Data availability

Data will be made available on request.

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